Prevalence, immunopathogenesis and antioxidant status of children with Acute Respiratory Infections due to Respiratory Syncytial Virus and Human Metapneumovirus and risk factors for disease severity in Yemen

Nagla'a Nasr Addin Alsonboli

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Dedication

To my parents who are always there for me

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This work could only take shape with help of many colleagues whom I really want to thank.

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List of abbreviations

AHSR	The Annual Health Statistical Report
AOR	Adjusted ODD Ratio
APPs	Acute phase proteins
APR	Acute phase response
APV	Avian pneumovirus
ARI	Acute respiratory tract infections
ARLI	Acute lower respiratory tract infections
ASD	Atrial Septal Defect
ATG	antithymocyte globulin
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
Bgl	Bacillus globigii
BO	bronchiolitis obliterans
BME	Beta-methabol
bp	Base Pair
BPD	Bronchopulmonary dysplasia
C3	complement factor 3
CHD	congenital heart disease
CI	Confidence interval
CLD	Chronic lung disease
CMV	Cytomegalovirus
CRP	C-reactive protein
CTL	cytotoxic lymphocytes
Cu	Copper
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DPT	Diphtheria, Pertussis and Tetanus
dTT	dithiothreitol
DW	Distilled water
E/A	Emergency and Accident unit
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	Ethylenediaminetetra-acetic acid
EIA	enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay

ESR	Erythrocyte sedimentation rate
FI-RSV	formalin-inactivated RSV
FITC	fluorescein isothiocyanate
GA	gestational age
GM	geometrical mean
GM-CSF	granulocyte-macrophage colony stimulating factor
Hae	Haemophillis aegyptius
HAZ	Height for age Z score
Hb	Haemoglobulin
HLA-DR	human leukocyte-associated antigen-DR
Hind	Haemophillius influenza Rd com-10
HMPV	Human Metapnuemovirus
HPLC	High Performance Liquid Chromatography
HSV-1	Herpes simplex virus type-1
ICAM-1	intracellular adhesion molecule-1
ICPMS	Inductive Coupled Plasma Mass Spectrometry
ICU	Intensive care unit
IFAV	Influenza A virus
IL	interleukin
IL-2R	Interlukin -2 receptor
INF-α/β	Interferon – α/β
INF-γ	Interferon-gamma
IVF	Intravenous fluid
kDa	kilo Dalton
LA	Luria plate
LB-Borth	Luria-Bertani –Borth
MCP-1	monocyte chemotactic protein-1
MHC-1	major histocomatibility complex-1
MIP-1α	macrophage inflammatory protein-1α
µmol/l	Micromol/Litre
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NGT	Nasogastric tube
NK	natural killer cells
NLF	nasal lavage fluid
No.	Number
NPA	Nasopharyngeal aspirate

NW	Nasal wash
OD	Optical Density
OPD	Out patient department
OR	Odds Ratio
ORFs	Open reading frames
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDA	Patent Ductus Arteriosus
pg	picogram
PG	prostaglandin
PICO	Paediatric intensive care unite
PMNCs	Polymorphonuclear cells
PMW	Pediatric Medical Ward
Pst	Providencia stuartii
PVM	pneumonia virus of mice
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RPM	Rotations per minute
RBP	retinol binding protein
RFLP	Restriction Fragment Length Polymorphism
RLT buffer	denaturing guanidine isothiocyanate GITC-containing buffer
RNA	ribonucleic acid
RNase	ribonuclease
ROC	Receiver Operation curve
RR	Relative risk
Rsa	Rhodopseudomonas sphaeroids
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription- polymerase chain reaction
SD	Standard deviation
Se	Selenium
SE	Standard error
SP-A	surfactant protein-A
Stat	signal transducer and activator for transcription
sTNFR	soluble TNF receptors
TA	tracheal aspirate
TBE	Tris Borate EDTA
TG	transgenic
Th1	T helper-1

Th2	T helper-2
TLR4	Toll-like receptor- 4
TNF-α	Tumor necrosis factor-α
TTR	transthyretin
URTI	Upper respiratory tract infection
UAE	United Arab Emirates
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VSD	Ventricular Septal Defect
WAZ	weight for age Z score
WHO	World Health Orgaisation
WHZ	Weight for height Z score
Zn	Zinc

Abstract

Aims: to establish the prevalence of RSV and HMPV in Yemeni children with ARI, describe the risk factors for disease severity, the cytokine (IL6, TNF-α, IL7, IL10, IL12 and INF-γ) and chemokine (IL8 and RANTES) concentrations in children with RSV and/or HMPV and their association with disease severity. To determine serum micronutrients and C- reactive protein concentrations in children with RSV and/or HMPV with mild and severe ARI.

Methodology: Children < 2 years old with signs and symptoms of ARI attending a reference hospital in Sana'a, Yemen, were enrolled during 2002 and 2003. RSV and HMPV were identified using RT-PCR. Children with mild/moderate ($pO_2 \geq 88\%$) hypoxia were compared to those with severe hypoxia ($pO_2 < 88\%$). Cytokines and chemokines were measured by ELISA. Inductive Coupled Plasma Mass Spectrometry was used to measure zinc, selenium and copper and a High Performance Liquid Chromatography to measure serum vitamins A and E concentrations.

Results: RSV was identified in 40%, HMPV in 7% and RSV/HMPV co-infections in 4% of the children. Group A was the predominating RSV. The period with high RSV and HMPV incidence occurred from December to March but both viruses were detected throughout the study. Single RSV and HMPV infections were undistinguishable clinically and dual infections were common. The factors independently associated with an increased risk of severe hypoxia due to RSV were age ≤ 3 months, the child not having his/her vaccines up to date, the presence of a smoker at home and using a cooking fuel other than gas. In contrast, only age ≤ 3 months, having a history of recurrent ARI and using a source of fuel other than gas were the only risk factors found to be independently associated with an increased risk of severe HMPV. RSV and HMPV infections in infants differ significantly in regard to the type of induced immune response elicited and coinfections modified the production of cytokines. Young age modifies immune responses against the infections. There was an inverse association of IL7 with hypoxia due to RSV. Micronutrients deficiency is widespread in our study population (especially with RSV) and was associated with disease severity and inflammatory stress with an inverse relationship between zinc and copper concentrations and zinc/copper ratios of children with RSV and HMPV.

Conclusion: RSV and HMPV are important causes for ARI in Yemen. This thesis describes the immunological mechanisms observed in RSV and HMPV infections and the risk factors associated with disease severity. This information can be used to inform the development of curative and preventive strategies for acute respiratory infections and for the monitoring of curative interventions. Micronutrient deficiencies are highly prevalent in children with ARI and micronutrient supplementation may decrease the risk for developing severe RSV and HMPV disease.

Chapter 1

Introduction

Acute respiratory infections (ARI) are the main infectious cause of death in children in developing countries (Vardas et al., 1999, Cashat-Cruz et al., 2005) and a large number of viruses and bacteria have been implicated (Calza et al., 2003, van den Hoogen et al., 2001, Weber et al., 1998b). Respiratory Syncytial Virus (RSV) is the most frequent cause of acute lower respiratory infections (ALRI) in children and is responsible for between 50% and 80% of infant ARI hospitalisations due to bronchiolitis (Shay et al., 1999). Although risk factors that predispose to severe RSV ALRI are well described in industrialised countries, there is paucity of information on whether these same factors operate in developing countries. In addition, other viruses such as Human Metapneumovirus (HMPV) have recently been reported to have clinical presentations resembling RSV (van den Hoogen et al., 2001). Despite its frequent occurrence, there is scanty information on the factors that predispose children with this virus to experience severe ALRI.

For the first time, we report here the prevalence of RSV and HMPV and the risk factors associated with severe ALRI associated to these two viruses alone or in combination in young children attending a referral hospital in Yemen.

It appears that an inadequate or over exuberant immune responses are implicated in the severity of illnesses associated with RSV infections (Hussell et al., 1997, Roman et al., 1997). Clinical and experimental evidence suggest an important role for cell mediated immunity (Scott et al., 1978), both for the resolution of infection and pathogenesis of severe RSV disease. RSV bronchiolitis is often considered to be immune mediated. The experience of the immunisation program with the formalin-inactivated RSV (FI-RSV) vaccine in the 1960s that resulted in RSV disease enhancement instead of protection (Kim et al., 1969), suggests that immunological mechanisms may be key to the severity of RSV bronchiolitis in infancy. There is very limited information on the immune responses in children with moderate and severe RSV in developing countries and this thesis explores the topic in the context of children in Yemen.

The pathogenesis of HMPV has not yet been explored. Most reports to date have examined the incidence or prevalence of HMPV in different populations and its range of clinical manifestations have been reported to be very similar to the range of manifestations caused by RSV. However, it is unclear whether similarities in signs and symptoms of infants infected with HMPV and RSV are associated with similar immunological mechanisms.

Better understanding of the immunological responses associated with the severity of this disease could help the research for interventions for its management and prevention. Micronutrient deficiencies have significant adverse consequences on key functioning aspects of the immune system and resistance to infection (Coutsoudis et al., 1991, Field et al., 2002). In developing countries, malnutrition and micronutrient deficiencies are associated with an increased incidence and severity of ARI (Victora et al., 1999). We have however a poor understanding of the role of specific micronutrient such as zinc, selenium, copper, vitamin A and E in relation to disease severity caused by RSV and other viral infections and there are no reports on micronutrient changes in children with HMPV.

This study describes the micronutrient status of children with RSV, HMPV and RSV/HMPV co-infections and their association with severity of disease.

Chapter 2
Methodology

This was a cross-sectional descriptive prospective study, conducted during the cold season in Sana'a, Yemen, from the 1st of October 2002 to the 15th of May 2003. Acute respiratory infections are highly prevalent in Sana'a during this period of the year, which correspond to the winter and the spring. The rainy season in Sana'a is mainly during the summer with scattered rainy days during the winter.

Objectives of the study

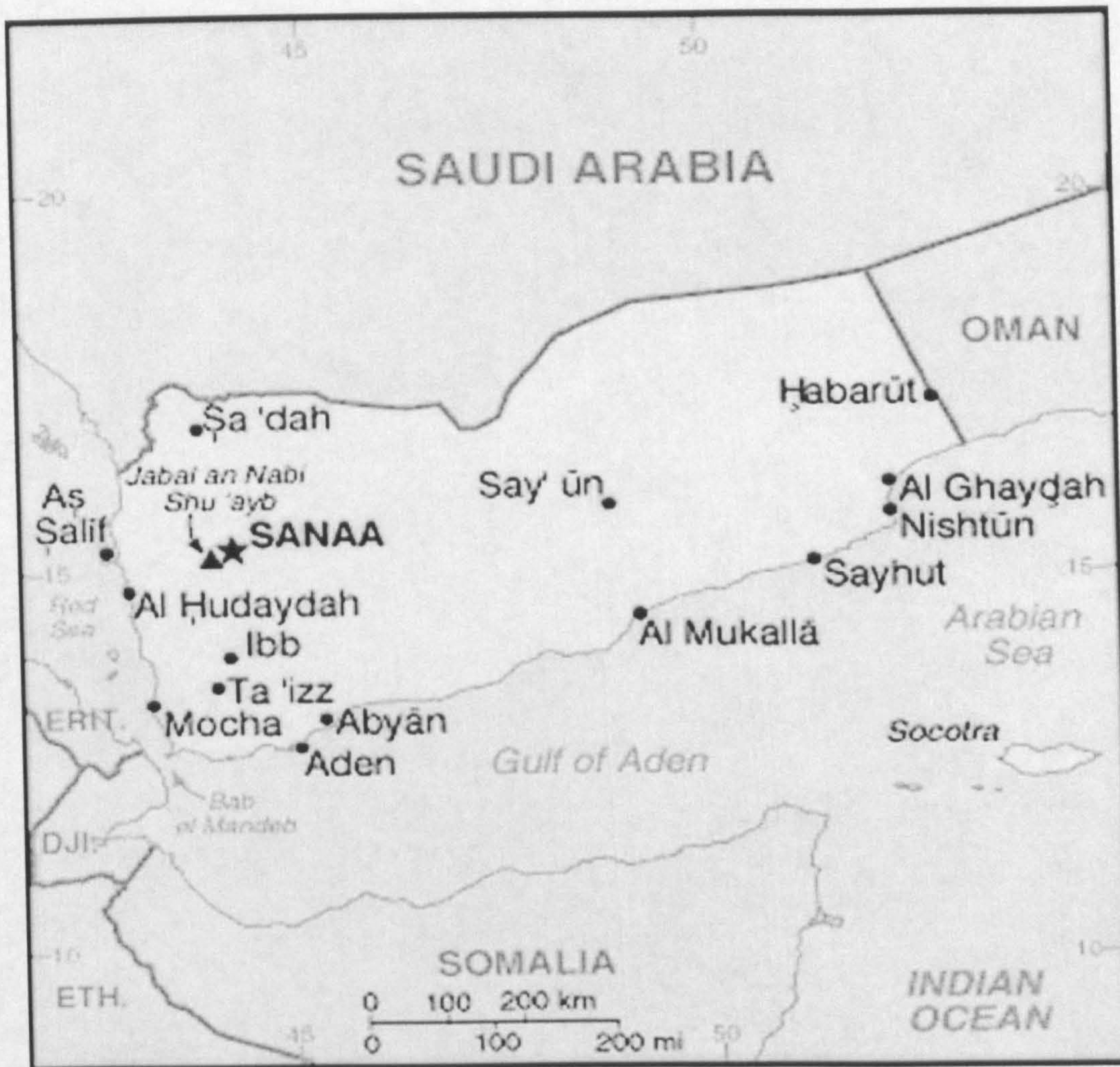
The objectives of the study were:

1. To establish the prevalence of RSV, HMPV and their genotypes in children with ARI.
2. To identify some epidemiological risk factors for disease severity in children with ARI.
3. To compare the NPA concentrations of selected cytokines (IL6, TNF-α, IL7, IL10, IL12 and INF-γ) and chemonkines (IL8 and RANTES) in children with ARI due to RSV and/or HMPV and their association with disease severity.
4. To determine the serum vitamins A and E, zinc, selenium, copper, and C- reactive protein concentrations in children with RSV and/or HMPV and to compare their concentrations in children with mild and severe ARI.

Study location

The study was based in Sana'a, capital of the Republic of Yemen. Sana'a is located at 2200 metres altitude above the sea level. The Republic of Yemen lies in the South of Arabia (The Arabian Peninsula), between latitudes 12° and 20° north of the equator, longitudes 41° and 54°. It is bordered on the north by Saudi Arabia, on the south by the Arab Sea and the Gulf of Aden, on the east by the Sultanate of Oman and on the west by the Red Sea as show in figure 2.1

Figure 2.1 Map of Yemen



According to the Annual Health Statistical Report (AHSR) of the Ministry of Health, the estimated population of Yemen was 18,862,999 in 2001 (AHSR, 2001) of which 26.5% was urban and 73.5% rural, with a population growth rate of 3.5%, and a life expectancy 60.7 years (58.8 years for males and 62.7 years for females). The total fertility rate is 5.8/1000 (4 in urban and 6.3 /1000 in rural areas). Forty six percent of the population is < 14 years of age, 44% between 15-44 years, 6.4% between 50-64 years and 3.9% > 65 years old.

The under 5 years mortality rate of the country is 106 per 1000 children under five for males and 83 per 1000 for females (94.5 for both). The infant mortality rate for the year 2000 was estimated to be 80 per 1000 live births for males, 82.4 for females and 81.2/1000 for both.

The government of Yemen utilizes a traditional three tier health system consisting of health units, health centres and hospitals. There are 360 health units, 928 health centres and 133 public hospitals with 18,538 beds with 2.16, 4.54 and 5.9 physicians, nurses and beds per 10000 population respectively. Sana'a has a population of 1,394,947 inhabitants.

The number of doctors who work in Sana'a city is 919 with 5.76 doctors per 10000 population. Sana'a has 6 public hospitals, 6 health centers and 124 primary health care centres.

Vaccination coverage for children < 1 year is 84.9% for the first dose of Diphteria-Pertussis-Tetanus (DPT), 80.9% for the second dose and 76% for the third dose of DPT, 74% for BCG, 78% for measles and 86.4%, 82.5% and 76% for first, second and third dose of poliomyelitis; 45.3% for the first dose, 32.2% for the second dose and 16.4% for the third dose of Hepatitis B. The most commonly reported diseases are malaria (317,807 cases per year for the year 2000), respiratory infections (301,145 cases for the same year), and diarrhoeal diseases (285,625 cases) (AHSR, 2001).

Study design

This was a prospective cross- sectional descriptive study with a case - control analysis for objective two to four.

Source of participants

Participants were recruited from the emergency and accident unit (E/A), the paediatrics outpatients clinic (OPD), the paediatric medical ward (PMW) and the private PMW (PPMW) of Alsabeen hospital for Women and Children. This is a tertiary referral hospital, which serves Sana'a city and Sana'a province and receives referred cases from other surrounding provinces. The PMW consists of 41 beds, and the infectious disease ward has 40 beds and an E/A unit with 20 beds. The PPMW has 20 beds. Six beds were dedicated for the patients participating in the study.

All the patients attending the Accident and Emergency and outpatient clinics of the hospital between 8 am and 1 pm with signs and symptoms of ARI were recruited consecutively at the time of consultation, independently of the severity of their illness. Patients admitted between 1 pm and 8 am were assessed early next morning. An assistant research pediatrician and doctors running a WHO multicentre study in the hospital to assess the efficacy of ampicillin and gentamycin vs. chloramphenicol to treat children with severe pneumonia collaborated to recruit patients during the afternoon and night periods. During the study period 1000 ARI cases were screened of these, 607 children met the inclusion criteria and were recruited for the study. Most of the children excluded did not fulfil the age for inclusion (i.e. > 2 years old) or their illness was of more than 7 days duration. About 7 cases also excluded because at that

time they were considered to be bacterial pneumonia given that they had lobar consolidation. With hind sight, it would have been better to include these patients.

Criteria for inclusion of patients

Patients aged from 1 to 24 months with a clinical diagnosis of ALRI or mild upper respiratory tract infections were selected.

Case Definition: The following case definitions were used:

Mild upper respiratory tract infection

Any child complaining of cough with absence of crackles or wheezing on auscultation and oxygen saturation ≥ 88% in air.

Bronchiolitis

Any child with cough, respiratory distress, crackles with or without wheezing on auscultation and with oxygen saturation < 88% in air.

Screening of the patients

Children were referred to the investigator by the hospital staff in either the E/A or outpatient clinic. The investigator screened all children using a screening form (appendix 2) to evaluate whether they fulfilled each of the inclusion criteria.

Baseline examination and laboratory data

After necessary attention to the airway, breathing and circulation, a baseline history and examination were obtained from the children enrolled to determine their baseline clinical status. This was done by the researcher as soon as possible. Patients who had attended during the night, were assessed early the following morning by the research doctor. Data included their general characteristics, presence and duration of symptoms prior to admission, a physical examination, including respiratory and heart rate while the child was sleeping or awake but not crying. Body temperature was measured with a Braun ThermoScan Pro 3000 tympanic thermometer. Body weight (in Kg) was recorded to the nearest 10 gm using a beam balance scale weight. Height was measured to the nearest centimetre. The level of consciousness respiratory, cardiovascular and neurological findings were measured. pO_2 was obtained by pulse oximetry (Nonin Medical, Inc MPL, MN USA, model 8500) before the use of oxygen or after discontinuation for 5 minutes if the child was already on oxygen. Oxygen

was reinstated if the pO_2 was <88% and a record of <88% was made. A standard form was used for all patients (Appendix 4).

Investigations

Following the physical examination the following investigations were done:

Chest x-ray

Chest x-ray was done to every child admitted to the hospital. These x rays were read by the hospital's radiologist, the principal investigator and the research assistant doctor independently. The presence of hyperinflation, consolidation, other pulmonary changes or heart changes were recorded in the baseline examination sheet (appendix 4).

Nasopharyngeal aspirates collection

NPA were obtained by direct aspiration via a mucus trap (Maesk Medical A/s) connected to a suction device. Immediately after collection of the NPA, the samples were transferred from the mucus traps into 2 ml cryotubes after addition of a small amount of sterile phosphate buffered saline (PBS) and vortex mixing (1:1 dilution). The samples were stored into two aliquots at 2 - 4°C within 1 hour of collection, then stored at – 70°C to be transported in frozen condition to the UK. Specimens were processed in the Faculty of Medicine, Liverpool University.

Blood sample collection

Two to 3 mls of blood were obtained by venepuncture and the serum was separated and stored in two aliquots then transferred to UK frozen at -70°C for measurement of the serum concentrations of vitamins A, E, zinc, selenium, copper, calcium and C reactive protein (CRP). One of these aliquoted samples were collected in brown tube to protect vitamin A from degradation by light.

Follow up

Admitted patients were reviewed daily until they were discharged according to the hospital staff's decision. Their vital signs, pO_2, difficulty of breathing, cyanosis, chest indrawing, crackles, wheeze, data regarding the need for and duration of intravenous fluids, nasogastric tube feeding, oxygen and mechanical ventilation were noted. The duration of the hospitalization period, death and outcome at discharge were recorded on follow up forms

(appendix 5). Patients received the standard hospital management (Appendix 6). Infants who were seen in the A/E department and discharged home were assessed only on one occasion but were advised to return if their symptoms deteriorated.

Sample size

Sample size for objective 1

The sample size was calculated assuming that the expected prevalence of RSV in children with respiratory symptoms is 70% (Kim et al., 1973), and the lowest expected frequency would be 62% (i.e. ± 8% error). Accordingly, the sample size required is 126 children. The expected prevalence of HMPV in bronchiolitis is unknown and we assumed a prevalence of 50%. A margin of error of 8% would require 150 participants.

The samples size required to establish the prevalence of RSV and HMPV therefore 150 children assuming an 80% power and 95% confidence interval and ± 8% error.

Sample size for objective 2 and 4

The sample size was calculated by obtaining the prevalence of the risk factor in the general childhood population from published studies and reviewing the literature to summarise the OR obtained by previous researches. These odd ratios (OR) and the prevalence in the general population were used to calculate the sample size for risk of prematurity, congenital heart disease, and micronutrients deficiencies. No data were available for some antioxidants (vitamin E and copper deficiencies). Since there are no studies reporting this information, we calculated the minimum OR that the sample size proposed will be able to detect. The relative risk (RR) was used to calculate the sample size required for other risk factors such as age less than 6 weeks, immunodeficiency and belonging to RSV group A or B (Table 2.1).

The sample size was selected with the use of data presented in table 2.1 and 2.2 to obtain 80% power level and a 95% confidence interval, for a significant difference in the prevalence of each risk factor.

Table 2.1 Sample size and the criteria used for its calculation

	Odds Ratio	Population prevalence	Required sample size Case &control (total)	Reference
Prematurity	3.8	12%	64 & 64 (128)	(Wang et al., 1995a)
Congenital heart disease	99.2	10%	7&7 (14)	(Kaneko et al., 2001)
RSV group A	90%	0	94 & 46 (138)	(Lukic-Grlic et al., 1998)
RSV group B	10%	0	94 & 46 (138)	(Lukic-Grlic et al., 1998)
Age less than 6 weeks	*28.7	7.3	<11 & 11 (22)	(Wang et al., 1995a)
Immunodeficiency	*19.1	7.3	<15 &15 (30)	(Wang et al., 1995a)

*RR

Table 2.2 Sample size of antioxidants and the criteria used for its calculation

	Odds Ratio	Population prevalence	Required sample size	Reference
Zinc deficiency	3.0	25%	65 & 65 (130)	(Clements et al., 2000)
Selenium deficiency	3.1	15%	80 & 80 (160)	(Patwari, 1999)

One control will be selected for each case. These assumptions required a sample size of 180 (cases and control). We decided to enrol 200 cases and 200 controls to allow for 10% of dropouts or refusal to participate.

Sample size for objective 3

Assumption for the sample size

The expected mean (SD) IL6, IL7, IL8, IL10 and TNF-α is not known. For this reason we assumed that their expected means (SD) are close to the expected means (SD) of INF-γ, MIP-1α, MCP-1 and RANTES published for children with severe and mild bronchiolitis. These levels are shown in table 2.3.

Table 2.3 Sample size: Cytokine and chemokine Levels

Cytokine/ chemokine	Mean cytokine/ chemokine level in controls	Mean cytokine/ chemokine level in cases	Standard Deviation	Sample size per group	Reference
INF-γ	1.59 pg/ml	1.09 pg/ml	0. 66	28	(Garofalo et al., 2001)
MIP-1α	2.13 pg/ml	2.50 pg/ml	0.59	40	(Garofalo et al., 2001)
MCP-1	1.59 pg/ml	2.0 pg/ml	0.86	41	(Garofalo et al., 2001)
RANTES	1.53 pg/ml	2.01 pg/ml	0.80	44	(Garofalo et al., 2001)

The study will enrol cases and controls with a ratio of 1:1. The chemokine RANTES is the chemokine that requires a larger sample size (44).

A sample size of 44 cases and 44 controls will have 80% power and, 95% confidence interval, will allow establishing a significant difference in the levels of IL6, IL7, IL8, IL10, IL12, TNF-α and IFN-γ. Data from all IL12 studies are represented as medians, which are unhelpful to calculate a sample size. With these assumptions, the sample size will be 44 cases and 44 controls.

Laboratory Investigations

RNA extraction

RNA was extracted from the NPA using the Qiagen Rneasy extraction method (Qiagen LTD, Crawley, UK) following the manufacturer's instructions. This is a novel technology for RNA isolation. It combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the Rneasy silica-gel-system. Nasopharyngeal aspirates were first lysed and homogenized in the presence of a denaturing guanidine isothiocyanate (GITC)-containing buffer (RLT buffer), which immediately inactivates Rneasy to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions and the sample is then applied to an Rneasy mini column where the total RNA binds to the membrane and contaminants are washed away using RW1 buffer which contains ethanol and RPE buffer. Both of these latter solutions are washing buffers. High-quality RNA is then eluted in 50 µl water. The Qiagen RNeasy extraction method required to thaw samples and prepare aliquots of 500µl NPA into 1.5ml eppendorf tubes. RLT buffer made up by adding 100µl of β-ME to

10ml RLT buffer working in the fume hood. Then 350µl of the RLT buffer was added to each tube and vortexed. 350µl of 70% ethanol was aliquoted to the homogenised lysate and mixed by pipetting up and down. The 2ml Qiagen mini columns and tubes supplied in the kit were labelled, 600µl of the sample were added to the mini column and the tube was closed gently and centrifuged at full speed for 15 seconds. The flow through was then discarded into a waste container. The collection tube was blotted onto an absorbent towel and the same step was repeated again. Seven hundred µl of RW1 wash buffer were added to the column, the tube was closed gently and centrifuged at full speed for 15 seconds, and the collection tubes were discarded. The columns were placed into clean collection tubes and 500µl of RPE buffer were pipetted into each column. The tubes were closed and centrifuged at full speed for 15 seconds and the flow through was discarded. 500µl RPE buffer were pipetted to each column and centrifuged at full speed for 2 minutes. The collection tubes were discarded and the columns were placed into clean 2ml collection tubes and centrifuged at full speed for 2 minutes. The columns were placed into clean; labelled 1.5ml collection tubes and the RNA were eluted by adding 50µl of HPLC water directly onto the silica membrane. The tubes were closed gently, incubated at room temperature for 1 minute, and centrifuged at full speed for 1 minute. The extracted RNA was pipetted into clean 0.5ml eppendorf tubes and store at -80°C.

HMPV/RSV Reverse transcription- polymerase chain reaction (RT-PCR)

PCR is a technique for amplifying a specific region of DNA, defined by a set of two "primers" at which DNA synthesis is initiated by a thermostable DNA polymerase. Usually, at least a million-fold increase of a specific section of a DNA molecule can be realised and the PCR product can be detected by gel electrophoresis. The regions amplified are usually between 150-3,000 bases pairs length. PCR was done to amplify parts of the N gene in RSV using specific primers amplifying the N gene between nucleotides 858-1135 giving 278bp product (Cane and Pringle 1991). These were RSV N1 primer 5'GGAACAAGTTGTTGAGGTTTATGAATATGC3' and RSV N2 primer 5'CTTCTGCTGTCAAGTCTAGTACACTGTAGT3' and to amplify parts of the M gene in HMPV using specific primers amplifying the M gene between nucleotides 212-331 giving 121bp product, they were HMPVMF1 5'AAGTGAATGCATCAGCCCAAG3' and HMPVMR1 5'CACAGACTGTGAGTTTGTCAAA3'. The HMPV/RSV RT-PCR mastermix per sample was composed of 19.0µl water, 5.0µl 10x PCR buffer, 6.0µl 25mM $MgCl_2$, 2.5µl 0.1M 1.4-Dithiothreitol (dTT) (Roche Diagnostics, Mannheim, Germany), 2.0µl 10mM dNTP's (Amersham Bioscience Corp., Piscataway, USA), 4.0µl primer mix (primer mix contains equal volumes of all four primers at a concentration of 20µM), 0.5µl RNase Inhibitor

(Promega, Madison, UK), 0.5μl MuLV RT (Applied Biosystems, Crawley, UK), and 0.5μl Amplitaq gold (Applied Biosystems PCR, Crawley, UK). 40μl of the RT-PCR mix was aliquoted into each of the 0.2μl PCR tubes and 10μl of the extracted RNA was added to the appropriate tube and mixed by pipetting up and down. The PCR tubes were then placed in the thermal cycler under the following RT-PCR programme with the following conditions: 1 cycle at 50°C for 30mins, 1 cycle at 94°C for 5mins, 40 cycles at 94°C for 1min and at 55°C for 1min and at 72°C for 1min and 1 cycle at 72°C for 10mins finally soaked under 4°C using a thermal cycler (GeneAmp, PCR System 2400, Perkin Elmer, Warrington, Cheshire, UK). When the programme was completed 8μl of the PCR product were run on a 2% agarose gel and viewed under ultraviolet (UV) light. Detection of a band at 278 bases pairs (bp) was regarded as positive for RSV and a band at 121 bp as positive for HMPV result.

Ten percent of positive M-gene ampilcons of HMPV were confirmed by doing RT-PCR using other primers that amplifying the polymerases protein gene (L). These were L6 CAT GCC CAC TAT AAA AGG TCAG and L7 CAC CCC AGT CTT TCT TGA AA, to confirm the results of the first PCR for HMPV.

Loading the PCR products into a 2% agarose gel

The gel tray was prepared by sealing both ends with tape and inserting a comb. Two gms of Gibco electrophoresis agarose (GIBCO, Paisley, Scotland) mixed to 100 mls of 0.5x TBE (5x Concentrate Tris Borate EDTA (TBE) Buffer (54gm Tris base, 27.5gm orthoboric acid, 20ml 0.5M EDTA pH 8.0) were put in a conical flask and microwaved for approx. 2 mins and left to cool slightly. Then 3μl of ethidium bromide were added, mixed with the agarose, poured into the prepared gel try and left to set at room temperature. The correct number of 0.5ml eppendorf tubes was labelled to correspond to the number of PCR products plus one (for DNA ladder) then 2μl of 10x loading buffer were added to each tube. One hundred or 120 bp DNA ladder (GIBCO, Paisley, Scotland) were diluted 1:5 into the extra tube (1.5μl DNA marker, 8.5μl distilled water to 2μl 10x loading buffer) to give a total volume of 10 μl. Then 10μl of each PCR product were added to the corresponding tube. The comb and tapewere removed from the gel tray and placed into a horizontal electrophoresis tank. The gel was ensured that it was fully immersed in 0.5x TBE buffer. Then the prepared PCR products and the DNA ladder were loaded into the wells and ran at 150 Volts for approximately 1 hour. Then viewed under UV light.

Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. Restriction endonuclease specifically cleaves DNA into different lengths depending on the number and position of recognition sequence. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another.

Restriction Endonucleases

Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme recognition sites are usually 4 to 6 bp in length. Generally, the shorter the recognition sequence, the greater the number of fragments generated. If molecule differs in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. Restriction enzymes are isolated from a wide variety of bacterial genera and are thought to be part of the cell's defences against invading bacterial viruses. These enzymes are named by using the letter of the genus, the first two letters of the species, and the order of discovery.

N gene restriction patterns

RSV positive samples were subjected directly to restriction enzyme digestion to be typed into NP genotypes on the bases of their restriction profiles (Cane and Pringle, 1991). Briefly, the reagent volumes for the number of tests and control plus two (to allow for loss during pipetting) were calculated. The restriction mastermix composed of 1.0µl 10x enzyme buffer, 0.5µl restriction enzyme and 0.5µl HPLC H_2O. The template was labelled and 8µl of PCR product pipetted into a microtitre plate. Then 2µl of each restriction mastermix were added to the appropriate well. The plate was sealed with a cover and mixed thoroughly. The microtitre plate incubated at 37°C (RSV taq enzyme incubation temp. 65°C). Two percent agarose gel prepared. After one hour incubation 2µl of 10x loading buffer was added to each well. Then 10µl of samples plus a molecular marker were loaded onto the gel and electrophorese at 100V for approximately 75mins. Then viewed under UV light. The enzymes that were used for restriction are Restriction Endonuclease Hind III (10U µl), Restriction Endonuclease Pst1 (10U/µl), Restriction Endonuclease Rsa1 (10U/µl), Restriction Endonuclease Bgl1 (10U µl) and Restriction Endonuclease Hae 111 (Roche Diagnostics, Mannheim, Germany). The

buffers supplied by the manufacturer were used. The following table shows the restriction patterns of the N gene.

Table 2.4 NP restriction pattern

	*Hind*III	*Pst*I	*Bgl*II	*Hae*III[1]	*Hae*III[2]	*Rsa*I[1]	*Rsa*2[2]
NP1	-	-	-	-	-	+	-
NP2	-	-	-	+	-	+	-
NP3	-	-	+	-	-	+	-
NP4	-	-	+	+	-	+	-
NP5	+	-	+	+	-	+	-
NP6	-	-	+	-	+	+	-
NP9	+	-	+	-	-	+	-
NP10	-	-	+	+	-	+	+

Source: (Cane and Pringle, 1991)

Purification of RSV /HMPV DNA

Purification of the DNA was done before sending 10% of the positive HMPV or RSV samples by PCR for sequencing to confirm the identity of the virus,. The DNA was purified by running it on a low melting temperature agarose Nusieve ®GTG®agarose (GIBCO, Paisley, Scotland). Then the DNA was extracted from the gel using QIAquick Gel Extraction kit following the manufacturer instructions (Qiagen LTD, Promega, Crawley, UK). QIAquick Gel Extraction kit used for the extraction of DNA fragments (70BP-10KB) from standard or low-melting agarose gels in TAE (Tris-acetate/EDTA) or TBE (Tris-barate / EDTA) buffer and DNA cleanup from enzymatic reactions. It is suitable for fast cleanup of up to 10 µg of DNA fragments. Then 25 µl of purified DNA was sent for sequencing.

Cloning

Selected PCR products were cloned into a TA cloning vector (Qiagen LTD, pGEM-T, Promega, Crawley, UK). Cloning involved the following steps:

a. Transformation of bacteria (Rubidium Chloride method)

The process of transferring exogenous DNA into cells, called "transformation", refers to any application in molecular genetics in which purified DNA is actively imported into host cells using nonviral methods. A technical breakthrough in molecular genetics came in 1970 when a simple DNA transformation protocol was developed that permitted the transfer, and stable inheritance, of exogenous λ bacteriophage and bacterial genomic DNA into E.*coli* cells. Soon afterward, plasmids encoding antibiotic resistance genes were used in DNA transformation

studies, and within 3 years, experiments based on recombinant DNA technology were reported.

Transformation of bacteria using the Rubidium Chloride method is as follows:
Overnight cultures were diluted 1 in 100 Luria-Bertani (LB)-Borth and grown at 37°C, 200 (round per minute) rpm to optic density (OD) 600 of between 0.3-0.8 [2 hours]. Five ml culture were needed for each transformation. Then incubated on ice for 15 minute and kept cold until heat shock, then spanned for 10 minutes (mins) at 4 ° 2000 rpm (j2). The pellet were then resuspended in 20 ml of RF1 per 50 ml of culture and incubated on ice for 15 mins. Then spanned for 10 mins at 4 ° 2000rpm and the pellet resuspended in 2 ml of RF2 per 5 ml culture and incubated on ice for 15 mins. At this stage the bacteria was competent. For each transformation, DNA were mixed with 200 µl of competent cells and left on ice for 1 hour (using falcon 2059 tubes). Then heat shock at 42 ° C for 45 seconds and incubated on ice for 5 mins then 800µl of LB Borth (preheated to 42° C) was added and incubated at 37 ° C for 45mins. The cells were then spread onto LB-agar plates (+antibiotic) and left at 37 ° C overnight. The composition of 500 ml RF1 solution is 6.05 g 100mM Rbcl 2, 1.47g 30mM K acetate, 0.735g 10mM CaCL2, 4.95 g 50mM Mn Cl2 (pH5.8 with acetic acid) and 75 glycerol (15%). The composition of 500ml RF2 is 1.05 g 100mM MOPS, 5.5 g 75mM CaCL2, 0.6g 10mM Rb Cl2 (pH 6.5 with KOH) and 75 glycerol (15%).

b. LA (Luria) plate preparation

400 ml bottle of LA was dissolved in a microwave oven on defrost for a maximum of 20 minutes, then the agar was cooled and 400µl ampicillin (100mg/ml, 1:1000 dilution in LA), 400µl X-gal (substrate) (prepared in dimethyleformamide, 1:1000 dilution -80mg/ml), and 200µl IPTG (inducer) (200mgl.ml 1:2000 dilution) were added. Then the plates were poured in a laminar flow cabinet and left to dry.

C. Ligation (promega pGEM-T Kit cat. No A1360)

The mastermix per sample was prepared from 0.5µl pGEM-T, 0.5 µl T4 ligase, 5 µl 2x buffer, then 7 µl were aliquoted into labeled 0.5 ml eppendorf tubes and 3 µl of the test sample were added. A negative control was made by adding 3 µl HPLC water instead of a sample. The positive control was diluted 1:3 (2 µl of control plus 1 µl of HPLC water). The mixtures stood at room temperature of a minimum of ½ hour. During this time a water bath was set to 42°C and enough LB was incubated. 800µl of the LB were added to all samples. Ten µl of ligation preparation were added after incubation to 14 ml Falcon tubes and store on ice. Two hundred µl of TG2 competent cells were added to the Falcon tubes then mixed and incubated on ice

for 1-1½ hour. (This allows the plasmid to stick to the cells). During this incubation the LA plates were dried in the laminar flow cabinet for approximately 45 minutes. If the plate turns white this indication that the B-galactosidae enzyme is working. If the plate turns blue, this means that the action of this enzyme is not good.

d. Heat shock

The 14 ml Falcon tubes were incubated at 42°C for 45 seconds (1 minute maximum). This allows the cells to take up the plasmid. Then cells were incubated on ice for 5 minutes to allow the cells to recover. Using a clean tip for each sample 800µl of the incubated LB Borth was run down the side of each tube. Then tubes were incubated on 37 °C shaking incubator for 45 minutes. Two LA plates per sample were labeled and 200µl were added to the first and 400µl to the second plates. The plates were spread with sterile glass spreader and incubated overnight at 37°C.

e. PCR screening

Ampicillin was added to LB Borth to give a final concentration of 1 µl/ml (1.e. 100 µl stock solution per 100 ml LB), then 5 ml were aliquoted in sterile universal tubes (3 tubes per sample). Three white colonies were selected for each sample. Sufficient PCR mastermix was prepared to test 3 colonies for each sample plus one extra using the M13 plasmid specific primers (20 µl total volume is sufficient for PCR screening). The PCR mastermix is composed of 15.6 µl, 2.0 µl 10xPEbuffer, 1.2 µl 25mM $MgCl^2$, 0.5 µl dNTP's, 0.5 µl primers, and 0.2 µl Amitaq. 20µl of the mix were aliquoted into labeled 0.2 ml PCR tubes and working on the bench, a small amount of the selected colonies were picked up by touching with a sterile pipette tip and dipped into the 0.2 ml PCR tube, then placed the tip into a labeled universal tube containing 5 ml LB Borth. The M13 programme was run under the following conditions : 1 hold 94°C for 1 minute, then 30 cycles at 94°C for 1 minute, at 55°C for 1 minute, at 72°C for 1 minute, then hold at 72°C for 1 minute then oak at 4°C for 1 minute. Five µl of the PCR product were run on 2% agarose gel. If the product of the right molecular weight was found, the corresponding LB Borth was incubated overnight in the 37°C –shaking incubator.

Plasmid purification

Plasmid purification was done using a Qiagen Spin miniprep Kit (Qiagen LTD, pGEM-T, Promega, Crawley, UK) purifying 1 Borth per sample. Briefly pelleted bacterial cells were resuspended in 250 µl buffer P1 (after addition of RNase to the P1 buffer) and transfered to a micro centrifuge tube. The 250 µl buffer P2 was added and the tube was inverted gently 4-6

times to mix. Three hundred and fifty μl buffer N3 were added and the tube immediately inverted gently 4-6 times. The solution turned cloudy and was centrifuged for 10 minutes at maximum speed in a tabletop microcentrifuge until a compact white pellet was formed. The supernatant was applied to the QIAprep spin column by pipetting and centrifuged for 30-60 seconds and the flow-though was discarded. The QIAprep spin column was washed by adding 0.75 ml of buffer PE and centrifuged for 30-60 seconds. The flow-through discarded and centrifuged for an additional 1 minute to remove any residual wash buffer. The QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 μl of water were added to the centre of each QIAprep spin column, and let to stand for 1 minute then centrifuged for 1 minute. The purified DNA was diluted to 1:200 (5 μl sample + 995 μl HPLC) and the spectrophotometre was used to determine the DNA concentration (260/280 nm). Then 20 μl of purified DNA was sent for sequence to confirm the identity of the virus detected.

Confirmation of HMPV and selected strange N restriction profiles of RSV samples

After RFLP typing of positive RSV, samples were grouped into sets that had identical N and P profiles (Cane and Pringle, 1992). Some samples had strange restriction NP profiles. These samples either purified directly (see above) or were cloned and then purified and send for sequence. Ten percent of the HMPV positive samples were selected and purified or cloned then purified and sent for sequence. Sequence analysis was performed by Lark Technologies Inc, Surrey, UK.

Measurement of serum vitamin A and E levels

The High Performance Liquid Chromatography (HLPC) method was used by the researcher to measure serum vitamins A, E levels. In brief first extraction was done as follows: 200 μl serum, 40 μl retinol acetate (Internal standard) and 150μl ethanol were put into micro-centrifuge tube and briefly vortexed. Then 1 ml hexane was added and vortexed for 2-3 minutes. The tube is spun for 2 minutes at low rpm (x1000), and the hexane layer is separated and put into fresh centrifuge for evaporation. A second 1 ml of hexane was added to the microcentrifuge tube for a further extraction and the process repeated. The second hexane fraction was pooled with the first and then evaporated to dryness under N_2.

The dried residue was reconstituted with 100 μl ethanol to be ready for injection. Ultra spectrophotometer 2000 (Amersham Bioscience UK LTD, Pollards, Nightingales lane Chalfont St. Giles Bucks HP8 45P, UK) was used to analyze and read the concentrations of vitamin A and E.

Table 2.5 The standard line for retinol and tocopherol assay

Std No	Vitamin A		Vitamin E	
	µg/L	µM/L	µg/L	µ/L
V	50	0.174	1000	2.32
W	100	0.348	2000	4.64
X	200	0.696	4000	9.28
Y	400	1.392	8000	18.56
Z	800	2.784	16000	37.12

Concentration of working solutions

Retinol	- 5 µg/L	Abs.325nm – 0.923 A.U
Tochopherol	- 200 µg/L	
Retinol acetate	- 2 µg/L	Abs.325nm -0.310 A.U.

Measurement of serum zinc, selenium and copper levels

The Inductive Coupled Plasma Mass Spectrometry (ICPMS) was used to measure zinc and selenium and copper.

Simultaneous measurement of serum copper, selenium and zinc using ICPMS

Instrumentation PQ ExCell (Thermo Elemental, Ion Path, Road Three, Winsford, Cheshire.)

Method Specimens were diluted 1:60 with diluent (containing internal standard) (1 in 61) and then aspirated (1.0ml/minute) into the instrument, utilising a CETAC ASX-500 sample changer. Gallium and Indium were used as internal standards.

Calibration 3 point calibration,

0 calibrant (diluent) = Cu 0 µmol/l
Se 0 µmol/l
Zn 0 µmol/l

Mid point calibrant = Cu 23.6µmol/l
Se 1.90µmol/l
Zn 22.95µmol/l

Hi point calibrant = Cu 47.2µmol/l
Se 3.80µmol/l
Zn 45.9µmol/l

Diluent Ultra pure water with added (AR grade)
Conc. Nitric acid (10ml/l)

Propan-2-ol (2ml/l)
Butan-1-ol (2ml/l)
Triton X-100 (to give a final concentration of 0.005%w/v).
ICPMS grade Gallium and Indium to give a final concentration of 10ppb.

Sample preparation/Run procedure

100µl of Calibrant/control/ sample was diluted with 6.0 ml of diluent. The calibrants were prepared as 4 of each level. Controls and samples were prepared in duplicate; calibrants were run as tests after every twenty two aspirations as a drift check. Controls were run at the start and finish of the specimen run.

Results Results were collected for Copper (63 and 65), Selenium (77 and 82) and Zinc (66 and 68). This was to check for possible interferences. The final results were taken as the copper 65, selenium 82 and zinc 66 values (if no interference was indicated).

Quality control

	Material	Assigned Value	Range
Copper			
	seronorm	19.0µmol/l	16.0-21.0
	UTAK Normal	20.0µmol/l	15.0-25.0
	UTAK Elevated	47.0µmol/l	36.0-56.0
Selenium			
	seronorm	1.05µmol/l	0.85-1.25
	UTAK Normal	1.60µmol/l	1.20-2.00
	UTAK Elevated	4.00µmol/l	3.20-4.80
Zinc			
	seronorm	20.0µmol/l	16.0-24.0
	UTAK Normal	17.0µmol/l	13.0-21.0
	UTAK Elevated	37.0µmol/l	30.0-44.0

CRPLX-Tinaquant C-Reactive Protein (Latex)

CRP assays were measured on the Roche Modular Analyser using the Roche CRPLX kit (Roche UK Ltd, Bell Lane, Lewes, East Sussex BN7 1LG). This assay is based on the

principle of particle-enhanced immunoturbidimetric methodology intended for use on automated clinical chemistry analysers. Buffer is added to the sample, then mixed, anti-CRP antibody-latex is then added to start the reaction. Anti-CRP antibodies coupled to latex microparticles react with the antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. Within batch precision is 3%, between batch precision is 5%.

ELISA test for identification of the cytokines and chemokines

The DuoSet ELISA kits (DuoSet ELISA development system) (R&D systems Europe, UK) was used for identification of the cytokines (IL6, TNF-α, IL7, IL10, IL12 and INF-γ) and the chemokines (IL8 and RANTES).

Preparation of specimens

Each sample of the NPA positive for RSV, HMPV or RSV/HMPV was diluted 1:1 in PBS and centrifuged for 5 minutes, then the supernatant was aspirated in small tubes and stored in -70C freezer.

Solutions required

PBS	137mM Nacl, 2.7mM Kcl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2- 7.4, and 0.2μm filtered.
Wash Buffer	0.05% Tween ® 20 in PBS, pH 7.2-7.4.
Block Buffer	1% BSA, 5% Sucrose in PBS with 0.05% NaN3.
Substrate Solution	1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbezidine) (R&D systems Cat DY999).
Stop solution	2N H2SO4
Reagent Diluent	Two types of Reagent Diluents were used:
Reagent Diluent - A	0.1% BSA. 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150mM NaCl), pH 7.2-7.4, 0.2 μm filtered. Used for measuring IL6, IL8, INF-γ
Reagent Diluent - B	0.1% BSA.in PBS, pH 7.2-7.4, 0.2 μm filtered. Used for measuring IL7, IL10, TNF-α and RANTES.

Each EISA kit composed of Capture antibody vial, Detection antibody vial, Standard vial, and Streptavidin-HRP vial.

Tables 2.6 and 2.7 identified the number of the ELISA kit and the composition of the Capture antibody, Detection antibody and the Standard that used for each cytokine and chemokine concentrations.

Streptavidin-HRP composition

1.0 ml of streptavidin conjugated to horseradish-peroxidase and stored at 2-8 C for up to 6 months (never to be frozen). Then dilute to the working concentration using Reagent Diluent.

General ELISA protocol

Plate preparation

1. The Capture Antibody was diluted to the working concentration in BPS without protein carrier and immediately coating a 96-well microplate with 100 µl per well of the diluted Capture Antibody. The plate was sealed and incubated overnight at room temperature.
2. Each well was aspirated and washed with Wash Buffer, repeating the process three times. The washing by filling each well with Wash Buffer (400µl) using autowasher machine (Opsys MW, Dynex Technologies), complete removal of liquid at each step was assured. After the last wash, any remaining Wash Buffer removed by aspiration using multi-channel pipette and then by inverting the plate and blotting it against clean paper towels.
3. The plate blocked by adding 300 µl Block Buffer to each well. Then the plate incubated at room temperature for a minimum of 1 hour.
4. The aspiration/wash repeated as in step 2. Then plates were now ready for sample addition.

Assay Procedure

1. 100µl of sample or Standard in Reagent Diluents were added per well.The plate was covered with adhesive strip and incubated for 2 hours at room temperature.
2. The aspiration/wash repeated as in step 2 of plate preparation.
3. 100µl of the Detection Antibody, diluted in Reagent Diluents were added to each well. The plate then was covered with new adhesive strip and incubated for 2 hours at room temperature.
4. The aspiration/wash repeated as in step 2 of plate preparation.
5. 100µl of working dilution of streptavidin-HRP were added to each well. The plate then was covered and incubated for 20 minutes at room temperature avoiding placing the plate in direct light.

6. The aspiration/wash repeated as in step 2 of plate preparation
7. 100µl of Substrate Solution were added to each well. The plate then was covered and incubated for 20 minutes at room temperature avoiding placing the plate in direct light.
8. 50 µl of Stop Solution were added to each well. Gently the plate was tapped to ensure thorough mixing.
9. The optical density of each well was determined immediately using a microplate reader set to 540 nm (Opsys MRT, Revalation Quick Link. Version 4.24, Thermolab system, Thermo Electronic business)

Table 2.6 Components of the ELISA kits for each cytokine and chemokine-A

Cytokine/chemokine	No of the Kit	Capture antibody	Detection Antibody
IL6	Dy 206	360 µg/ml of mouse anti-human IL6 reconstituted with 1.0 ml PBS with working dilution of 2 µg/ml in PBS	36 µg/ml of biotiylated goat anti – human IL6 reconstituted with 1.0 ml RD. with working concentration of 200 µg/ml in RD.
TNF-α	Dy 210	720 µg/ml of mouse anti-human TNF-α reconstituted with 1.0 ml PBS with working dilution of 4 µg/ml in PBS	54 µg/ml of biotiylated goat anti – human TNF-α reconstituted with 1.0 ml RD. with working concentration of 300 µg/ml in RD.
IL7	Dy 207	360 µg/ml of mouse anti-human IL7 reconstituted with 1.0 ml PBS with working dilution of 2 µg/ml in PBS	9 µg/ml of biotiylated goat anti – human IL7 reconstituted with 1.0 ml RD. with working concentration of 50 µg/ml in RD.
IL10	Dy 217	720 µg/ml of mouse anti-human IL10 reconstituted with 1.0 ml PBS with working dilution of 4 µg/ml in PBS	108 µg/ml of biotiylated goat anti – human IL10 reconstituted with 1.0 ml RD. with working concentration of 400 µg/ml in RD.
IL12	Dy 270	720 µg/ml of mouse anti-human IL12 p70 reconstituted with 1.0 ml PBS with working dilution of 4 µg/ml in PBS	54 µg/ml of biotiylated goat anti – human IL12 reconstituted with 1.0 ml RD. with working concentration of 4 µg/ml in RD.
INF-γ	Dy 285	720 µg/ml of mouse anti-human INF-γ reconstituted with 1.0 ml PBS with working dilution of 4 µg/ml in PBS	18 µg/ml of biotiylated goat anti – human INF-γ reconstituted with 1.0 ml RD. with working concentration of 400 µg/ml in RD.
IL8	Dy 208	720 µg/ml of mouse anti-human IL8 reconstituted with 1.0 ml PBS with working dilution of 4 µg/ml in PBS	3.6 µg/ml of biotiylated goat anti – human IL8 reconstituted with 1.0 ml RD. with working concentration of 20 µg/ml in R D.
RANTES	Dy 278	360 µg/ml of mouse anti-human RANTES reconstituted with 1.0 ml PBS with working dilution of 2 µg/ml in PBS	3.6 µg/ml of biotiylated goat anti – human RANTES reconstituted with 1.0 ml RD. with working concentration of 400 µg/ml in RD.

RD = Reagent Diluent

Table 2.7 Components of the ELISA kits for each cytokine and chemokine-B

Cytokine/chemokine	No of the Kit	Standard
IL6	Dy 206	30 ng/ml of recombinant human IL6 reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 300 ng/ml
TNF-α	Dy 210	310 ng/ml of recombinant human TNF-α reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 1000 ng/ml
IL7	Dy 207	70 ng/ml of recombinant human IL7 reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 500 ng/ml
IL10	Dy 217	70 ng/ml of recombinant human IL10 reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 4000 ng/ml
IL12	Dy 270	70 ng/ml of recombinant human IL12 p70 reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 2000 ng/ml
INF-γ	Dy 285	30 ng/ml of recombinant human INF-γ reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 1000 ng/ml
IL8	Dy 208	90 ng/ml of recombinant human IL8 reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 2000 ng/ml
RANTES	Dy 278	70 ng/ml of recombinant human RANTES reconstituted with 0.5 ml RD
		A 7 point SDT curve using 2 fold serial dilutions in RD and a high SDT of 300 ng/ml

DW= distilled water; SDT= standard, RD = Reagent Diluent

Ethical issues

This study approved by the Ethical Committee of Liverpool School of Tropical Medicine, and the Ethical Committee of Al-Sabeen Hospital for Women and Children.

Informed Consent

The patients were enrolled after obtaining signed written informed consent from the parents/legal guardian. An informed consent form (appendix 3) indicating the general purpose and procedures of study were read out to the parent/guardian of the child by the investigator. The researcher ascertained that the parent/ guardian understood the information and answered any questions or enquiry before obtaining their signature.

Analysis

Data were entered into computer database and analysed by Epi-info 2002. Analysis of categoral data was performed with X^2 test for significance tests. A student's two-tailed independent t-test was used to compare means of normally distributed data.

Risk factors that make the child susceptible to develop severe disease were likely to be associated with each other. For this reason, logistic regression was used to identify variants that were confounders and adjusted odd ratios were obtained. To achieve this, all the variables with a p value <0.20 were recoded and the Epi info REGRESS program was used.

Geometrical means of log-transformed data were calculated for data positively skewed. Non-parametric tests (Mann-Whitney's test) were used if the variance of the mean was statistically different by the Barlett's test. A value of <0.05 was considered statistically significant. However, to adjust for the effects of multiple comparisons, Bonferroni corrections were made within each analysis carried out. Thus, if n comparisons were made for a particular variable, the significance level for each individual comparison was set at 0.05/n (e.g. for 3 comparisons, significance was set at 0.05/3=0.02). Differences that were significant at a level between the Bonferroni adjusted value and 0.05 were nevertheless still considered to be important. Corrections were kept to a minimum, as suggested by (Perneger, 1998).

Chapter 3
RSV and HMPV in children with respiratory infections

Literature review

Respiratory syncytial virus

RSV is the single most important cause of viral lower respiratory tract infections in infants during infancy and early childhood world-wide. It causes annual epidemics together with repeated infection of individuals. Ninety percent of infants and young children are infected by 24 months of age (Holberg et al., 1991). Peak rates of infection occur in infants aged 6 weeks to 6 months but are most prevalent in those under 3 months of age (Simoes, 1999). Severe RSV disease as measured by the requirement to hospitalisation, is most common among infants aged 1-3 months (Henderson et al., 1979). Re-infection with RSV occurs regularly throughout life although infants are unlikely to get recurrent bronchiolitis (Hall, 1999).

Classification

RSV is a single-stranded nonsegmented negative-sense RNA virus; it is a member of the order (Superfamily) *Mononegavirales.* RSV is the type species for the genus *Pneumovirus* in the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (Collins et al., 1999).

Historical Background

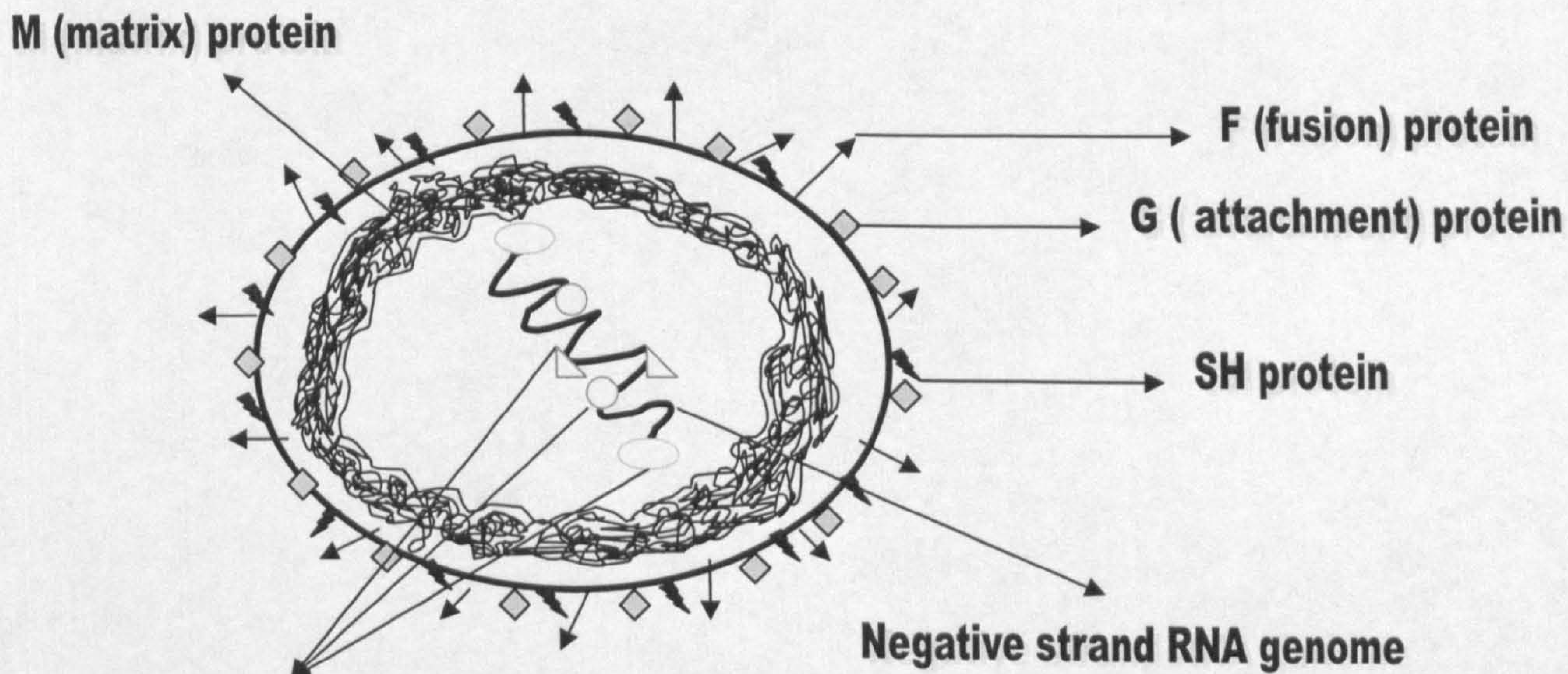
In 1956 Blount et al discovered RSV as a cause of respiratory disease in chimpanzees (Blount et al., 1956) and soon thereafter it was identified as a cause of respiratory disease in human infants. One isolate, from a 17-month-old boy (Long) with pneumonia in Maryland, USA, was selected as the representative or prototype strain (Chanock and Finberg, 1957) and the virus was named according to the syncytial cytopathogenic effect it caused in tissue culture (Chanock et al., 1961). Antigenic differences were soon noted. The CH-18537 strain (presently group B prototype) isolated in Washington, D.C. in 1962 was shown to be different from the Long strain by cross-neutralisation test (Coates et al., 1963).

RSV Structure

RSV is a medium sized enveloped RNA paramyxovirus. Its virion is about 200 nm in size and consists of a nucleocapsid within a lipid envelope. The nucleocapsid is a symmetrical helix with a

helical diameter of 12-15 nm. The lipid bilayer is derived from the host cell plasma membrane and contains virally encoded transmembrane surface glycoproteins which are 11-20 nm in size and closely spaced at interval of 6-10 nm (Hacking and Hull, 2002) (Figure 3.1).

Figure 3.1 Respiratory syncytial viral structure



Source: (Hacking and Hull, 2002)

The RSV genome is 15,222 nucleotides in length (in the case of strain A2). The RSV genome is transcribed into 10 subgenomic messengers RNA (mRNA) transcripts, encoding 11 proteins (Collins et al., 1999). Figure 3.2 show the genomic map of RSV and table 3.1 shows its structural proteins.

Figure 3.2 Genetic map of RSV

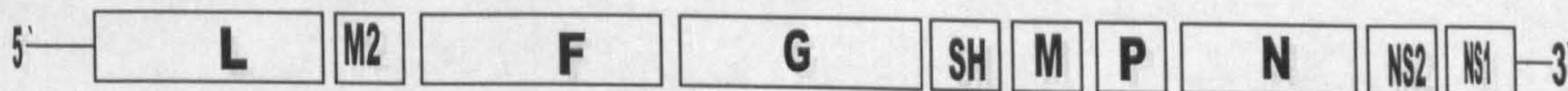


Table 3.1 Structure of RSV

	Gene	Nucleotide location*[1]	Nucleotide	Amino acids	Type of protein	Function	Reference
NS1	Non – structural protein	99... 518	532	139	Non-structural protein	Putative negative regulatory factor for replication and transcription	(Atreya et al., 1998)
NS2	Non-structural protein	628.. 1002	503	124	Non-structural protein	Unknown	(Collins et al., 1999)
N	Nucleo-protein	1140.. 2315	1203	391	Nucleocapsid associated	Binds tightly to genomic and antigenomic RNA	(Collins et al., 1999)
P	Phospho-protein	2348.. 3073	914	241	Nucleocapsid associated	Maintains free N and L proteins in soluble form. Polymerase cofactor.	(Collins et al., 1999)
M	Matrix	3233.. 4003	958	256	Non-glycosylated	Mediate virion assembly	(Collins et al., 1999)
SH	Small hydrophobic	4274.. 4468	410	64*[2]	glycoprotein	Stabilization of the viral envelope or viral encoded virulence factor	(Chen et al., 2000)
G	Attachment protein	4659.. 5555	923	298*[3]	glycoprotein	Mediate viral attachment	(Collins et al., 1999)
F	fusion	5632.. 7356	1903	574	glycoprotein	Mediate viral fusion	(Collins et al., 1999)
(M2)*[4]	Nucleocapsid	7576.. 8430	961		Nucleocapsid associated		
M2-1		7576.. 8181	594	194		Transcription elongation	(Collins et al., 1999)
M2-2		8156.. 8430	270	83-90		Putative negative regulatory factor for replication and transcription	(Bermingham and Collins, 1999)
L	Polymerase	8468.. 14965	6578	2165	Nucleocapsid associated	Major polymerase subunit	(Collins et al., 1999)

*[1]; nucleotide positions correspond to A2 prototype. There are intergenetic regions between each gene. *[2]; 64 in group A or 65 in group B. *[3]; the lengths in some strains are between 289 and 297. *[4]; M2 is divided into M2-1 and M2-2

Four are nucleocapsid proteins, the nucleoprotein (N), a phosphoprotein (P), the polymerase (L), and M2-1. The N protein binds tightly along the entire length of the genomic RNA. P is thought to associate with free N and L to maintain them in soluble form. L is the major polymerase subunit. The N, P and L proteins form the minimum unit for RNA replication. RNA replication involves the synthesis of a positive-sense, exact-copy, encapsidated, replicative intermediate called the antigenome, which serves, in turn as the template for progeny genome. Replication needs in addition M2-1, which is transcription antitermination protein, being involved in transcription as an elongation factor. As in other nonsegmented negative-stranded RNA viruses, the RSV proteins are encoded by individual subgenomic mRNAs which are synthesised by sequential stop-start transcription guided by the gene-start and gene-end motif, the regulatory elements for viral RNA-dependent RNA polymerase (Wertz and Moudy, 2004). M2 mRNA contains two overlapping translational open reading frames (ORFs), which each express a protein. The one in the upstream region expresses the M2-1 protein. The other downstream region encodes the M2-2 protein, which mediates a regulatory switch from transcription to RNA replication (Wertz and Moudy, 2004). The matrix protein (M) plays a major role in virion assembly. It is thought to mediate interaction between the nuceocapsid and envelope proteins during morphogenesis (Schmidt et al., 2001).

There are two non-structural proteins, NS1 and NS2, whose functions are unknown, although NS1 appear to be a negative regulatory factor for RNA synthesis (Atreya et al., 1998). RSV encodes three surface envelope proteins, the small hydrophobic protein (SH), the fusion protein (F), and the attachment (G) protein. They assemble separately into homo-oligomers that make up the membrane spikes; F and G assemble into either trimers or tetramers, and SH might be a pentamer (Kochva et al., 2003). The F glycoprotein mediates membrane fusion, which is responsible for viral penetration and syncytium formation. The G glycoprotein mediates viral attachment. The SH protein is associated with stabilisation of the viral envelope or is a viral encoded virulence factor (Chen et al., 2000).

Attachment and entry into the cell

The first critical step in the RSV infection process is entry of the virus into the cell. Thereafter, the virus replicates is packaged and leaves the cell, either through fusion with adjacent cells or following cell rupture.

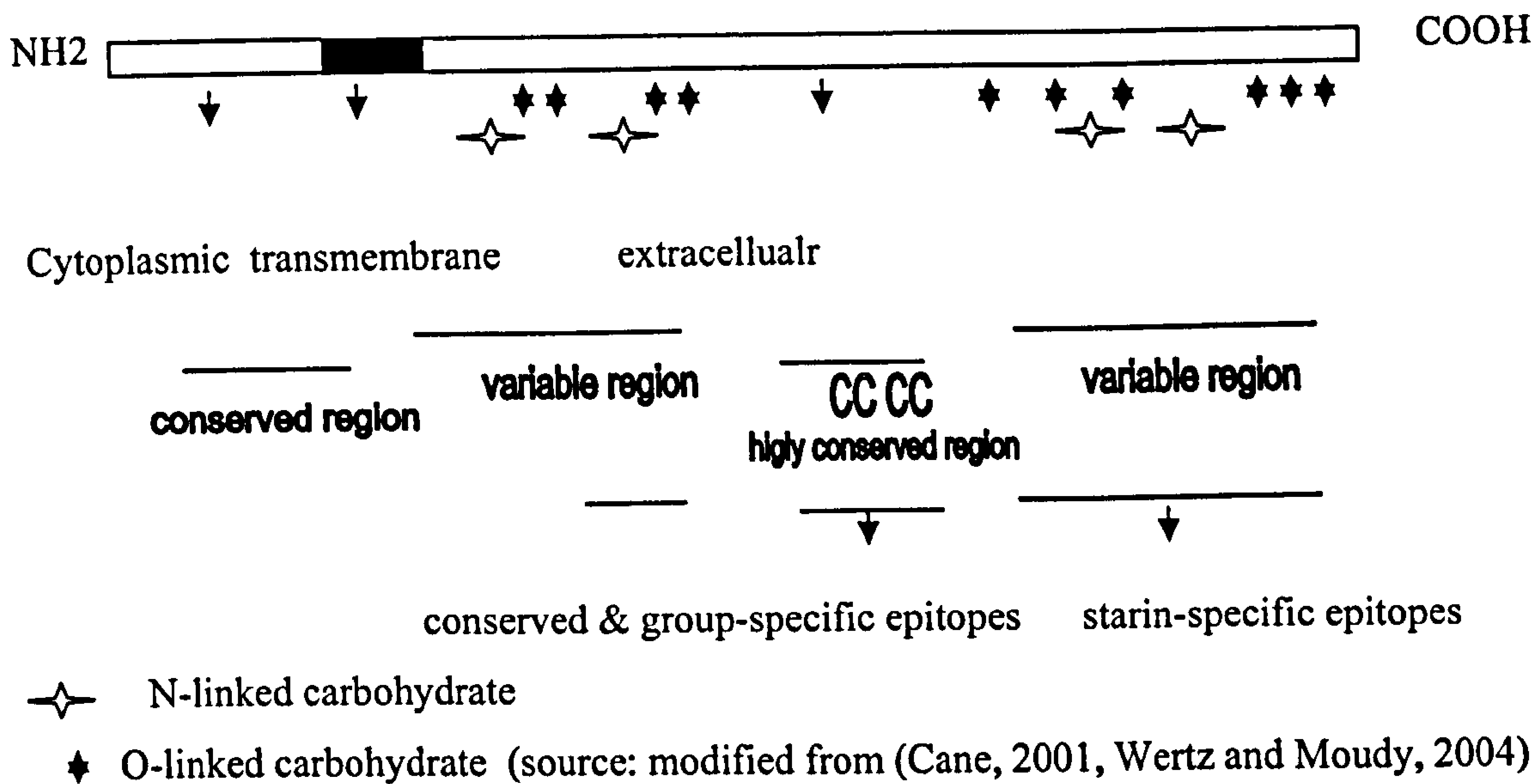
The attachment G gene and proteins

Sequencing studies have shown that the G gene and protein is the most variable protein between the groups of RSV, with only 53% aminoacid identity between the G proteins of the A and B prototype strains ((Johnson et al., 1987). However, within the groups there appear to be much greater levels of similarity, with 94% aminoacid identity reported between group A strains (Johnson et al., 1987) and 98% amino acid identity between group B strains (Sullender et al., 1990). It has unusual structural immunological features and is a major target for the major neutralising and protective antibody response. Despite this variability, the ectodomain of the G protein has a highly conserved 50 amino-acid central region, including four cysteine residues which are conserved in all isolates, flanked by two regions that show a high level of sequence variation (Cane, 2001). The central conserved region is slightly hydrophobic and has been proposed as the putative host receptor site for binding (Johnson et al., 1987). The G protein is a glycosylated type II transmembrane protein of 289-299 aminoacids depending on the viral strain. It consists of three regions an intracellular domain or cytoplasmic tail (aminoacid residues 1-37 near the N-terminus), a transmembrane domain (residue 38-66) and a large extracellular domain or ectodomain (residues 67 to the end) in the C terminal domain. The transmembrane domain is a single hydrophobic domain, which serves as both signal sequence and transmembrane anchor. The ectodomain is composed of two highly glycosylated mucin-like variable regions that sandwich a conserved region (residues 143-197) as shown in figure 3.3. The ectodomain has been found to be formed at times as non – virion- associated (soluble) forms of the G protein. The ectodomain contains three to eight potential sites for N- glycosylation, joined to asparagines, depending on the viral strain, and 25-70 potential sites for O-glycosylation, joined to serine or thereonine residues, which comprise 30% of the amino acid residues on the RSV G protein (Wertz et al., 1985).

Many of the epitopes recognised by the host antibody response lie in the C terminal variable region. The virus may have the potential of changing the antigenic profile of this region of the G protein in order to evade the host response (Cane, 2001). N-glycosylation that masks the epitopes could be another means of evading immune recognition (Cane, 1997). In addition to the membrane bound G protein an N-terminal truncated soluble and secreted form is produced. It was suggested that the soluble G protein served to redirect the immune response away from infected cells and released virus (Cane, 2001). Using murine monoclonal antibodies, three types of epitopes were identified: 1) conserved epitopes that are present in all isolates; 2) group-specific

epitopes that are shared by viruses of group A or B; and 3) strain-specific or variable epitopes that are present in some isolates only (Figure 3.3) (Cane, 2001).

Figure 3.3 Structural features and antigenic domins of RSV G protein



N-linked carbohydrate

O-linked carbohydrate (source: modified from (Cane, 2001, Wertz and Moudy, 2004)

Some studies have shown that RSV strains lacking the membrane bound G protein but expressing the soluble G protein had efficient replication *in vitro* and were only slightly attenuated in vivo (Teng et al., 2001). This may mean that the soluble form can remain associated with the virus in order to mediate cell entry. Surprisingly, recombinant RSV in which the secreted and membrane bound G protein have both been deleted are still able to infect some cell types as efficiently as wild type virus. This mutant form of the virus has a much lower efficiency of infection of human airway cells and the airways of mice in vivo (Teng et al., 2001, Karron et al., 1997). This indicates that the G protein is not essential for cell attachment, but acts as an accessory protein which increases the efficiency of the process (Teng and Collins, 1998).

The fusion protein (F)

The F protein is a type 1 transmembrane glycoprotein with a cleaved N-terminal signal sequence and a transmembrane anchor near the C-terminus. Its inactivated form, F^{0} comprises 574 aa and has trimeric coiled-coiled structure, similar to other viral fusion proteins. After synthesis and modification by the addition of N-linked sugars, it is activated and cleaved into two subunits, F^{1} and F^{2} that are linked by disulphide bonds (Meanwell and Krystal, 2000). The F protein promotes both fusion of viral and cell membranes resulting in the transfer of viral genetic material, and

fusion of infected and adjacent cell membranes causing the formation of syncytia (Domachowske and Rosenberg, 1999). These syncytia are the hallmark of the RSV cytopathic effect and are necessary for cell-to-cell viral transmission. Syncytia formation may be associated with the expression of cytokeratin-17 by RSV-infected respiratory epithelial cells. Cytokeratins are among the major components of the filament networks that make up the cytoskeleton. Cytokeratin-17 expression in RSV infection is neutralised by anti-RSV F protein antibody (Domachowske et al., 2000).

The small hydrophobic protein (SH)

It comprises of 64 aminoacids. Its precise role is unknown at the moment. It is a channel-like homopentamer whose transmembrane pore diameter is 1.46 °A and it may act as ion channel (Kochva et al., 2003). SH is not required for viral replication or syncytium formation although it does facilitate fusion (Domachowske and Rosenberg, 1999). Recombinant RSV without the SH gene, when inoculated intranasally into mice, is indistinguishable from wild-type with regard to replication in the lower respiratory tract, but replication is restricted 10-fold in the upper respiratory tract (Bukreyev et al., 1997). This site-specific attenuation of viral replication may have implications for future vaccine development. The SH protein exists in at least four different forms in infected cells (Olmsted and Collins, 1989). Both B and T epitopes have been identified in the SH protein and it may be involved in the immune response (Nicholas et al., 1988).

RSV antigenic and genetic diversity

RSV was initially thought to be a monotypic virus until 1985 when it was shown that isolates can be divided into two antigenic groups and each group had genotypes or lineages. The variability of human RSV isolates can be examined both at the antigenic level using monoclonocal antibodies and at the genetic level using RT-PCR and RFLP, RNase A mismatch cleavage and nucleotide sequencing.

Antigenic variation

RSV isolates can be divided into two antigenic groups, A and B, on the basis of their reaction with panels of monoclonal antibodies particularly that are directed against G and F proteins (Anderson et al., 1985, Mufson et al., 1985). Reactivity varies among viruses within individual groups. Then nucleotide sequencing showed that these antigenic groups are consistently distinct entities (Cane et al., 1991).

The degree of sequence identity between strains of group A and B has been determined for most genes: inferred aminoacids identity ranged from 53% for the G protein to 96% for the N protein (Johnson et al., 1987).

Genetic variations

Isolates of groups A and B of RSV can be further subdivided into distinct genotypes. Genetic variability and evidence of intragroup strains has been demonstrated by many studies. Initially by ribonuclease protection (RNase) assays (Storch et al., 1989), then by analysing restriction patterns of PCR-amplified cDNA, and nucleotide sequences (Cane and Pringle, 1991) showing the intragroup genetic variability of group A and B. By sequencing, the G protein showed up to 20% amino acid variability for group A isolates and 9% for group B isolates (Cane and Pringle, 1991, Sullender et al., 1991).

Monoclonal antibodies and ribonuclease protection have suggested that there may heterogeneity within group A RSV. This heterogeneity was particularly evident in testing with G – protein monoclonal antibodies, in which some monoclonal antibodies showed a strain –specific reactivity (Garcia-Barreno et al., 1989). In addition, isolates from Cuba (Valdivia et al., 1997), the Gambia (Cane et al., 1991) and South Africa (Nusgen, 2000) were used to demonstrate the genetic variability of group A. Sullender et al. studied isolates from Sweden and described the genetic variability of group B (Sullender et al., 1990). Cane and Pringle showed that restriction mapping of N gene products also revealed intragroup genetic variability (Cane and Pringle, 1991). Discrete genetic lineages of the viruses were observed in a single epidemic period. They found that the N gene can be used to divide RSV into genotypes nominated as NP1-8 based on different restriction patterns of the 278 base pair (bp) PCR product between nucleotide 858 and 1135 from the N gene using five restriction enzymes (Cane and Pringle, 1991). NP1, NP3, NP6 pattern are classified as group B and NP2, NP4 and NP5 as group A. NP7 and NP8 have been defined for bovine RSV and pneumonia virus of mice (PVM) respectively. Furthermore, using SH gene sequence analysis particularly from the 5` non-coding regions group A strain of RSV were subdivided into a number of genotypes, which have been nominated SH1-6 (Cane and Pringle, 1991). Cane et al. analysed the G – protein gene of group A RSV using nucleotide sequence determination and they found differences of up to 20% among the group A RSV G proteins (Cane et al., 1991). Subsequently, Cane and Pringle proposed a classification of the RSV group A genotype based on restriction mappings of N and nucleotide sequencing of part of the SH and G genes as the

restriction pattern and sequencing of the G gene gave concordant results to these classification (Cane and Pringle, 1992). Later, with some isolates from The Gambia, (Cane et al., 1999) noted that there was discordance between the N and G gene variability. Nusgen and Venter et al. also noted discordance between them for isolates from South Africa (Venter et al., 2001, Nusgen, 2000).

Epidemiology of RSV

The epidemiological pattern of RSV is well delineated as a predictable, regular outbreak occurring each year with peak rates of infection occurring annually in the cold season in temperate climates and in the rainy season, as temperature falls, in tropical climates. The duration of the outbreaks may vary from year to year but averages 22 weeks (CDC, 1997). The situation of islands and areas of the inner tropics with perennial high rainfall is less clear-cut.

Weber et al. reviewed 36 studies from the developing world and observed that the age group mainly affected by RSV in developing countries was in children under 6 months of age (mean 39% of hospital patient with RSV). RSV-ALRI is slightly more common in boys than in girls and very scanty information is available about the mortality of children infected with RSV and the frequency of bacterial co-infection in the developing world (Weber et al., 1998b).

Incidence and prevalence of RSV in industrialised area

RSV remains an important cause of morbidity in industrialised countries. In the United Kingdom about 1-2% of infants infected with RSV require admission to hospital. In the USA it is an important cause of hospitalisation with approximately 100,000 infants hospitalised every year at a cost of at least 300 million dollars per year (Hall, 1999). In Germany 12% of children hospitalised for ARI have RSV (Weigl et al., 2001) and in Italy RSV was found in 50% of children hospitalised with ARI (Gerna et al., 2005). In Denmark the incidence of RSV infection requiring hospitalisation among infants < 6 months of the age is 34/1000/season (Kristensen et al., 1998). In the developed world about 2% of infants and children admitted to hospital with RSV infection require assisted ventilation (Behrendt et al., 1998). The estimated annual incidence of RSV infection requiring hospitalisation in Hong Kong is 2.5/1000 children <5 years old with a mortality of 0.15% among hospitalised children and on average, 248 children are admitted each year (Chan et al., 1999).

Incidence and prevalence of RSV in non-industrialised areas

Little information is available about the role of RSV infection in the developing world. In a review by (Vardas et al., 1999) of published hospital-based studies about the incidence and outcome of RSV infection in South African children, the incidence varied from 3% to 18% and the mortality rates varied between 12% and 43%. In Sudan, however the mortality rate was lower (2%) and the incidence was 28% of hospitalised children (Salih et al., 1994). In the Gambia Weber et al (2002) did surveillance for RSV in the western region of the Gambia for the period from 1993 to 1997. They started by hospital surveillance in the only three hospitals in the western region of the Gambia, where any child < 2 years old admitted with symptoms or signs of ARI was screened for RSV disease and NPA samples were taken for investigations. Then community surveillance was started by visiting the compound of the admitted case (index case) within one week and screening for ARI in any child < 5 years old was done if there was, then NPA and serum samples were taken in the initial visit and on a final visit six weeks later. During 1993, only the compound in which the index case lived was investigated in this way. During 1994, a neighbouring compound was investigated in addition to the index cases's compound, to obtain information about spread of RSV between compounds. RSV was detected by immunofluorescence. Serum samples were assessed for neutralizing antibodies against RSV with a micronutralization assay against group A RSV (Tracy strain) and B (strain 18537). Incidence rates were calculated as the number of hospital admissions per year divided by an estimate of the population at risk. Between 1994 and 1996, the observed incidence rate for ALRI in 100 children younger than one year living close to hospital was 9.6 cases per year; for severe RSV associated respiratory illness 0.83; and for hypoxaemic RSV associated respiratory illness, 0.089. Overall, 18.7% of children with ALRI from who nasopharyngeal aspirates were taken, 47% of cases tested positive for RSV. The incidence rate was significantly higher in males, younger children, and children from rural settlements ($p<0.05$). Generally, 41% of children younger than five years in compounds in which cases lived and 42% in control compounds had evidence of RSV infection during the surveillance period (Weber et al., 2002). An epidemiological study from Zambia during January to December 1996 was conducted. Patients were recruited from two urban health centres in Lusaka. Throat swabs and nasal washings were collected from 2424 and 736 children with ARI respectively; RSV was detected by enzyme immunoassay (EIA) and virus isolation. It was isolated from 62 (4.1%) out of 1496 specimens collected from March to September and the highest isolation rate was 8.1% in June 1996. RSV antigen was detected in 99 (16.3%) out of 609 nasal washings collected from March to November 1996. The RSV antigen positivity rate by EIA

was 13.5% and the highest monthly rate was 30.5% in June. The lowest RSV antibody positivity rate was at 1 year of age and it increased with age. The antibody positivity rate was 60-80% among children > 4 years (Saijo et al., 1998).

The incidence of hospitalization due to RSV was found to be 26.9% and 25.3% in two studies from Argentina (Carballal et al., 2000, Carballal et al., 2001); 21% and 17.4% in two studies from Brazil (Straliotto et al., 2001, Moura et al., 2003), 22.1% and 23% in two Korean studies (Kim et al., 2000, Ahn et al., 1999) and 18.4% in Malaysia (Chan and Goh, 1999).

In the Middle East, RSV also represents a major health problem representing between 23 and 29% of causes of viral ALRI in hospitalised children (Table 3.2).

Table 3.2 RSV infection among hospitalised children in the Middle East

Country	Hospitalisation rate due to RSV	Reference
Turkey	29.2%	(Dereli et al., 1994)
United Arab Emirates	28.5%	(Uduman et al., 1996)
Sudan	28%	(Salih et al., 1994)
Jordan	25.5%	(Bdour, 2001)
Saudi Arabia	23%	(al-Hajjar et al., 1998)

In southern Israel, patients with RSV bronchiolitis represented 18% of all hospitalised infants ≤ 9 months and 35% of all hospitalisation for respiratory problems of infants ≤ 9 months old. The yearly incidence of hospitalisation for RSV bronchiolitis was 5.4/1000 live births for Jews and 18/1000 live births for Bedouins (Dagan et al., 1993). These figures reflect the socio-economic conditions in each of the Jewish and Arab communities. In a recent study from Tunisia, RSV infection was present in 46 (23%) out of 268 hospitalised neonates with ALRI. Among the 46 documented RSV positive neonates, 17 (37%) had a nosocomially acquired RSV infection whereas 29 were hospitalised because of community acquired RSV ALRI. Twenty five (54%) had an underlying condition predisposing to severe disease and 13% died (Fodha et al., 2004).

Molecular epidemiology

RSV is unusual in that even natural infections do not protect against subsequent re-infections although their clinical intensity may be diminished, and in that infection of babies occurs despite the presence of maternal antibody. A possible partial explanation of the ability of the virus to re-infect individuals is that the virus is variable and it is this aspect that has encouraged the analysis of the genetic variation and molecular epidemiology of the virus.

Epidemiology of group A and B RSV

Group A predominates in most RSV outbreaks in different geographical regions (Hall et al., 1990). Group B is less frequent but occasionally becomes predominant in an epidemic. The two antigenic groups are usually found to circulate concurrently. The higher variability of group A may reflect its worldwide predominance (Hall et al., 1990).

Data on the frequency of group A and B in the Middle East and Africa have been scarce. In Tunisia group B RSV had a higher prevalence than group A during two seasons from June 2000 to August 2002 (Fodha et al., 2004) while in Jordan, group A predominated over 3 consecutive years from January 1997 to May 1999 (Bdour, 2001). In South Africa group A predominated in two seasons during 1997-2000 while groups A and B co-circulated approximately equally in the other seasons of the study (Venter et al., 2001). Another study from South Africa during the year 2000 showed that 92.5% of the isolated RSV groups were group A while during 2001 group A and B were nearly equally co-circulating. However the sample size of the isolates during 2001 was small, it was only 9 (Madhi et al., 2003b).

Group A also predominated in a study conducted in Vienna and Zagreb (Lukic-Grlic et al., 1998). In Liverpool, UK, group A predominated for two seasons over two years from 1991 to1992 (Fletcher et al., 1997).

This is however not universal, as group B RSV was the most frequently isolated genotype (85% versus 15%) in Mozambique from children under 5 years of age during 1998-1999 (Roca et al., 2001). In a US study of the circulation patterns of genetically distinct group A and B strains of RSV in Rochester was done by sequencing a variable region of the G protein gene of 204 RSV isolates collected from children < 3 years old with ALRI during five consecutive epidemic seasons (1990/91 – 1994/95), the relative ratio of group A and B strains shifted each year. Group B strains predominated during the first and third years, group A strains predominated during the second and fourth years, and group A and B strains were present at nearly equal proportions during the last year (Peret et al., 1998).

In the summer of 1987, only group B strains were isolated in Hawaii from 522 hospitalised children with ALRI over a period of 32 months (January 1987 to August 1989) while in the summer of 1988, only group A was isolated. Group B strains predominated in the first half of 1987, while group A strains predominated throughout most of 1988 and 1989. The prevalence of group B strains decreased from 1987 to 1989 (Reese and Marchette, 1991).

The distribution of RSV group A and B strains during 7 consecutive years (1990-96) were studied in two cities of Argentina. RSV was detected by indirect immunofluoresence in 352 (26.9%) out of 1304 children < 2 years of age hospitalised with ALRI. RSV epidemics were detected every year, detection rates ranged from 42.8% in 1991 to 20% in 1995. Of 195 samples, 174 (89%) were identified as group A strains and 21 (11%) as group B. Both strains cocirculated during 5 of 7 years with group A predominating. The proportional occurrence of group A strains to group B was approximately 8:1 in all years except for 1994-95 where lowest number of group A (53%) were detected and the highest frequency for group B (48%) was observed (Carballal et al., 2000).

Table 3.3 show the temporal and geographical epidemic patterns of RSV groups A and B. It was clear that group A predominates in the Americas and Africa, while in Europe epidemics are mixed with A/B being more common.

Table 3.3 Epidemiology of group A and B

Continent	Year	Predominant group	Reference
1	1993-6	A-B-A/B-A	(Venter et al., 2001)
1	1999	B	(Roca et al., 2001)
1	2000 -2	B	(Fodha et al., 2004)
2	1984-6	B-A-A	(Russi et al., 1989)
2	1990-8	A-A-A/B-A/B-A/B-A/B-A/B-A/B-B	(Straliotto et al., 2001)
2	1998	A	(Moura et al., 2003)
2	1993-5	A-A-B	(Coggins et al., 1998)
2	1990-5	B-A-B-A-A A+B	(Peret et al., 1998)
3	1979-5	A-B-B-B-A	(Tsutsumi et al., 1988)
3	1991-9	A/B-A-A-B-A-A-A/B-A/B-A/B	(Choi and Lee, 2000)
4	1991-2	A-A	(Fletcher et al., 1997)
4	1982-9	A/B-B-A/B-A/B-A/B-A-A/B-B	(Freymuth et al., 1991)
4	1988-93	A-A/B-A-A-B	(Cane et al., 1994)

1= Africa, 2= Americas, 3= Asia, 4= Europe

Group A or B is notated when at represents more than 75% of isolates of an annual epidemic. Year is expressed as the first year when an epidemic ranged two consecutive years for one winter season, for example 1987/1988 is expressed as 1987.

Prevalence of RSV genotypes

During the RSV outbreak in 2000 in Mozambique, the molecular characterization of viral RNA in NPAs from 45 infected children < 1 year of age indicated a high level of genetic uniformity among the infecting viruses, all of which belonged to a single genotype of RSV group A which was NP2 (Roca et al., 2003).

Analysis of the SH and N genes from a series of 40 isolates submitted to the Regional Virus Laboratory, Birmingham, UK during the 1989-90 epidemic allowed the differentiation of the isolates into four group A genotypes (SHL1-4) and two group B genotypes (NP1 and NP3) (Cane and Pringle, 1991). Two further group A genotypes were detected from 1988-89 and 1990-91 epidemics (SHL5 and SHL6, respectively).

In a hospital based study, Cane et al followed the relative prevalence of the different genotypes in the south Birmingham (UK) area through five epidemic periods from 1988 to 1993 (Cane et al., 1994). Genotype A: SH5 predominated the first epidemic 1988-89, A: SH1, 3,4 and B: NP1 predominated during the second epidemic period 1989-90, genotype A: SH2 predominated in the third and fourth epidemics (1990-91 and 1991-92, respectively) while group B genotype NP3 predominated the last epidemic period 1992-93. They found that each epidemic showed a different mix of the genotypes, with those predominating in one epidemic appearing to decline in significance in the next epidemic, for example, group A genotype SH5 predominated in the study area during 1988-89 epidemic but then declined to undetectable levels in subsequent epidemics with the exception of one isolate from 1990-91. Similarly, genotype A: SH2 was not detected in the first epidemic, then built up until it was the most frequently detected genotype in the epidemic of 1991-92. These data suggest that the prevalence of a particular genotype of RSV could be to some extent be influenced by herd immunity to that genotype.

A comparison of isolates from the same epidemic periods in Liverpool; Newcastle and Birmingham, UK showed that genetically very similar viruses were circulating at the same times but their relative proportions could vary from city to city. For example in a comparison of the relative levels of the various genotypes in Birmingham and Liverpool, UK during two epidemics (1990-91 and 1991-92), the first of these epidemics was very much a mixture in both cities; the second epidemic was much more homogeneous in Liverpool than in Birmingham, although the same genotype (A: SH2) was the most prevalent in both cities during this epidemic which was particularly severe throughout the UK. In this case although the individual epidemics appear to be local in structure, since these epidemics can be considered to be made up of multiple mini-epidemics, it may be that the mini-epidemics are, to some extent at least, regional in nature (Cane and Pringle, 1995b). In a study of 30 infants with severe RSV LRT in Liverpool, NP4 group A was the predominant genotype (Greensill et al., 2003).

On the other hand (Peret et al., 2000) undertook an analysis of the variability of RSV during one epidemic season (1994-95) in 5 communities representing geographically diverse locations in

North America (Rochester, Houston, Birmingham, St. Louis and Winnipeg). A total of 220 isolates were studied. Each community had 5-7 genotypes identified and 1 or 2 dominant genotypes that included 1 predominant G gene sequence or several closely related G gene sequence. However, this study showed that the predominant strains and overall patterns of circulating genotypes were distinct for three of the five communities. This shows that there was no wide regional spread of one predominant outbreak genotype.

Patterns of RSV strains circulating during community outbreaks are complex, consistent with the fact that outbreak strains are determined at the community level not the regional or national levels. Local factors such as previous strain-specific immunity to the virus and possibly the relative fitness of the circulating RSV dictate which strains (present endemically in a community or introduced from other communities) will predominate in a given season.

NPAs were obtained from 114 infants with RSV ARI admitted to the Royal Liverpool Children's hospital, Alder Hey, Liverpool, UK, over the period from January 1991 to March 1992 (covering two separate RSV epidemics) and were included in a study by (Fletcher et al., 1997). The majority was found to be infected with group A genotypes NP2 and NP4 being detected in 36 and 66 children respectively. In the first epidemic (January – July 1991), the NP2 and NP4 genotypes were evenly distributed (31 and 34 patients respectively). In the second epidemic, (September 1991 to March 1992) the majority were NP4 (32 cases) with the minority being NP2 and NP3 genotypes. No NP6 genotypes were detected in either epidemic. On basis of G genotypes all the NP2 genotypes fell into three closely related lineages (SHL1, 3,4) and the NP4 types fell into two lineages (SHL2 and SHL6). In the first epidemic SHL2 and SHL6 were found in equal numbers, but in the second epidemic almost all were SHL2.

A study of nine consecutive epidemics has been reported from Seoul, Korea. As in Birmingham, it was found that different genotypes predominated in each epidemic and were then replaced in subsequent epidemics. Interestingly, the group A genotype SH5 was also not detected in this study which commenced in 1990-91 (Choi and Lee, 2000). Similarly a study from Rochester, USA, over 5 years from 1990-1995, showed multiple genotypes in each epidemic with no genotype predominating for more than one epidemic, but group A genotype A: SH5 was abundant in the 1994-95 epidemic in Rochester (Peret et al., 1998).

A study from Japan during the period from December 1980-1995, which included 15 successive epidemics, 125 group A RSV were isolated. Isolates from the first two seasons were all shown to

have the NP5 restriction pattern; however, this strain was never isolated subsequently. NP4 was predominated from the third season to the last, except that four NP2 strains were isolated in the 1990-91 and 1991-92 seasons. Using G protein gene RFLP, seven distinct patterns were observed. In all 10 different patterns were observed in the strains when NP and G protein gene RFLP analysis were combined and it was shown clearly that the dominant lineages were replaced by others after every one to three seasons. Thus, the dominant strains changed five times with no recurrence of the former predominant genotypes (Seki et al., 2001).

This is consistent with Choi and Lee who reported that changes of predominant genotypes of G gene of RSV group A strains occurred every 2-4 consecutive epidemics (Choi and Lee, 2000). The complete disappearance of NP5, after the 1982-83 season might have been caused by resistance to some G genotype that was closely linked to NP5 genotype, however, NP5 strains were minor isolates after the second half of the 1980s suggesting that the decline of NP5 strains may also be a worldwide phenomenon (Cane et al., 1991). Frequent shifts of predominant genotype of RSV group A strains over successive epidemics was thought to be a universal phenomenon

In a South African study over four consecutive epidemics from 1997 to 2000 225 isolates were grouped by RT-PCR and sequencing the C terminus of the G protein gene (Venter et al., 2001). Group A RSV predominate in two seasons, while A and B co-circulated approximately equally in the other seasons. One new South African group A (SAA1) and three new group B (SAB1, SAB2 and SAB3) genotypes were identified. Similarly, different genotypes co-circulated each year, with certain genotypes becoming dominant and then declining before being replaced with a different dominant genotype. Some genotypes were found throughout the 4 years of study (GA5, GB3 and SAB3) while others were found at a low frequency in some seasons. Different circulation patterns were identified for group A and B genotypes. More diversity was found among group A isolates during the 4 years study period than group B, supporting the findings by (Choi and Lee, 2000). Group A RSV showed a gradual build-up and then replacement of dominant genotypes, e.g. GA5 (1997) was replaced by SAA1 (1998), which was then replaced by GA2 (1999). GA2 predominated for more than one season, increasing from a prevalence of 42% (1999) to 78% (2000). The prevalence of group B was more consistent, and two group B genotypes (GB3 and SAB3) remained co-dominant throughout all 4 years. It has been suggested that this higher variability may be advantageous to group A RSV and could contribute to its higher worldwide predominance (Hall et al., 1990). Previous studies on group A RSV have reported that a new genotype becomes dominant each year (Cane et al., 1994, Peret et al., 2000).

Although this is true for the first three years of this South African study, the same genotype, GA2, remained dominant during the last 2 years (1999 and 2000). This suggests that successful variants may be able to persist and remain dominant for more than one season.

Clinical presentation of RSV infection

RSV infection is manifest as a clinical spectrum from mild to severe respiratory disease. The variations in clinical manifestations relate partly to known host factors such as age and underlying conditions but definite factors that may predispose an individual to severe disease remain largely unknown. The best described and most often recognized clinical manifestation of RSV infection is ALRI disease in infants that require hospitalisation.

Two patterns of severe disease are observed with lung function tests: in about two-thirds of cases there is obstructive small airways disease (bronchiolitis) and in the remainder there is a restrictive pattern (pneumonia). Most of the latter cases meet the criteria for acute respiratory distress syndrome.

During the peak of an RSV outbreak, between 40-90% of bronchiolitis cases are associated with RSV (Hall, 1999). In addition, RSV is responsible for at least 50% of pneumonia cases in the first 2 years of life (Murphy et al., 1981).

Signs of URTI commonly precede those of the lower respiratory tract by few days. Fever, when present, is usually low grade. Lower respiratory tract involvement subsequently develops, often indicated by a worsening "croupy" cough, tachypnoea and non-specific signs such as irritability, malaise and anorexia. Fever may no longer be present at the time of hospital admission, despite worsening of the disease. Dyspnoea, lower chest indrawing and difficulty in feeding characterize ALRI. In bronchiolitis, wheeze may be audible with or without the stethoscope and a prolonged expiratory phase and crackles are characteristic. Air trapping results in very fast breathing, a palpable liver and spleen and a typical radiographic pattern of hyperinflation with diffuse interstitial marking and peribronchial thickening. Segmental atelectasis, which usually clears spontaneously, is often seen. A consolidated area, which may represent atelectasis, occurs in approximately one quarter of infected children (Rice and Loda, 1966). This finding raises concern about bacterial infection and may lead to the administration of antibiotics. Children with RSV

pneumonia have fine crackles and a radiographic pattern of alveolar, segmental or lobar consolidation. The illness commonly appears more severe than the roenterographic picture would indicate the differential diagnosis from bacterial pneumonia is difficult.

RSV has been isolated from around 11% of URTI, 10% - 30% of tracheobronchitis and 3-10% of croup cases (Dereli et al., 1994). Acute otitis media occurs in up to a third of the children with RSV and is found in 40% of cases of chronic otitis media with effusion (Moyse et al., 2000). Asymptomatic RSV infection is uncommon in young children (<1%).

In older children and adults, RSV is associated with milder disease, mostly URTI, tracheobronchitis and otitis media. However RSV URTI tends to be more severe than those caused by other viral agents that cause common colds even in normal older children and adults (Hall, 1999). A case-control study compared two groups of patients with (n=37) and without RSV (n=82) and classified illness presentation into acute or late. Late illness was defined as any illness occurring on the first day after the acute phase until the first asymptomatic day. In the RSV infected group, there was higher percentage of patients with fever during the acute illness phase (27% vs. 13%), nasal congestion (59.5% vs. 26%) cough (46% vs. 21%) and earache (14% vs. 2%) than in patients without RSV. In the late stage, there was higher percentage of patients with asymptomatic illness in the RSV negative group than in the RSV – positive group (74% vs. 34%) demonstrating that RSV is more severe than other viruses (Hall, 1999).

Geographical variability in disease presentation in children with RSV

There is a significant geographical variability of RSV associated disease presentation in children. The ratio of children with primary presentation as bronchiolitis to those with primary presentation as pneumonia is about three to one in USA and Europe (Behrendt et al., 1998, Walsh et al., 1997). The exact opposite (one to three) has been reported from the Gambia (Weber et al., 1998a). These differences may be attributed to socio-economic, nutritional and environmental factors, but genetic and viral factors may also be important. For example in New York, severe RSV LRTI was associated with group A (Behrendt et al., 1998), whereas group B caused longer hospital stay in Gambian children (Weber et al., 1998a).

There are also geographical differences in developed countries. For example, a comparison of children admitted to the hospitals in Rotterdam, the Netherlands and Geneva showed significant differences in disease presentation. The mean respiratory rates were 51/ min and 59/min in the Netherlands and Geneva, respectively; the proportion of children with wheezing was 29% and 66%; apnoea was present in 24% and 2% of the children and the rates of admission to intensive care were 28% versus 4% for Rotterdam and Geneva respectively (Brandenburg et al., 1997b).

Human Metapneumovirus

In the past few decades, many new aetiological agents causing respiratory tract illnesses have been identified. However, a proportion of these illnesses still cannot be attributed to known pathogens. Recently, van den Hoogen et al. isolated a new virus from nasopharyngeal aspirate samples of 28 young children from the Netherlands suffering from respiratory tract infections during the past 20 years (van den Hoogen et al., 2001).

Classification

The virus exhibits a paramyxovirus-like morphology in negative contrast electron microscopy (van den Hoogen et al., 2001). The virus is a new member of the *Metapneumovirus* genus. It displayed a high percentage of sequence identity with and has a genomic organization similar to avian pneumovirus (APV), the aetiological agent of upper respiratory tract disease in turkeys (Cook, 2000). APV, also known as turkey rhinotracheitis virus, belong to the *Metapneumovirus* genus, which together with *Pneumovirus* genus constitutes the *Pneumovirinae* subfamily in the *Paramyxoviridae* family (Viruses, 2000). The *Pneumovirus* genus contains the mammalian respiratory syncytial viruses (human, ovine, bovine RSV) and pneumovirus of mice (PVM). Until the discovery of HMPV, APV was the sole member of the Metapneumovirus genus. The classification of the two genera is based primarily on the gene constellation; Metapneumoviruses lack nonstructural proteins NS1 and NS2 and the gene order is different from that of pneumoviruses (RSV, 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'; APV, 3'-N-P-M-F-M2-SH-G-L-5'). Sequence analysis of the nucleoprotein (N), phosphoprotein (P), matrix protein (M), and fusion protein (F) genes of HMPV revealed the highest percentage of sequence identity with APV serotype C, the avian pneumovirus found primarily in birds in The United States. These analyses also revealed the absence of NS1 and NS2 at the 3' end of the viral genome and positioning of F immediately adjacent to M (van den Hoogen et al., 2001, van den Hoogen et al., 2002).

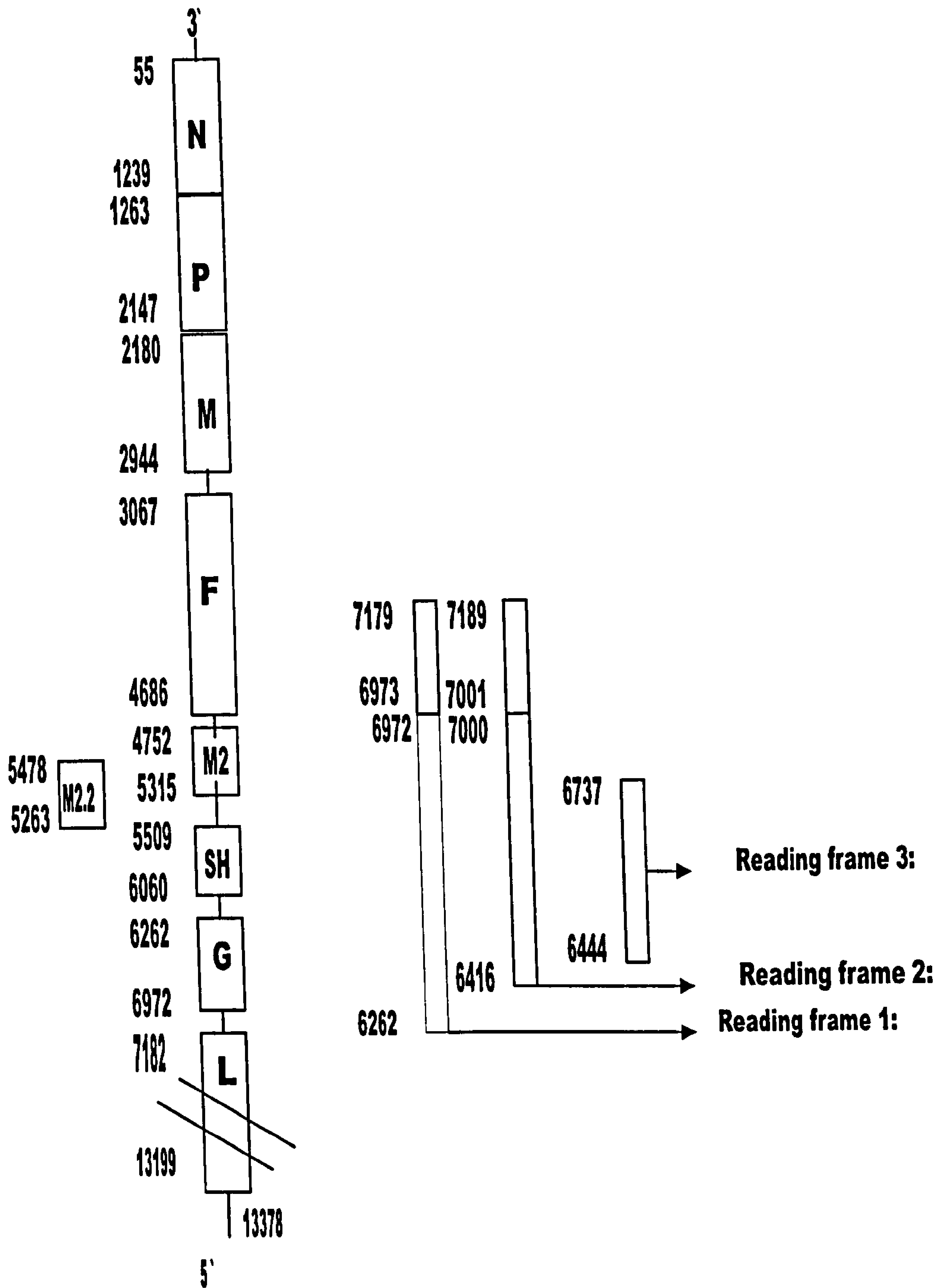
HMPV Structure

The analysis of the sequences of all HMPV ORFs and intergenic sequences as well as partial sequences of the genomic termini was described by (Jartti et al., 2002, van den Hoogen et al., 2001) and figure 3.4 demonstrate the genomic map of HMPV.

The nucleoprotein (N) ORF

It is the first gene in the genome map of HMPV (Jartti et al., 2002, van den Hoogen et al., 2001) and codes for a 394 aminoacid protein, which resembles the N protein of other pneumoviruses. The length of N ORF is identical to the length of the N ORF of APV-C (Jartti et al., 2002) and is smaller than those of other paramyxoviruses (Barr et al., 1991). Analysis of the aminoacid sequence revealed the highest percentage of sequence identity with APV-C (88%) and only 7-11% with other paramyxoviruses Barr et al. identified three regions of similarity between viruses belonging to the order Mononegavirales: A, B and C. Here aminoacid residues regarded areas similar when they are replaced by aminoacid residues from a group sharing some physical or chemical properties. Although similarities are highest within a virus family, these regions are highly conserved between virus families (Barr et al., 1991). In all three regions HMPV revealed 99.3% aminoacid sequence similarity with APV-C, 98.6% with APV-A, 95.3% with APV-B, and 78-92% with RSV and PVM (van den Hoogen et al., 2002). The region between aminoacid residues 160 and 340 appears to be highly conserved among metapneumovirus and to a somewhat lesser extent in pneumoviridae (Barr et al., 1991). This is in agreement with HMPV being metapneumovirus, showing 99% similarity with APV-C (van den Hoogen et al., 2002).

Figure 3.4 Genomic map of HMPV isolate 001



Source: modified from (van den Hoogen et al., 2002).

The putative ORFs and the nt positions of the start and stop codons are indicated. The double lines crossing the L ORF indicate the shortened representation of the L gene. Note that the length of the genome is still uncertain since the sequence of the genomic termini reflects primer sequences. The three reading frames beside the map indicate the primary G ORF (nt 6262 -6972) and overlapping potential secondaries ORFs.

The Phosphoprotein (P) ORF

The second ORF in the genome map code for a 294-aminoacid protein which shares 68% sequence identity with the P protein of APV-C and only 22-24% with the P protein of RSV (van den Hoogen et al., 2002). The putative P gene of HMPV contains one substantial ORF and in that respect is similar to P from many other paramyxoviruses (Sedlmeier and Neubert, 1998, Lamb, 1996). In contrast to APV-A and –B and PVM and similar to RSV and APV-C the HMPV P ORF lacks cysteine residues. The C-terminus of the HMPV P protein is rich in glutamate residues as has been described for APVs (Ling et al., 1995). Ling et al. suggested that a region of high similarity between all pneumoviruses (aminoacid 185-241) plays a role either in RNA synthesis process or in maintaining the structural integrity of the nucleocapsid complex. This conserved region may represent the domain that interacts with the polymerase protein (Ling et al., 1995). This region of high similarity is also found in HMPV. Showing 100% similarity with APV-C, 93% with APV-A and –B, and approximately 81% with RSV (van den Hoogen et al., 2002).

The matrix protein (M) ORF

The third ORF of the HMPV genome encodes a 254- aminoacid protein, which resembles the M ORFs of other pneumoviruses. The putative M ORF of HMPV has exactly the same size as the M ORFs of other Metapneumoviruses (van den Hoogen et al., 2002) and shows high aminoacid sequence identity with the matrix proteins of APV (76-87%), lower identity with those of RSV and PVM (37-38%), and 10% or less identity with those of other paramyxoviruses. Easton and Chambers compared the sequences of matrix proteins of all pneumoviruses and found a conserved hexapeptide at residues 14 to 19 that is also conserved in HMPV (Easton and Chambers, 1997). For RSV, PVM and APV small secondary ORFs within or overlapping with the major ORF of M have been identified (52-51 aminoacid in bovine RSV, 75 aminoacid in human RSV, 46 aminoacid in PVM, and 51 aminoacid in APV (Samal and Zamora, 1991, Satake and Venkatesan, 1984). van den Hoogen et al noticed two small secondary ORFs in the M ORF of HMPV. One small ORF of 54 aminoacid residues was found within the major M ORF, starting at nt 2281, and one small ORF of 33 aminoacid residues was found overlapping with the major ORF of M, starting at nt 2983. Similar to the secondary ORFs of RSV and APV there is no significant sequence identity between these secondary ORFs of the other pneumoviruses, and apparent start and stop signals are lacking. Evidence for the synthesis of proteins corresponding to these secondary ORFs of APV and RSV has not been reported (van den Hoogen et al., 2002).

The fusion protein (F) ORF

The putative F ORF of HMPV is located adjacent to the putative M ORF, which is characteristic for members of the *Metapneumovirus* genus. The F gene of HMPV encodes a 539 aminoacid protein, which is 2 aminoacid residues longer than F of APV-C. Analysis of the aminoacid sequence revealed 81% sequence identity with APV-C, 67% with APV-A and –B, 33-38% with other pneumovirus F proteins, and only 10-18% with other paramyxoviruses (Jartti et al., 2002). One of the conserved features among F proteins of paramyxoviruses and also seen in HMPV is the distribution of cysteine residues (Morrison, 1988, Yu et al., 1991). The metapneumoviruses share 12 cysteine residues in F1 (7 are conserved among all paramyxoviruses), and 2 in F2 (1 is conserved among all paramyxoviruses). Of the three potential N –linked glycosylation sites present in the F ORF of HMPV, none are shared with RSV and two are shared with APV. The third, unique potential N- linked glycosylation site for HMPV is located at position 206 (van den Hoogen et al., 2002).

Despite the relatively low percentage of sequence identity with other paramyxoviruses, the F protein of HMPV revealed typical fusion protein characteristics consistent with those described for the F proteins of other paramyxoviridae family (Morrison, 1988). F proteins of members of paramyxoviridae family members are synthesized as inactive precursors (F^0) that are cleaved by host cells proteases, which generate N-terminal F^2 subunits and large C-terminal F^1 subunits. The cleavage site is conserved among all members of the *Paramyxoviridae* family (Collins et al., 1996). Both arginine residues are shared with APV and RSV, but the glutamine and serine residues are shared with other paramyxoviruses such as human parainfluenza virus type 1, Sendai virus, and morbilliviruses. In RSV a second cleavage site was found which is separated from the first cleavage site immediately upstream of the fusion peptide by a stretch of 27 aminoacid. Cleavage at both sites is required for the acquisition of membrane fusion potential of the F protein. The F proteins of the other pneumoviruses such as APV and PVM as well as other paramyxoviruses lack the sequence between the two cleavage sites (Zimmer et al., 2001, Gonzalez-Reyes et al., 2001). This second cleavage site and the connecting peptide are absent in the HMPV F protein. The hydrophobic region at the N terminus of F1 functions as the membrane fusion domain and displays a high degree of sequence similarity among paramyxoviruses and morbilliviruses and to a lesser extent among the pneumoviruses (Morrison, 1988, Horvath and Kellie, 1990). This hydrophobic region (position 137-159) is conserved between HMPV and APV-C, which is in agreement with this region being highly conserved among the metapneumoviruses (Naylor et al., 1998). Adjacent to the fusion peptide and transmembrane

segment are two regions that contain heptad repeats (HRA and HRB) which are relatively poor in glycines, contain no helix-breaking prolines, and contain charged aminoacid side chains in all heptad positions except a and d. These heptad regions necessary for viral fusion are also found in the HMPV F gene (Chambers et al., 1990, Lamb, 1993, Russell et al., 2001). Whereas a high percentage of sequence identity is found between HRA of HMPV and all other pneumoviruses (especially in positions a and d of the repeat), a high level of sequence identity in HRB is restricted to the Metapneumoviruses. Furthermore, for RSV and APV, the signal peptide and anchor domain were found to be conserved within subtypes and displayed high variability between subtypes (Naylor et al., 1998). At the N-terminus of F2 of HMPV, 11 of 18 aminoacid residues are identical to those of APV-C and lower sequence identity is observed with the signal peptide of other APV or RSV F ORFs. Much more variability is seen in the membrane anchor domain at the C-terminus of F1, although some sequence identity is still seen with APV-C.

The 22 K protein ORF

The M2 gene is unique to the members of the *Pneumovirinae* subfamily and two overlapping ORFs have been observed in all pneumoviruses. The first major ORF represents the M2-1 protein which enhances the processitivity of the viral polymerase (Collins et al., 1996, Collins et al., 1995) and its read-through of intergenic regions (Fearns and Collins, 1999, Hardy and Wertz, 1998). The putative M2-1 gene for HMPV is located adjacent to the F gene, encodes a 187-aminoacid protein (van den Hoogen et al., 2002) and reveals the highest percentage of sequence identity with M2-1 of APV-C (84%). Comparison of all pneumovirus M2-1 proteins revealed the highest level of conservation in the N- terminal half of the protein (Collins et al., 1990, Zamora and Samal, 1992b, Ahmadian et al., 1999) . Which is in agreement with the observation that HMPV displays 100% similarity with APV-C in the first 30 aminoacid residues that are conserved among all pneumoviruses. Such a concentration of cysteines is frequently found in zinc-binding proteins (Ahmadian et al., 1999, Cuesta et al., 2000).

The secondary ORFs (M2-2) that overlap with the M2-1 ORFs of Pneumoviruses are conserved in location but not in sequence and are thought to be involved in the control of the switch between virus RNA replication and transcription (Collins et al., 1985, Collins et al., 1990, Collins et al., 1999, Bermingham and Collins, 1999, Zamora and Samal, 1992a, Ahmadian et al., 1999). For the HMPV, the putative M2-2 ORF starts at nt 512 in the M2-1 ORF, which is exactly the same start position as APV-C. The lengths of M2-2 ORFs are the same for APV-C and HMPV, 71 aminoacid residues. Sequence comparison of the M2-2 ORF revealed 56% aminoacid sequence

identity between HMPV and APV-C and only 26-27% aminoacid sequence identity between HMPV and APV-A and –B (van den Hoogen et al., 2002).

The small hydrophobic protein (SH) ORF

The gene located adjacent to M2 of HMPV probably encodes a 183 aminoacid SH protein. There is no discernible sequence identity between this ORF and other RNA virus genes or genes products. This is not surprising since sequence similarity between pneumovirus SH proteins is generally low. The putative SH ORF of HMPV is the longest SH ORF known to date (van den Hoogen et al., 2002). The aminoacid composition of the SH ORF is relatively similar to that of APV, RSV and PVM, with a high percentage of threonine and serine residues (22%, 18%, 19%, 20%, 21%, and 28% for HMPV, APV, RSV A, RSV B, bRSV, and PVM, respectively). The SH ORF of HMPV contains 10 cysteine residues, whereas APV SH contains 16 cysteine residues. The SH ORF of HMPV contains two potential N – linked glycosylation sites (aminoacid 76 and 121). Whereas APV has one, RSV has two or three, and PVM has four.

The hydrophilicity profiles for the putative HMPV SH protein and SH of APV and RSV revealed similar characteristics. The SH ORFs of APV and HMPV have a hydrophilic N-terminus, a central hydrophobic domain which can serve as a potential membrane –spanning domain (aminoacid 30-53 for HMPV), a second hydrophobic domain (aminoacid 155-170), and a hydrophilic C-terminus. In contrast, RSV SH appears to lack the C-terminal part of the APV and HMPV ORFs. In all pneumovirus SH proteins the hydrophobic domain is flanked by basic aa residues, which are also found in the SH ORF for HMPV (aminoacid 29 and 54) (van den Hoogen et al., 2002).

The attachment glycoprotein (G) ORF

The putative G ORF of HMPV is located adjacent to the putative SH gene and encodes a 236 aminoacid protein (nt 6262-6972). A secondary small ORF is found immediately following this ORF, potentially encoding for 68 aminoacid residues (nt 6973-7179) but lacking a start codon. A third potential ORF in the second breading frame of 194 aminoacid residues overlaps both of these ORFs but also lacks a start codon (nt 6416-7000). This ORF is followed by a potential fourth ORF of 65 aminoacid residues in the same codon. Finally, a potential ORF of 97 aminoacid residues (but lacking a start codon) is found in the third reading frame (nt 6444-6737) (van den Hoogen et al., 2002). Unlike the first ORF, the other ORFs do not have apparent gene start or gene end sequences. Although the 236 aminoacid G ORF probably represents at least a

part of the HMPV attachment protein, the possibility cannot be excluded that the additional coding sequences are expressed as separate proteins or as part of the attachment protein through some RNA editing event. It should be noted that for APV and RSV no secondary ORFs after the primary G ORF have been identified but that both APV and RSV have secondary ORFs within the major ORF of G. However, evidence for expression of these ORFs is lacking and there is no sequence identity between the predicted aminoacid sequences for different viruses (Ling et al., 1992). The secondary ORFs in HMPV G do not reveal characteristics of other G proteins and whether the additional ORFs are expressed requires further investigations.

Blast analysis with all ORFs revealed no discernible sequence identity at the nucleotide or aminoacid sequence level with other known virus genes or gene products. This is in agreement with the low percentage of sequence identity found for other G proteins such as those of RSV A and B (53%) (Johnson et al., 1987) and APV-A and –B (38%) (Juhasz and Easton, 1994).

Whereas most of the HMPV ORFs resemble those of APV in both length and sequence, the putative G ORF of 236 aminoacid residues of HMPV is considerably smaller than the G ORF of APV (van den Hoogen et al., 2002). The aminoacid sequence revealed a serine and therionine content of 34%, which is even higher than the 32% for RSV and 24% for APV. The putative G ORF also contains 8.5% proline residues, which is higher than 8% for RSV and 7% for APV. The unusual abundance of proline residues in the G proteins of APV, RSV and HMPV has also been observed in glycoproteins of mucinous origin where it is a major determinant of the proteins' three-dimensional structures (Collins and Wertz, 1983, Wertz et al., 1985, Jentoft, 1990). The G ORF of HMPV contains five potential N-linked glycosylation sites, whereas RSV has seven, bovine RSV has five, and APV has three to five (van den Hoogen et al., 2002).

The predicted hydrophilicity profile of HMPV G revealed characteristics similar to those of other pneumoviruses. The N-terminus contains a hydrophilic region followed by a short hydrophobic area (aminoacid 33-53 for HMPV) and a mainly hydrophilic C-terminus. This overall organization is consistent with that of an anchored type II transmembrane protein and corresponds well with these regions in the G protein of APV and RSV. The putative G ORF of HMPV contains only one cysteine residue in contrast to RSV and APV (5 and 20 respectively). Of note, only two of the four secondary ORFs in the G gene contained 1 additional cysteine residue and these four potential ORFs revealed 12-20% serine and thereonine residues and 6-11% proline residues (van den Hoogen et al., 2002).

The polymerase protein (L) ORF

In analogy to other negatively –stranded viruses, the last ORF of the HMPV genome is the RNA – dependent RNA polymerase component of the replication and transcription complexes. L gene of HMPV encodes a 2005-aminoacid protein, which is one residue longer than the APV-A L protein (van den Hoogen et al., 2002). The L protein of HMPV shares 64% aminoacid sequence identity with APV-A, 44% with RSV, 13-15% with other paramyxoviruses . Poch et al. identified six conserved domains within the L proteins of nonsegmented negative-strand RNA viruses, from which domain III contained the four core polymerase motifs that are thought to be essential for polymerase function (Poch et al., 1990, Poch et al., 1989). These motifs (A, B, C, and D) are well conserved in the HMPV L protein: HMPV shares nearly 100% aminoacid sequence similarity with other pneumoviruses. For the entire domain III (aminoacid 625-847 in the HMPV L ORF), HMPV shares 83% aminoacid sequence identity with APV, 67-68% with RSV, and 26-30% with other paramyxoviruses (van den Hoogen et al., 2002). In addition to these polymerase motifs the pneumovirus L proteins contain a sequence which conforms to a consensus ATP-binding motif, $K(X)_{21}GEGAGN(X)_{20}K$ (Stec et al., 1991). The HMPV L ORF contains a similar motif as APV, in which the spacing of the intermediate residues is off by 1: $K(X)_{22}GEGAGN(X)_{19}K$ (van den Hoogen et al., 2002).

Diversity of the virus

Sequence analysis based on the N (300nt), F (405 nt) and L (102nt) genes gave comparable results, they identified two major groups or lineages. An overall nucleotide comparison for the isolates revealed 93% - 100% similarity between isolates in the same group and 83%-85% similarity between 2 groups. The predicted aminoacid sequence was less distinct; they showed 95%-97% similarity between the distinct groups (Peret et al., 2002).

By nucleotide comparison of 22 HMPV F gene sequences Boivin et al. revealed 2 major phylogenetic groups with 80.2%-83.3% similarity among groups versus 94.2%-100% (group 1) and 92.6-100% (group 2) similarity within groups. At the aminoacid level, similarity varied from 94% - 96.5% between groups and 96.8%-100 (group1) and 97.2 - 100% (group2) among isolates within the same group. Strains from both groups cocirculated during certain years (2000 and 2001), and HMPV sequences from different years were found in the same subclusters (Boivin et al., 2003). A Canadian study of 445 specimens from all age groups, found that 14.8% of them were positive for HMPV. The phylogenetic analysis of the F gene showed that two HMPV

genetic clusters were cocirculating in the 2001-2002 season, and comparison with earlier studies suggests a temporal evolutionary pattern of HMPV isolates (Bastien et al., 2003). These two groups had been noted in the original report from the Netherlands (van den Hoogen et al., 2001).

Epidemiology of HMPV

HMPV has epidemic nature, it seems that it has a seasonal pattern during the cold season in temperate climate (van den Hoogen et al., 2001, Peret et al., 2002, Falsey et al., 2003b) or the rainy season in tropical climates (Cuevas et al., 2003). HMPV seems to have regular outbreaks, occurring annually with peak rates of infection. Studies from the Northern hemisphere HMPV has been reported to peak in the first months of the year (Esper et al., 2004), while in the Southern hemisphere the peaks of HMPV have most often been reported in April and May (Cuevas et al., 2003) or in September, October and November (Galiano et al., 2004). However, in New York, RSV and HMPV peaked at different times of a two year study (Williams et al., 2004) and in Brazil, RSV and HMPV coincided temporally in 2002 but not in the 2003 peak RSV season (Serafino et al., 2004). HMPV seems to affect all age groups but affects predominantly children and the elderly (Falsey et al., 2003a, Boivin et al., 2003, Peret et al., 2002, van den Hoogen et al., 2001). In a study from Canada, children aged <5 years and elderly subjects aged >65 years represented 35.1% and 45.9% of the HMPV-infected cases, respectively (Boivin et al., 2003).

HMPV-ARI is slightly more common in boys than girls (van den Hoogen et al., 2001, Williams et al., 2004), however in other studies, females were more commonly affected (Esper et al., 2004). Others reported similar numbers of females and males affected by HMPV (Bastien et al., 2003).

Reports on seroprevalence in the general population described a 100% serpositive rate of all children aged over 5 years in the Netherland (van den Hoogen et al., 2001) and all children aged over 10 years in Japan (Ebihara et al., 2003). The seroprevalence of HMPV was significantly lower than that of RSV in children over 4-months-old (43% vs. 60%) and the difference was particularly notable between the ages of 4 months and 1 year (11% vs. 48%). The results suggest that primary infection with HMPV occurs somewhat later than that with RSV (Ebihara et al., 2004b).

A decline in seropositivity rate of HMPV was observed up to 10 months of age in 22% of USA children, partially explained by the clearance of maternally derived antibodies. From 10 months

onwards the seropositivity rate increased to 92% in children aged 24-36 months (Esper et al., 2004).

Reinfection by HMPV was reported by Peret et al. when they isolated two different strains each belonging to a different group from a child. The second isolate was found 10 months after the first (Peret et al., 2002).

Some reports on ARI in adult outpatients noted rates of HMPV infection in 2 to 7% (Falsey et al., 2003a, Osterhaus and Fouchier, 2003, Stockton et al., 2002). The lower rates found in adults may reflect decreased levels of viral shedding or methodologic differences among the reports.

Little information is available about morbidity and mortality of children infected with HMPV.

Prevalence and incidence of HMPV

The prevalence of HMPV is unknown, very limited data were known about the epidemiology of HMPV. Few studies have been published from some geographical areas to establish its wide-world presence and to determine its clinical spectrum and its epidemiology. From Australia (Nissen et al., 2002, Howe, 2002), North America (Falsey et al., 2003a, Boivin et al., 2002, Peret et al., 2002) variuos parts of Europe, from the Netherlands (van den Hoogen et al., 2001), France (Freymouth et al., 2003) United Kingdom (Greensill et al., 2003) Finland (Jartti et al., 2002), Italy (Gerna et al., 2005) from Latin America (Cuevas et al., 2003) and recently from Africa (IJpma et al., 2004).

Infected patients were identified either retrospectively (Boivin et al., 2002, Peret et al., 2002, Stockton et al., 2002, van den Hoogen et al., 2001, Galiano et al., 2004) or by random screening of respiratory infection (Nissen et al., 2002). The reports of HMPV in most of the continents suggest worldwide distribution; however, the complete epidemiology of HMPV remains to be defined.

The detection rates of HMPV varied from one country to another, in the Argentina (11%), Netherlands (10%) Australia (9.7%), France (7%) and Hong Kong (6%) (Mackay et al., 2003, Galiano et al., 2004, Freymouth et al., 2003, Peiris et al., 2003, van den Hoogen et al., 2001). A study from Norway detected HMPV in 21% of hospitalised children which is the highest reported rate up to date (Dollner et al., 2004). However, lower detection rates was reported from Canada

(2.3%); England (2.2%), and USA (4.5%) which included children as well as young adults and elderly adults (Boivin et al., 2002, Falsey et al., 2003a, Stockton et al., 2002). This evidence strongly suggests that HMPV is a common pathogen with a wide geographical distribution, which should be included in the routine diagnosis of respiratory viruses in young children and elderly individuals.

Epidemiology of HMPV groups

Two genetic lineages of HMPV, group A and group B have been identified (Esper et al., 2003, van den Hoogen et al., 2001, van den Hoogen et al., 2002). Scanty information known about HMPV groups. The virological features and clinical findings associated with HMPV were examined retrospectively in Canadian patients hospitalized for various respiratory conditions since 1993 (Boivin et al., 2002). Thirty eight previously unidentified respiratory viruses isolated from rhesus monkey kidney cells found to be positive for HMPV by RT-PCR, and those strains clustered in 2 phylogenetic groups. In another study by Boivin et al. they described several strains of HMPV during a brief period and they divided them into two major groups with one clearly predominating, accounting for 58% of all infection (Boivin et al., 2003). All HMPV virus strains from Hong Kong belonged to one of the two genetic lineages previously described (Peiris et al., 2003). A recent study from Italy over three seasons from 2001 to 2004, different types and subtypes were identified by sequencing and phylogenetic analysis of 2 fragments of both genes F and N of HMPV. They were classified as types A and B, and subtypes A1 - A2 and B1 - B2. Although all 4 subtypes were represented during each season, the relative distribution changed significantly in different years. In 2001-2002 subtype A1 was predominant (59%,7/12 strains), while subtypes B1 and B2 were circulating at a predominant comparable proportion (36.5%, 4/11 strains each) in 2002 – 2003, and subtype A2 was by far predominant (72%, 26/36 strains) in 2003 – 2004 (Gerna et al., 2005). This study demonstrated that HMPV strains circulate at a different rate in different years and changes in prevalence of HMPV groups and subgroups occur yearly. Such viral heterogeneity may allow multiple reinfections throughout life, especially in elderly persons and immunocompermised patients, (Boivin et al., 2002, Pelletier et al., 2002). Similar to Gerna et al study a study from Denmark identified the two HMPV genetic groups and their four lineages A1, A2 and B1, B2. They also investigate the inter and intra-patient sequence diversity of the genetic lineage A HMPV fusion gene. They found that the average intergenetic inter-patient sequence diversity was 1.68% and 1.64% at nucleotide and amino acid levels, respectively. Then they divided the samples into two groups on the bases of intra-patient sequence diversity. In group 1 (4 children) the intra-patient sequence diversity was low

(nucleotide: 0.26-0.39%, amino acid: 0.51 – 0.94) whereas group B (6 children) had a higher intra-patient sequence diversity (nucleotide: 0.85-1.98%, amino acid: 1.08 – 2.22). Phylogenetic analysis showed that group 1 children has lineage A1 only while group 2 children has both A1 and A2 lineages, indicating that they had been infected with at least two viruses (Winther et al., 2005). Several independent viruses contained premature stop codons in exactly identical positions resulting in truncated fusion proteins. Possibly this is a mechanism for immune system evasion. The F protein is a major antigenetic determinant, and the limited sequence diversity observed lay emphasis on the HMPV F gene as a putative target for future vaccine analysis (Winther et al., 2005).

Clinical presentation of HMPV infection

Although data on the clinical presentation of HMPV is still scanty, it seems to be largely similar although less severe, to that of RSV, ranging from mild respiratory problems to severe bronchiolitis and pneumonia.

In hospitalised and immunocompromised children and the elderly, HMPV disease tends to be more severe (Falsey et al., 2003a, Hamelin et al., 2004, Pelletier et al., 2002).

Symptoms of upper and lower respiratory tract has been reported from these patients (cough, rhinorrhoea, wheeze, dyspnoea and chest retraction). Table 3.4 shows the frequency of signs and symptoms of HMPV in selected studies.

Table 3.4 Signs and symptoms of children with HMPV

Signs & Symptoms	(Esper et al., 2004) (USA) N=53 (%)	(Dollner et al., 2004) (Norway) N= 50 (%)	(Williams et al., 2004) (USA) N=49 (%)	(Cuevas et al., 2003) (Brazil) N=19 (%)	(Peiris et al., 2003) (Hong Kong) N=32 (%)
Cough	36 (68%)	45 (90%)	44 (90%)	19 (100%)	
Fever	41 (77%)	43 (86%)	25 (52%)	15 (79%)	
Tachypnoea	41 (77%)	40 (80%)			
Wheeze	27 (51%)	28 (56%)	25 (52%)	8 (41%)	9 (28%)
Crepitation			10 (20%)		18 (56%)
Chest indrawing	28 (53%)			4 (21%)	
O_2 therapy	20 (38%)	14 (28%)			

Community acquired HMPV infection in adults usually present with relatively mild common cold-like respiratory symptoms (cough, rhinorrhoea, hoarseness, sore throat and sometimes fever) (Falsey et al., 2003b, Stockton et al., 2002).

A range of diagnoses has been reported because of HMPV infection. Fifteen percent of children with URTI and 5% -25% of cases of otitis media have been associated with HMPV (Freymouth et al., 2003, Hamelin et al., 2004). HMPV was identified in between 23-84% of bronchiolitis cases (Bastien et al., 2003, Freymouth et al., 2003), in 17% to 36% of pneumonia cases (Boivin et al., 2003, Peiris et al., 2003), in 25%- 67% of pneumonitis cases (Hamelin et al., 2004, Boivin et al., 2003) and has been linked with asthma exacerbations in up to 23% of the children (Peiris et al., 2003).

HMPV has been described as a cause of community- acquired respiratory illnesses, as a severe disease in immunocompromised children and elderly and in healthy children in whom the infection progress from mild URTI to acute respiratory failure and advanced fibrotic phase of acute respiratory distress syndrome and death and in elderly patients (Pelletier et al., 2002, Stockton et al., 2002, Vicente et al., 2003, Hamelin et al., 2004, Falsey et al., 2003a).

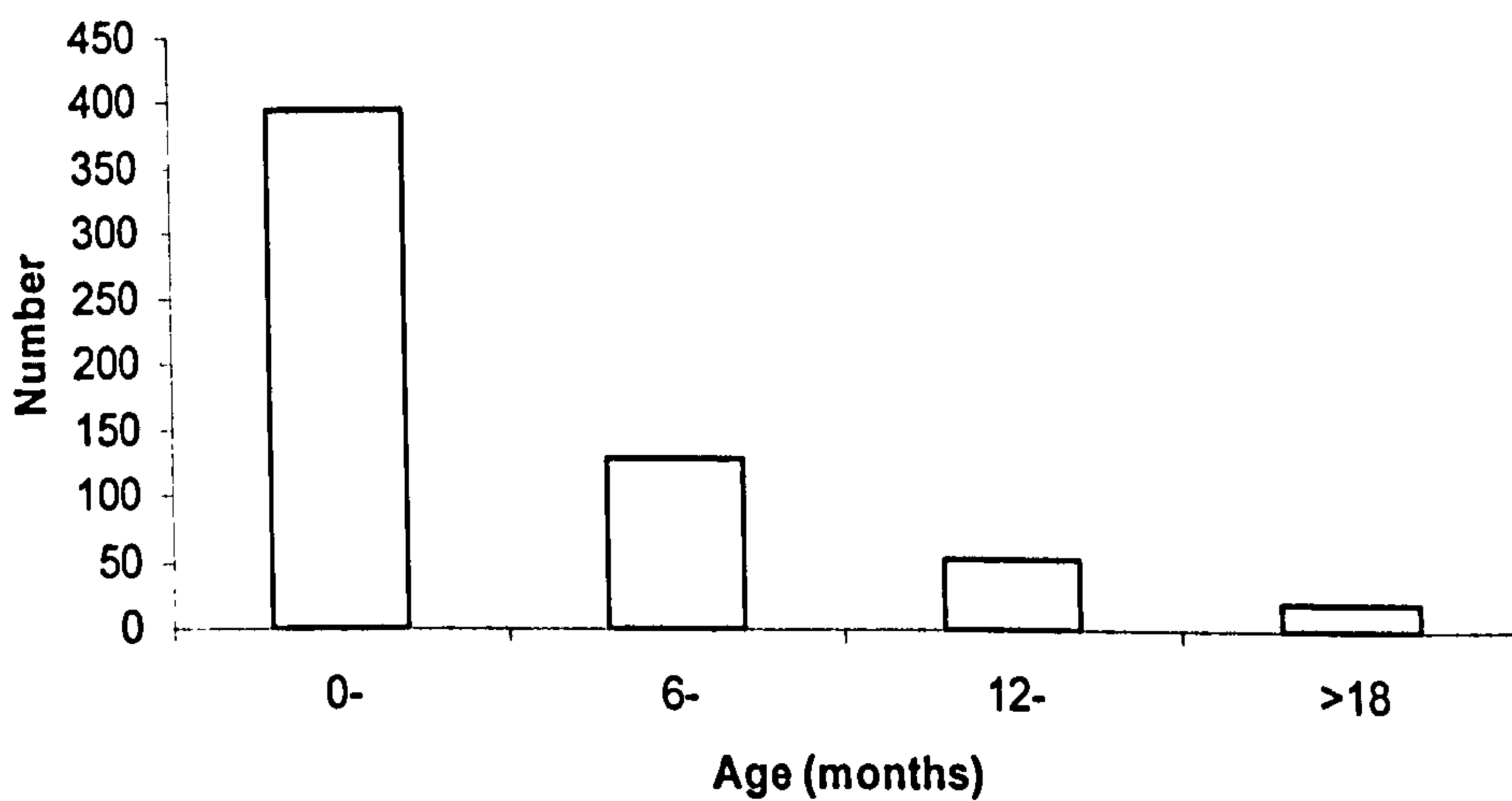
Results

General characteristics of the children

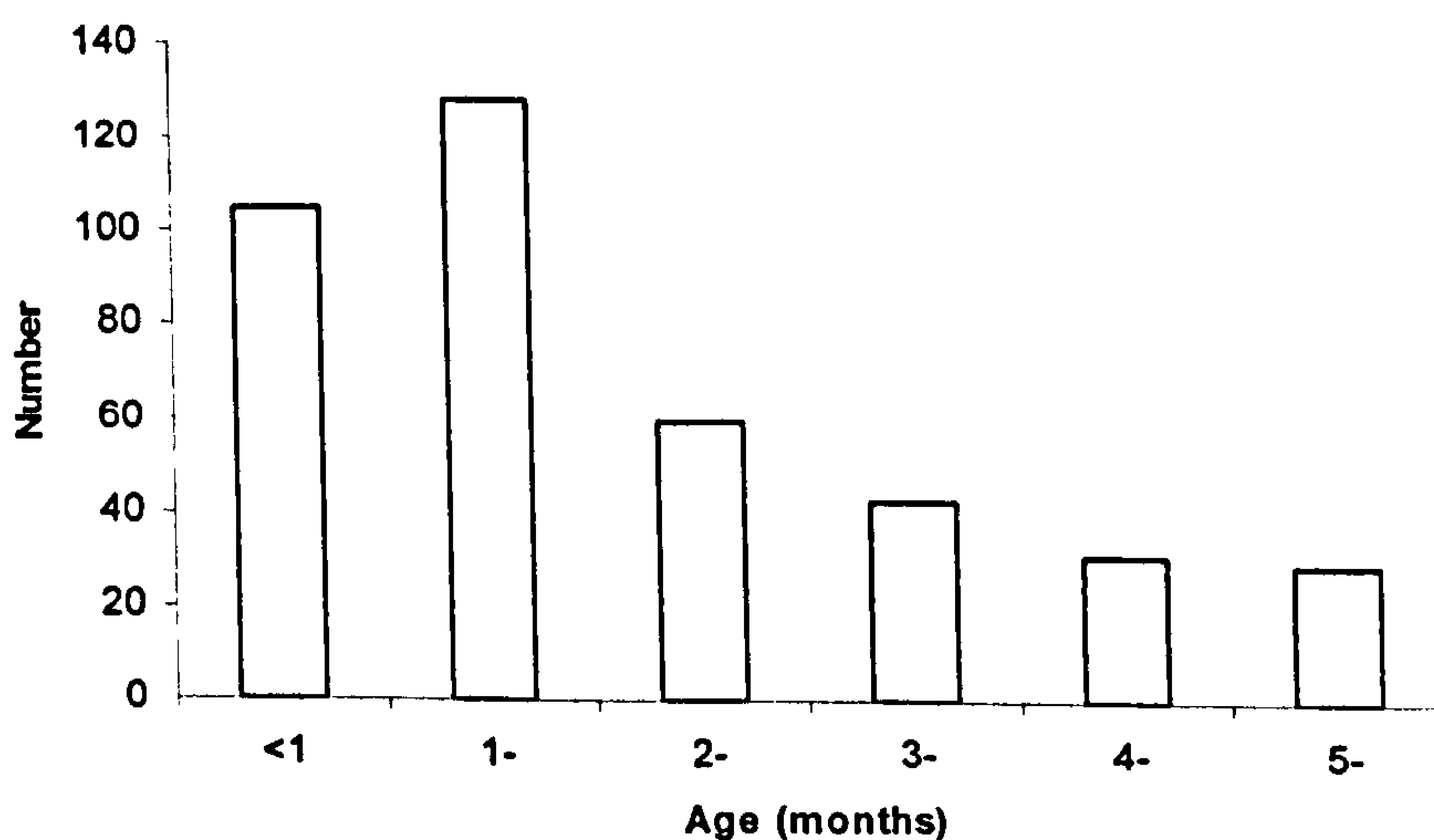
A total of 607 children were recruited during the study period. The nasopharyngeal aspirates of three were lost during processing; and the clinical data of three from the remaining 604 children were incomplete thus those children were excluded from the analysis. The age of the remaining, 601 children ranged from <1 - 24 months with a mean (SD) of 5.7 (5.5) months and a median of 4 months. The age distribution of the children is shown in figure 3.5. Three hundred and ninety five (66%) children were male and 206 (34%) female.

Figure 3.5 a) Age distribution of all children, b) Age distribution of children ≤ 6 months old

a.



b.



Nutritional status of the children

Two hundred and fifty eight (43%) children had acute severe malnutrition by anthropometry with weight for age Z scores below - 2 and only 29 (5%) of the children had Z score above 0 in contrast, nearly all the children had weight for height Z scores > 0. This indicates that most of those children had suffered acute malnutrition. Table 3.5 shows the nutritional indicators of the children.

Table 3.5 Nutritional indicators of the children

Nutritional indicators		N=601 (%)	*Local demographic Health survey data
Mean (SD) body weight (kgs)		5.2 (2)	
Mean (SD) WAZ		-2.0 (1.84)	
WAZ	< -2	258 (43%)	38%
	-2 to -1	193 (32%)	
	-1 to 0	121 (20%)	
	> 0	29 (5%)	
Mean (SD) WHZ		60.7 (23.5)	
WHZ	> 0	598 (99%)	84%

*(CSO, 1996)

Clinical history on enrolment

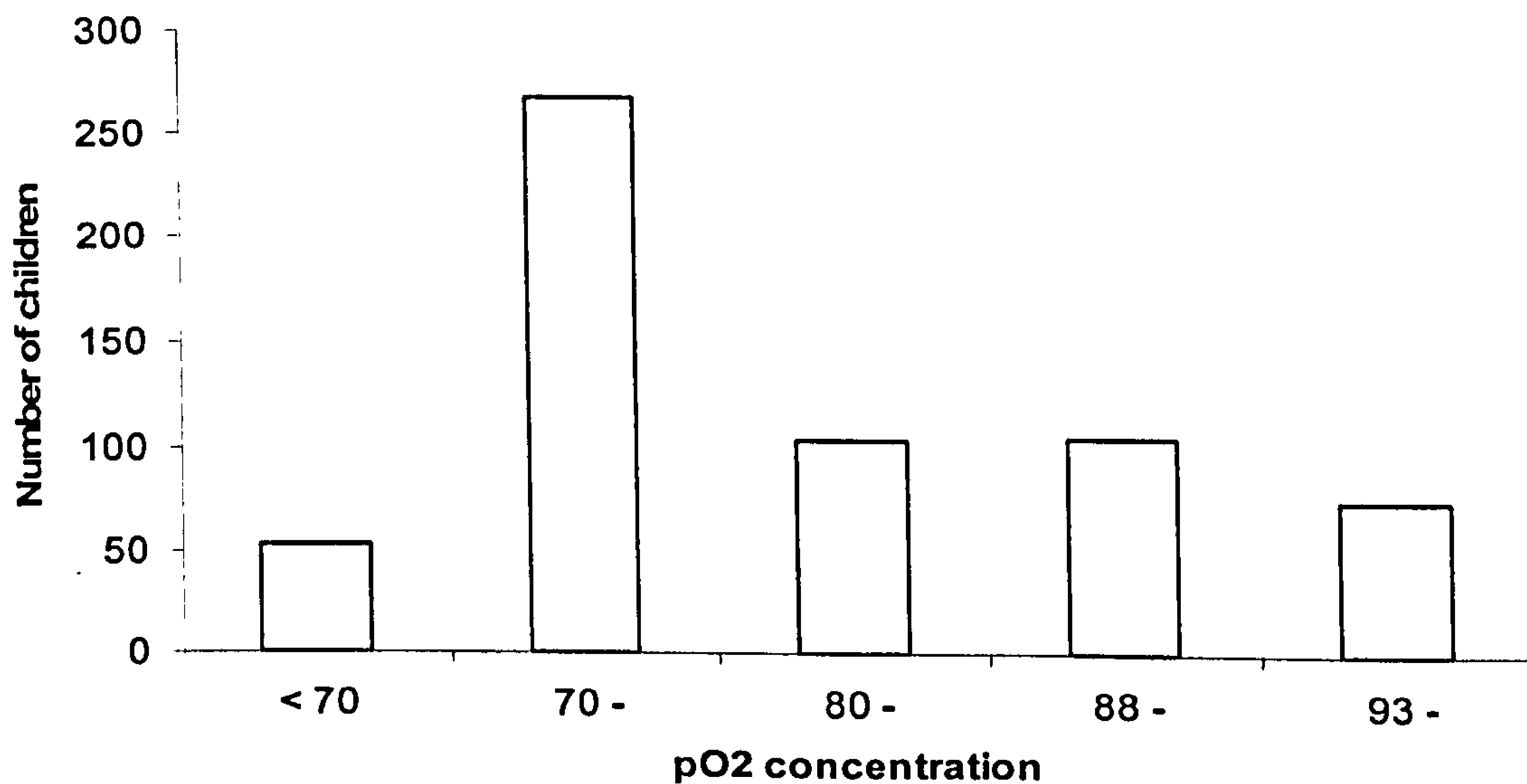
The most frequent complaints on presentation were cough in 600 (100%) children, tachypnoea in 450 (75%), fever 428 (71%), having blue lips in 353 (59%), wheezing in 194 (32%) and being unable to drink 114 (19%). Twenty five (4%) parents thought that their child had stopped breathing for longer than a few seconds. On clinical examination crackles were found in 400 (67%) children, cyanosis in 361 (60%), chest indrawing in 326 (54%) and 175 (29%) had wheezing on auscultation as shown in table 3.6.

Patients were classified as having mild ARI if their oxygen saturation (pO_2) was ≥ 88% in room air or severe ARI if they had pO_2 concentration < 88% in room air. The pO_2 cut off point of ≥ 88% was chosen as Sana'a is situated in a mountain valley at an altitude of 2,200 metres above the sea level (Duke et al., 2002). One hundred and seventy eight children (30%) were diagnosed as having mild ARI and 423 (70%) severe ARI. This unusual distribution reflects the characteristics of a tertiary hospital receiving more patients with severe than mild ARI as the

health centres treat most children with mild infections and families often come late to the hospital. The distribution of the pO_2 saturations of the children is shown in figure 3.6.

Figure 3.6 Oxygen concentration of the 601 children enrolled

a.



b.

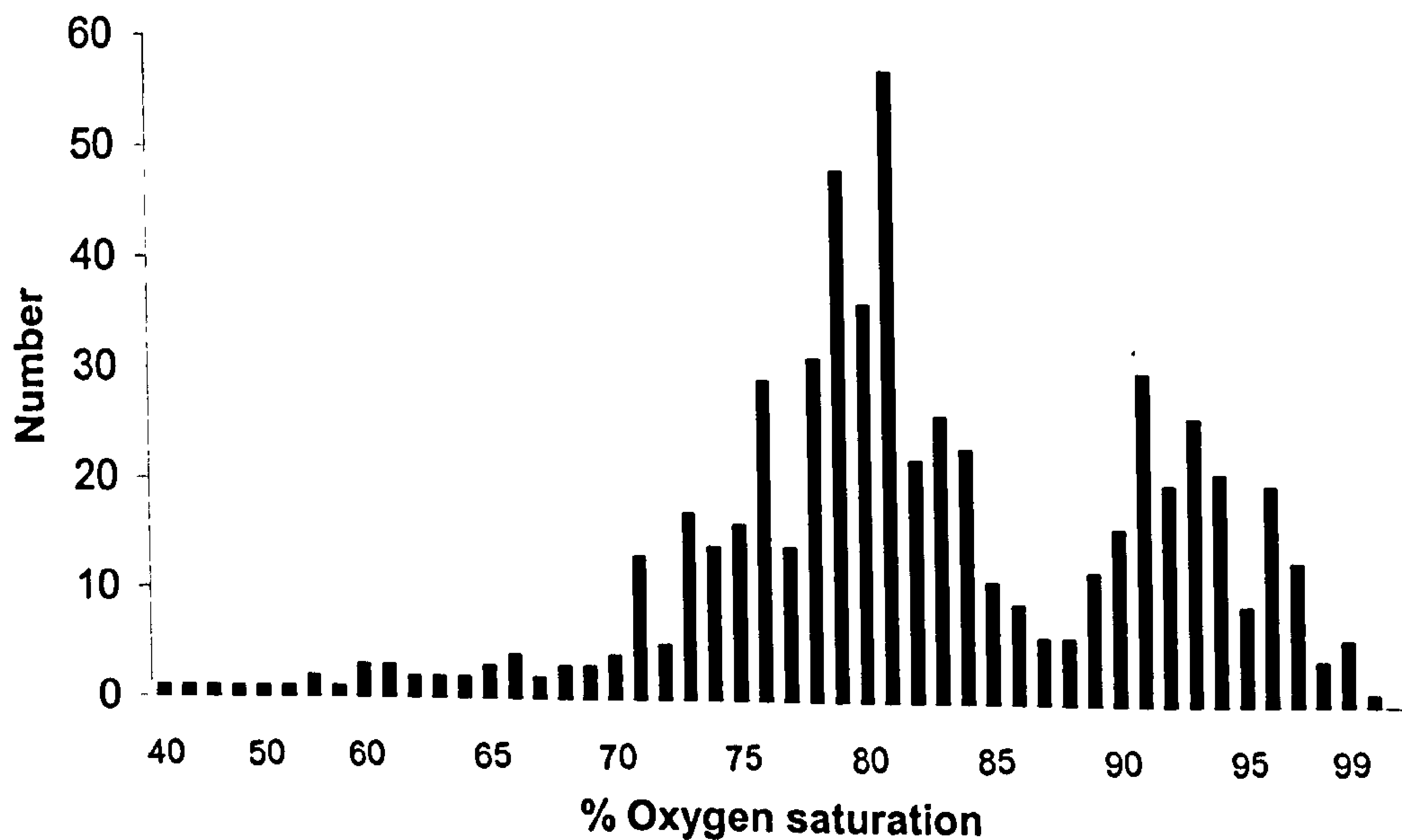


Table 3.6 History physical examination and clinical diagnosis of the children on enrolment

Characteristics		N= 601 (%)
History of:		
Cough		600 (100%)
Tachypnoea		450 (75%)
Fever		428 (71%)
Blue lips		353 (59%)
Wheeze		194 (32%)
Unable to drink		114 (19%)
Difficulty to awake		45 (7%)
Apnoea		25 (4%)
Skin rash		23 (4%)
Physical examination		
Crackles		400 (67%)
Cyanosis		361 (60%)
Chest indrawing		326 (54%)
Wheeze		175 (29%)
Fever (>37.5°C)		152 (25%)
Mean (SD) respiratory rate/minute		56 (14.4)
Hepatomegaly		31 (5%)
pO_2	Mean (SD)	81% (9.2)
	≤ 87%	423 (70%)
	88-90%	58 (10%)
	> 90%	120 (20%)
Clinical diagnosis		
	Pneumonia	220 (36%)
	Mild ARI	196 (33%)
	Bronchiolitis	185 (31%)
Chest x-ray on admission		416 (69%)

Hospital management

During the study period 1000 ARI cases were screened of these, 607 children met the inclusion criteria and were recruited for the study. Most of the children excluded did not fulfil the age for inclusion (i.e. > 2 years old) or their illness was of more than 7 days duration. About 7 cases also excluded because at that time they were considered to be bacterial pneumonia given that they had lobar consolidation. With hind sight, it would have been better to include these patients. Three hundred and twenty five (54%) children were recruited from the A&E department, 235 (39%) from the outpatient clinic; 38 (6%) from the wards and 3 from the nursery. Three hundred and seventy two (62%) children were admitted for a short time to the A&E unit until their general

condition stabilised and were then transferred to the paediatric ward. The total number of admitted and followed children was 372 of these, 2 children were admitted to the intensive care unit and ventilated.

One child was ventilated for one day and the other for two days. Three hundred and forty nine (58%) children were given antibiotics and 28 (5%) received intravenous fluid (IVF) therapy. Fourteen (2%) children had IVF for one day, 8 (1%) for 2 days, 2 for 3 days and 4 for more than 3 days. Nasogastric tube feeding (NGT) was used in 3 children. Three hundred and thirty four (56%) of the children did not receive oxygen, 59 (10%) received oxygen for one day, 60 (10%) for 2 days, 42 (7%) for three days, 41 (7%) for 4 days and 65 (11%) for five or more days. The number of days the children required oxygen ranged from 1 to 26 days. Table 3.7 shows the medications received by the children.

Table 3.7 Hospital management of the children

Children receiving	N= 601 (%)
Any treatment	400 (67%)
Antibiotics	349 (58%)
Anti-cough syrups	315 (52%)
Oxygen supplementation	267 (44%)
Antipyretics	266 (44%)
Intravenous fluids	28 (5%)
Bronchodilators	17 (3%)
Steroids	5 (1%)

Medical background

Five hundred and eighty (97%) of the children were term and twenty one infants (3%) had been born prematurely (gestational age < 37 weeks). The mean (SD) gestational age of these 21 children was 31.9 (2.4) weeks with a range of (28-35) weeks. Four (19%) out of the 21 were born at 28 weeks, 6 (28%) at 32 weeks, 1 (5%) at 33 weeks, 5 (24%) at 34 weeks and 1 (5%) at 35 weeks gestation. Table 3.8 describes the medical background of the children. Five hundred and twenty eight (88%) of the children were being breast fed at time of enrolment, 66 (11%) were not being breast fed in the past and only 7 had never been breast fed. Only 201 children (33%) had their vaccines up to date at the time of consultation and 397 (66%) were not up to date.

Fifteen (2.5%) children were classified as immunodeficient, although their HIV status was not tested. Seven had severe malnutrition and one severe malnutrition with rickets. Seven infants had severe rickets. In addition, 8 children had Down's syndrome.

Table 3.8 Medical background of the children

Characteristics	N=601 (%)
Previously breastfed	66 (11%)
Breast feeding on enrolment	528 (88%)
Partial breast feeding	289 (56%)
Exclusive breast feeding	239 (45%)
Never breast fed	7 (1%)
Mean (SD) age when weaned (months)	4 (1.6)
Vaccinations up to date	201 (33%)

Past medical history of the children enrolled

One hundred and twenty six children (21%) had a history of recurrent chest infections. Five (1%) children had a history of past bronchial asthma diagnosed by a doctor. In addition, 29 (5%) children had a history of recurrent wheeze and 24 (4%) had a history of eczema. Five children (1%) had congenital heart disease. These five patients had each Ventricular Septal Defects (VSD), VSD and Patent Ductus Arteriosus (PDA), Patent Foramen Ovale, moderate Atrial Septal Defect (ASD) with pericardial effusion and the defect was not recorded in one child. All the children had associated pulmonary hypertension. A summary of the past medical history of the patients is shown in table 3.9.

Table 3.9 Past medical history of the children

Past history of	N=601 (%)
Respiratory infections	126 (21%)
Recent wheeze	37 (6%)
Recurrent wheeze	29 (5%)
Eczema	24 (4%)
Asthma	5 (1%)
Congenital heart disease associated with pulmonary hypertension	5 (1%)

Family history of the children

Three hundred and sixty eight (61%) children had other members of the family with chest infections at the time of enrolment and 10 (2%) had a family history of tuberculosis. Sixty seven (11%) children had a family history of allergies. Six (9%) out of the 67 children with a family history of allergies had allergic conjunctivitis, 4 (6%) allergic dermatitis, 9 (13%) allergic rhinitis,

5 (8%) drug allergies, 37 (55%) food allergies, 2 (3%) had urticaria, and was not recorded in 4 (6%). Fifty seven (10%) and 37 (6%) children had a family history of asthma and eczema respectively. Four infants (1%) had a family history of congenital heart disease. A summary of the family medical history of the children is shown in table 3.10.

Table 3.10 Family medical history of the children

Family medical history	N= 601 (%)
Family member with ARI on enrolment.	368 (61%)
Allergies	67 (11%)
Asthma	57 (9%)
Eczema	37 (6%)
Tuberculosis	10 (2%)
Congenital heart disease	4 (1%)

Housing conditions of the children

Two hundred and eighty two (47%) children lived in families with a relative who smoked. In 227 (80%) cases, the smoker was the father in 2 (1%) cases the smokers were the father and the uncle and in 5 (2%) cases both the father and the mother smoked. Only in five (2%) cases was the mother the sole smoker. In three (1%) cases the whole family smoked. The mean (SD) number of rooms of the houses where the children lived was 3 (2.5) with a range of 1-36 rooms and median of three rooms. There was a mean (SD) number of 4 adults (2.7) per household with a range of 1-17 persons and a median of 3 persons. The mean (SD) number of children living in the household was 4 (3.4), with a range of 1-50 and a median of four children.

Two hundreds (33%) children were living in houses with indoor animals. Three hundred and twenty nine children (55%) lived in houses where gas was the main fuel for cooking, twenty eight (5%) used wood, two hundred and thirty four (39%) used both (gas and wood). Four households used petrol and 6 used a mixture of fuels.

The main source of water was the government's water project for 326 (54%) children. One hundred and thirty five (22%) used private water and twenty six (5%) used both types of water. One hundred and fourteen (19%) used natural sources of water (e.g. rain and spring water). Table 3.11 describe the housing conditions of the children.

Table 3.11 Housing condition of the children

Characteristics of the household		N=601
Smoker at home		282 (47%)
Mean (SD) number of	rooms	3 (2.5)
	children	5 (3.4)
	adults	4 (2.7)
Indoor pets		200 (33%)
Outdoor pets		159 (26%)
Type of fuel	gas	329 (55%)
	wood	28 (5%)
	both	234 (39%)
	others	10 (2%)
Type of water	government	326 (54%)
	private	135 (22%)
	both	26 (4%)
	natural sources	114 (19%)

Frequency of RSV, HMPV and RSV/HMPV

Six hundred and four NPA were analysed. Of these, 244 (40%) were infected with RSV; 41 (7%) with HMPV and 25 (4%) had RSV/HMPV co-infection. Two hundred and ninety four (49%) were negative for both viruses as shown in figure 3.7. The clinical data for three patients with RSV were lost, and 241 patients with RSV were analysed. Male children seem to be more frequently affected by these viruses than females. The proportion of children who were males (%) number for RSV; HMPV and RSV/HMPV co-infection were 158 (66%), 29 (71%), and 18 (72%) respectively (p= 0.7).

The number of children who had mild, moderate and severe ARI due to RSV, HMPV, RSV/HMPV co-infection and negative for both RSV/HMPV is shown in figure 3.8. Thirteen (5%) children with RSV had mild, 18 (8%) moderate and 210 (87%) severe ARI. Similarly, 12 (29%) children with HMPV infection had mild, 5 (12%) moderate and 24 (59%) severe ARI. Among the children with RSV/HMPV co-infection, 2 (8%) had mild 1 (4%) moderate and 22 (88%) severe ARI. Ninety three (32%) of the children who were both RSV and HMPV negative had mild, 34 (11 %) moderate and 167 (57%) severe ARI.

Figure 3.7 Number of children with RSV, RSV/HMPV co-infection, HMPV and negative for both viruses

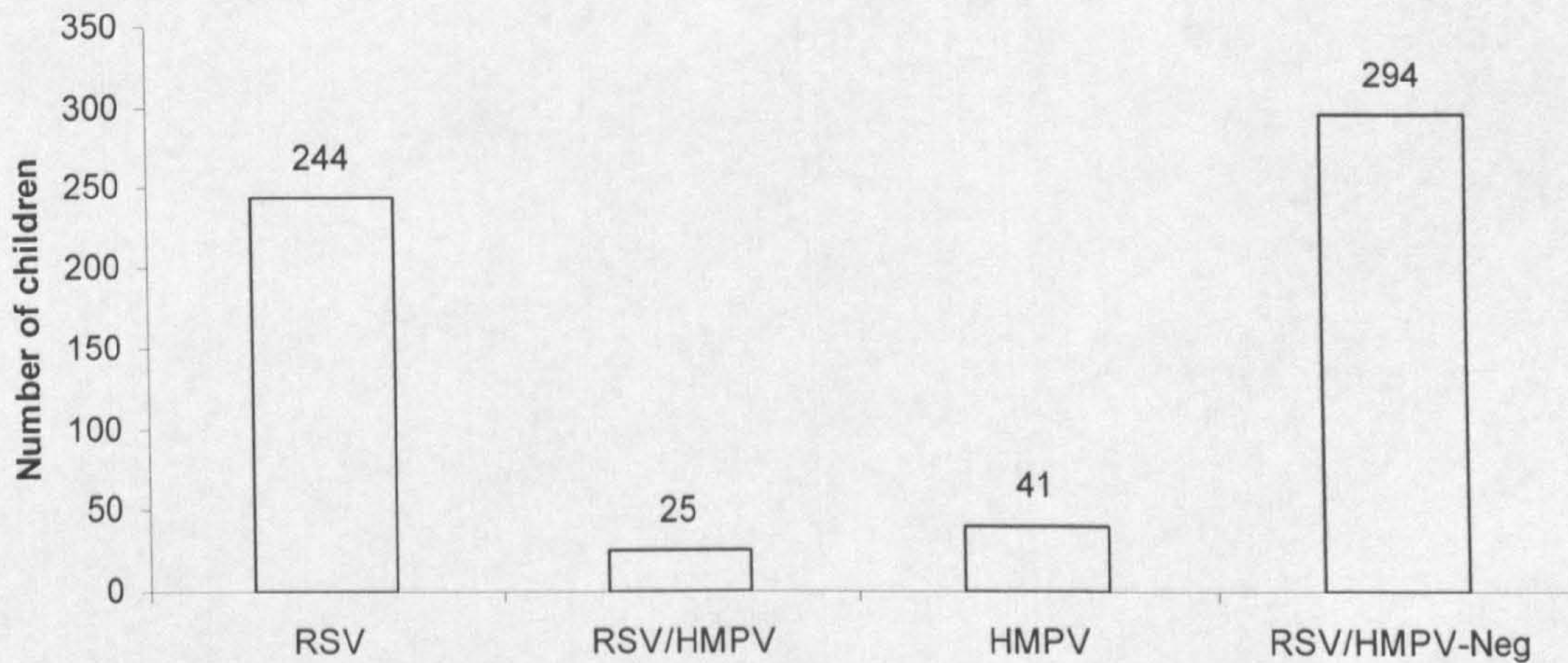
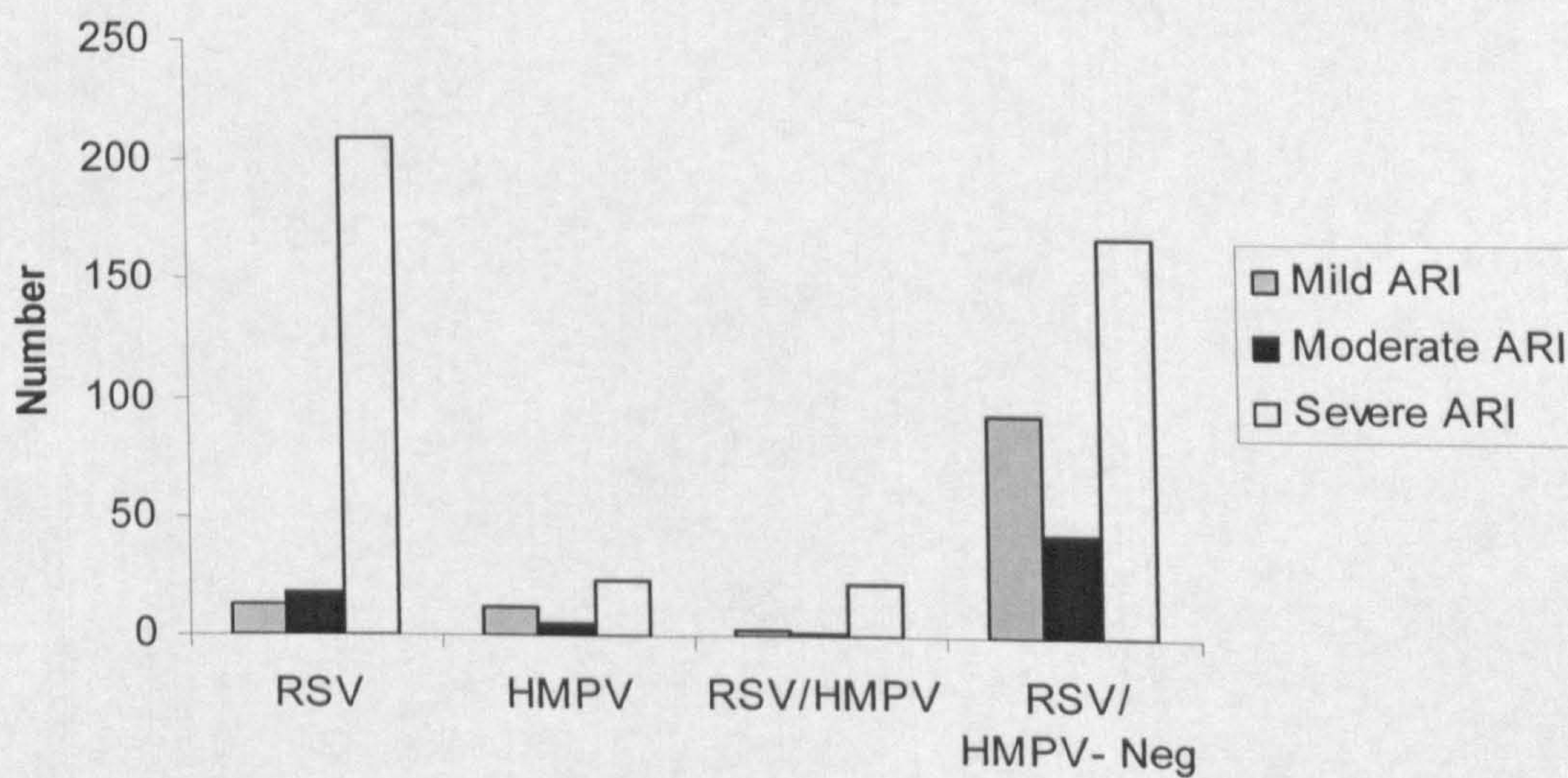


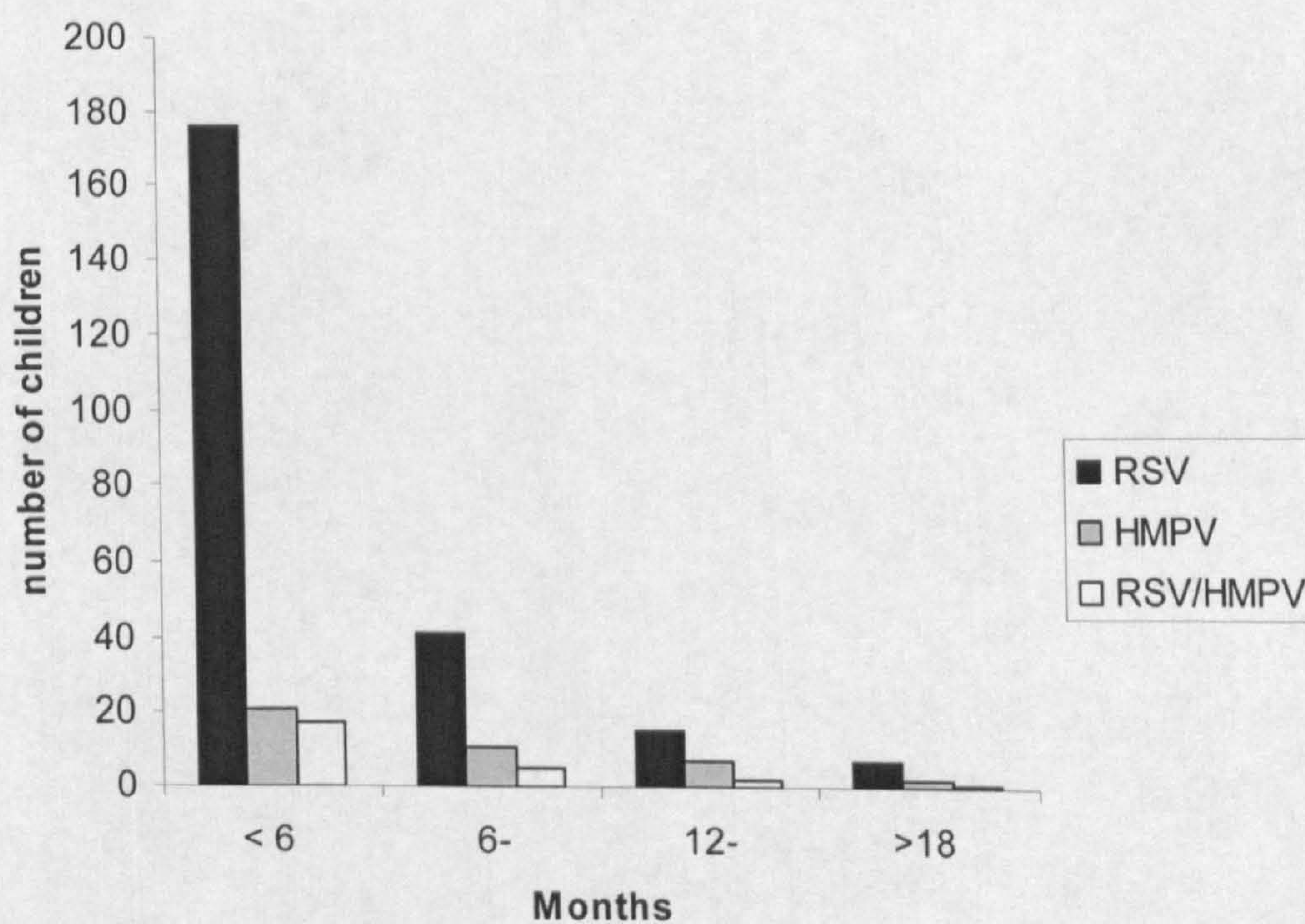
Figure 3.8 Number of children with mild, moderate and severe ARI due to RSV, HMPV, RSV/HMPV co-infection and negative for both



Age distribution of the children with RSV, HMPV, RSV/HMPV co-infection and negative for both

Children infected with HMPV were older than children with RSV or RSV/HMPV co-infection (median ages 6, 2 and 3 months respectively) ($p<0.01$). The distribution of the infants infected with RSV, HMPV, and RSV/HMPV co-infection by age is shown in figure 3.9. RSV was identified more frequently in children under 6 months of age (74%) and its prevalence decreased after this age. In contrast, HMPV infection occurred more frequently in older children, and 49% of HMPV infections occurred in children > 6 months of age. The mean (SD) of age of children with RSV, HMPV and RSV/HMPV co-infection was 4.7 (5), 7.5 (5.9) and 5.6 (5.5) months respectively ($p=0.001$).

Figure 3.9 Number of children with RSV, HMPV and RSV/HMPV co-infection by age



Seasonal variation of the viruses

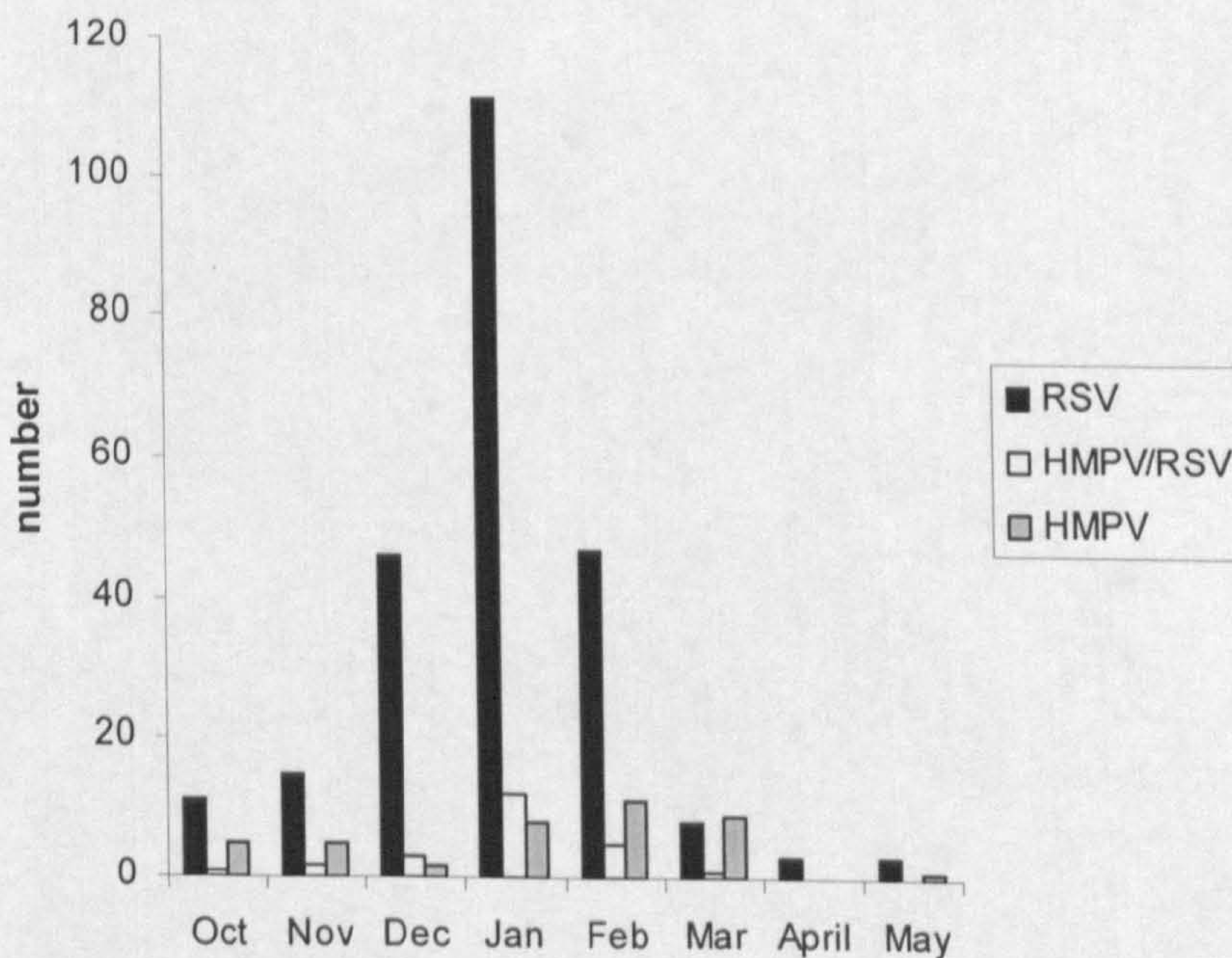
ALRI had a strong seasonal variation, increasing from October and reaching a peak in January. RSV and HMPV infections seemed to coincide in time during the study period, although HMPV

seemed to peak later than RSV. RSV infections peaked in January while HMPV peaked in February and March (Figure.3.10).

Clinical history on admission

The characteristics of the children with RSV, HMPV and RSV/HMPV co-infection are described in table 3.13. Patients infected with either RSV or HMPV had similar frequencies of cough and fever before consultation. The mothers of the children with RSV infection however reported that their children were wheezing in (42%) had blue lips (73%) and tachypnoea (90%), more frequently than mothers of children with HMPV who reported wheeze in (15%), blue lips (56%) and tachypneoa (59%) and the difference is statistical significant ($p < 0.05$).

Figure 3.10 Number of children with RSV, HMPV, RSV/HMPV co-infection by month (2002-2003)



Nutritional Status

The nutritional indicators of children with RSV, HMPV, RSV/HMPV and RSV/HMPV-Negative are shown on table 3.12. It showed that 92 (39%), 15 (37%), 8 (32%) and 143 (49%) of children with RSV, HMPV, RSV/HMPV co-infection and RSV/HMPV-Negative respectively their weight

for age Z-score were less than 2 (p=0.04), indicating that about the third of children with RSV, HMPV and RSV/HMPV coinfection had acute malnutrition. The mean weight for age Z score was -1.9, -2, -1:6 and -2 for children with RSV, HMPV, RSV/HMPV co-infection and RSV/HMPV-Negative respectively (P=0.06).

Table 3.12 Nutritional indicators of the children by RSV, HMPV and RSV/HMPV co-infection

Nutritional indicators		RSV N=241	HMPV N=41	RSV/HMPV Co infection N=25	Negative PCR N=294	*Local demographic Health survey data
Full term		230 (95%)	38 (93%)	24 (96%)	288 (98%)	
Mean (SD) body weight		4.9 (1.8)	6.1 (1.9)	5.5 (2.1)	5.5 (2)	
Mean (SD) WAZ score		-1.9 (2.68)	-2.0 (2.1)	-1.6 (1.3)	-2 (3.9)	
WAZ	< -2	92 (39%)	15 (37%)	8 (32%)	143 (49%)	38%
	-2 –to -1	83 (34%)	14 (34%)	9 (36%)	87 (29%)	
	-1 to 0	53 (22%)	11 (27%)	7 (28%)	50 (17%)	
	> 0	13 (5%)	1 (2%)	1 (4%)	14 (5%)	
Mean height (SD)		61 (35.2)	63 (10.5)	60 (9.6)	60.5 (9.6)	
Mean WHZ (SD) score		-1.7 (3.4)	-9.4 (3.5)	-7.3 (2.1)	-1.3 (3.5)	
WHZ	> 0	239 (99%)	41 (100%)	25 (100%)	293 (100%)	84%

*(CSO, 1996)

Table 3.13 Characteristics of the children with HMPV, RSV and RSV/HMPV co-infections

History of:	RSV N=241	HMPV N=41	RSV/HMPV Co infection N=25	Negative PCR N=294
Cough	241 (100%)	40 (98%)	25 (100%)	294 (100%)
Difficulty of breathing*	220 (91%)	26 (63%)	22 (88%)	183 (62%)
Fever	178 (74%)	33 (80%)	19 (76%)	198 (67%)
Blue lips*	177 (73%)	23 (56%)	19 (76%)	134 (46%)
Wheeze *	102 (42%)	6 (15%)	14 (56%)	72 (24%)
Difficulty to awake	23 (10%)	2 (5%)	3 (12%)	17 (6%)
Unable to drink	54 (22%)	6 (15%)	4 (16%)	50 (17%)
Apnoea	15 (6%)	0 (0%)	2 (8%)	8 (3%)
Skin rash	11 (5%)	1 (2%)	1 (4%)	10 (3%)

*p<0.05

Physical examination on admission

A summary of the clinical finding on examination on admission are shown in table 3.14. Children with RSV have faster respiratory rate (61 breaths / minute) than children with HMPV

(50) and children with RSV/ HMPV infection, (57 breaths / minute). The clinical diagnoses made by the attending doctors reflected these observations as 46% and 39% of the patients with RSV alone were diagnosed as having pneumonia and bronchiolitis compared to 20% and 29% of the patients with HMPV infection alone respectively ($p<0.001$ for both). These diagnoses were in agreement with the degree of hypoxia, as children with RSV and RSV/ HMPV co-infection had more severe disease than children with HMPV alone. Children with HMPV/RSV co-infections or single RSV infection had very low oxygen saturation with means of 76% and 79 % respectively compared to 84% in children with HMPV infection (p 0.0000). Cyanosis, chest indrawing, wheeze, crackles and pallor were recorded more frequently in children with RSV/HMPV and RSV infection than in children with HMPV infection. These differences are statistically significant.

Table 3.14 Clinical characteristics of the children with RSV, HMPV, and RSV/HMPV co-infection

Physical examination		RSV N=241	HMPV N=41	RSV/HMPV Co infection N=25	Negative PCR N=294
Tachypnoea		220 (91%)	26 (63%)	22 (88%)	182 (62%)
Crackles*		201 (83%)	21 (51%)	22 (88%)	156 (53%)
Cyanosis*		186 (77%)	17 (41%)	21 (84%)	137 (47%)
Chest indrawing*		173 (72%)	17 (41%)	19 (76%)	117 (40%)
Wheeze *		94 (39%)	9 (22%)	9 (36%)	63 (21%)
Fever (>37.5°C)		182 (76%)	30 (73%)	21 (84%)	216 (74%)
Mean (SD) respiratory rate/minute*		61 (13)	50 (15)	57 (15)	54 (14)
Hepatomegaly		9 (4%)	3 (7%)	1 (4%)	18 (6%)
Splenomegaly		3 (1%)	0 (0%)	1 (4%)	9 (3%)
Pallor*		164 (68%)	16 (39%)	18 (72%)	124 (42%)
pO_2	Mean (SD)*	79% (8)	84% (8.2)	76% (8.6)	83 % (9.6)
	≤ 87%	210 (87%)	24 (59%)	22 (88%)	167 (57%)
	88-90%	18 (8%)	5 (12%)	1 (4%)	34 (11%)
	> 90%	13 (5%)	12 (29%)	2 (8%)	93 (32%)
Clinical diagnosis					
	Bronchiolitis*	93 (39%)	12 (29%)	8 (32%)	72 (24%)
	Pneumonia*	111 (46%)	8 (20%)	14 (56%)	87 (30%)
	Mild ARI*	37 (15%)	21 (51%)	3 (12%)	135 (46%)
Chest x-ray	taken (%) *	205 (85%)	23 (56%)	22 (88%)	166 (56%)
	Hyperinflation	165 (82%)	18 (78%)	16 (73%)	122 (74%)
	Patchy change	192 (94%)	19 (83%)	22 (100%)	152 (92%)

*$p<0.05$

Hospital management

One hundred and eighty six (77%) children with RSV infection, 20 (80%) with RSV/HMPV co-infection but only 19 (46%) with HMPV were admitted to the hospital and followed up ($p<0.01$). Table 3.15 shows the treatment that children receive. Twenty (80%) children with RSV/HMPV co-infection received antibiotics compared to 174 (72%) of the children with RSV infection, 18 (44%) of children with HMPV and 137 (47%) of children negative for both viruses.

The case fatality rate between each group of these admitted cases were 6 (2%) children with RSV infection, 1 (2%) child with HMPV infection, none in children with RSV/HMPV co-infection, and 5 (2%) children with negative PCR for both viruses, confirming that HMPV can also cause fatalities in young children.

Table 3.15 Hospital management of the children with RSV, HMPV and RSV/HMPV co infection

Children receiving	RSV N=241	HMPV N=41	RSV/HMPV N=25	RSV/HMPV Negative N=294
Any treatment	166 (69%)	23 (56%)	15 (60%)	196 (67%)
Antibiotics *	174 (72%)	18 (44%)	20 (80%)	137 (47%)
Anti-cough	136 (56%)	21 (51%)	10 (40%)	148 (50%)
Oxygen supplementation	134 (56%)	13 (32%)	14 (56%)	106 (36%)
Antipyretics	104 (43%)	13 (32%)	12 (48%)	137 (47%)
Intravenous fluid therapy	16 (7%)	0 (0%)	0 (0%)	12 (4%)
Bronchodilators	6 (2%)	1 (2%)	0 (0%)	10 (3%)
Steroids	4 (2%)	1 (2%)	0 (0%)	0 (0%)

* $p<0.05$

Medical background of the children with RSV, HMPV and RSV/HMPV infection

The medical background of the children with RSV, HMPV and RSV/HMPV co-infection were shown in table 3.16. There are significant differences between the numbers of children who exclusively breast fed, less number of children with HMPV infection 12 (33%) who were breast fed exclusively in comparison to 116 (52%) children with RSV infection, 8 (36%) children with RSV/HMPV co-infection and 103 (41%) children negative for both viruses. Only 72 (30%) of children with RSV infection were vaccinated compared to 24 (59%) children with HMPV infection, 10 (40%) children with RSV/HMPV co-infection and 98 (33%) children negative for both viruses. These differences reach statistical significance.

Table 3.17 revealed the history of children with RSV, HMPV and RSV/HMPV infection. A history of asthma was recorded more frequently in children with HMPV infection 3 (7%) children, compared to 2 (1%) children with RSV infection and none in the children with RSV/HMPV co-infection ($p<0.001$).

Table 3.16 Medical background of the children with RSV, HMPV and RSV/HMPV infection

Characteristics	RSV N=241	HMPV N=41	RSV/HMPV N=25	RSV/HMPV Negative N=294
Previously breastfed	15 (6%)	4 (10%)	3 (12%)	44 (15%)
Breast feeding on enrolment	222 (92%)	36 (88%)	22 (88%)	248 (84%)
Partial breast feeding	106 (48%)	24 (67%)	14 (64%)	145 (59%)
Exclusive breast feeding	116 (52%)	12 (33%)	8 (36%)	103 (41%)
Never breast fed	4 (2%)	1 (2%)	0 (0%)	2 (1%)
Mean (SD) age when weaned (months)	3.46 (1.54)	3.36 (2.6)	3.75 (1.48)	3.60 (1.71)
Vaccinations up to date*	71 (30%)	22 (54%)	10 (40%)	98 (33%)

*p<0.5

Table 3.17 Past histories of the children with RSV, HMPV and RSV/HMPV co-infection

Past history of	RSV N=241	HMPV N=41	RSV/HMPV N=25	RSV/HMPV Negative N=294
Recurrent Respiratory infections	41 (17%)	12 (29%)	5 (20%)	68 (23%)
Recent wheeze	12 (5%)	4 (10%)	2 (8%)	19 (6%)
Recurrent wheeze	11 (5%)	3 (7%)	2 (8%)	13 (4%)
Eczema	11 (5%)	1 (2%)	1 (4%)	11 (4%)
Asthma *	2 (1%)	3 (7%)	0 (0%)	0 (0%)
Congenital heart disease associated with PH	2 (1%)	0 (0%)	0 (0%)	1 (1%)

*PH= pulmonary hypertension, *p<0.05

Family history of children with RSV, HMPV and RSV/HMPV infection

The family history of the children was shown in table 3.18. A family history of other members with ARI in the time of enrolment of the child in the study were recorded more in children with RSV and RSV/HMPV 69%, 64% respectively compared to 46% in children with HMPV infection.

Table 3.18 Family history of the children with RSV, HMPV and RSV/HMPV co-infection

Family medical history	RSV N=241	HMPV N=41	RSV/HMPV N=25	RSV/HMPV Negative N=294
Family member with ARI *	167 (69%)	19 (46%)	16 (64%)	166 (56%)
Allergies	31 (13%)	7 (17%)	1 (4%)	28 (10%)
Asthma	27 (11%)	4 (10%)	2 (8%)	24 (8%)
Eczema	13 (5%)	5 (12%)	1 (4%)	18 (6%)
Tuberculosis	5 (2%)	0 (0%)	0 (0%)	5 (2%)
Congenital heart disease	2 (1%)	0 (0%)	1 (0%)	1 (0.3%)

*p<0.05

Table 3.19 describe the social history of the participating children. There were no significant differences despite the fact that there were higher numbers of children and adults living in the same houses of children with RSV and RSV/HMPV than in children with HMPV. The indoor and outdoor animal contact was observed more frequently in children with RSV and RSV/HMPV co-infection in comparison to children with HMPV. Generally, the housing conditions of children with HMPV were much better than those of children with RSV or RSV/HMPV co-infection. This may explain to some extent why severe ARI is more common in RSV or RSV/HMPV co-infection than HMPV infection.

Table 3.19 Housing condition of the children

Characteristics of the household		RSV N=241	HMPV N=41	RSV/HMPV N=25	RSV/HMPV Negative N=294
Mean (SD) number of	rooms	3.5 (3%)	3 (2%)	3.3 (1%)	2.3 (2%)
	children	4.7 (4%)	5 (4%)	4.4 (2%)	4.2 (3%)
	adults	3.9 (3%)	3.5 (3%)	4.1 (2%)	3.7 (3%)
Smoker at home		113 (47%)	22 (54%)	16 (64%)	130 (44%)
Indoor pets		96 (40%)	9 (22%)	10 (40%)	85 (29%)
Outdoor pets		81 (34%)	6 (15%)	9 (36%)	63 (21%)

The RSV N genotypes were determined in 208 (77%) of the samples; of these 171 (82%) were group A and 37 (18%) group B. One hundred fifty one out of the 171 (81%) were group A in single RSV infection and 35 (19%) group B. In comparison, 20 (91%) out of the 25 patients with RSV/HMPV co-infection were group A RSV and 2 (9%) with group B (p=0.8) (Figure 3.11).

Group A RSV predominated this outbreak. The distribution of the group A and B over mild, moderate or severe infection was demonstrated in figure 3.12 and table 3.20. There is no statistically significant difference. Children with severe ALRI seemed to have a higher prevalence of group B than group A, but this was not statistically significant.

Table 3.20 Frequency of group A and B in children with mild, moderate and severe ARI due to RSV and HMPV/RSV infections

		Group A	Group B	P value
RSV	mild	10 (7%)	2 (6%)	0.7
	moderate	13 (8%)	3 (8%)	
	severe	128 (85%)	30 (86%)	
RSV/HMPV co-infection	mild	2 (10%)	0 (0%)	0.9
	moderate	1 (5%)	0 (0%)	
	severe	17 (85%)	2 (100%)	

Figure 3.11 Number of children with group A or B RSV

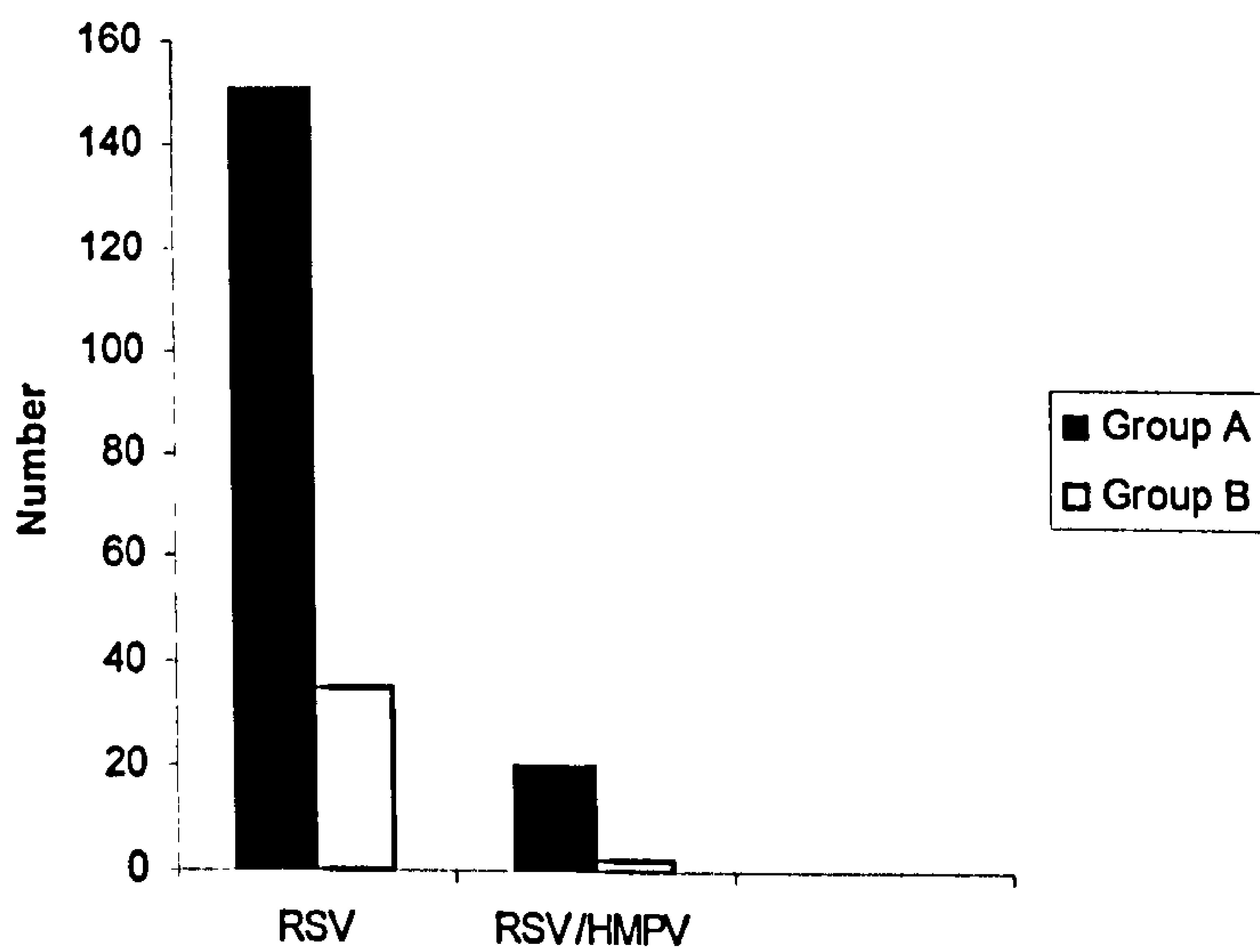
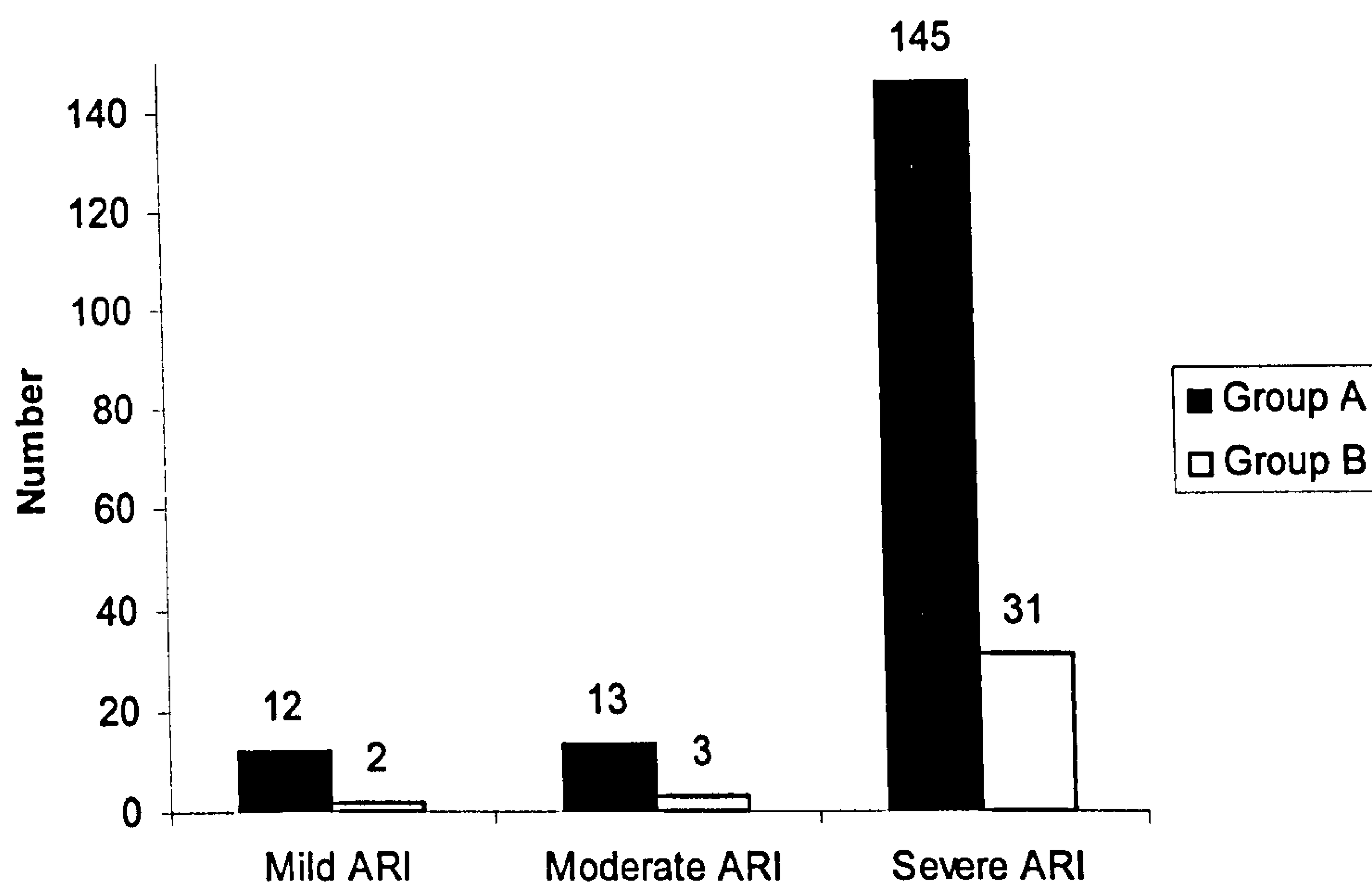


Figure 3.12 Proportion of children with group A and B in children with mild, moderate and severe ARI



NP genotypes were established for 208 (77%) of the 269 RSV positive samples. Eleven (5%) were RSV NP1, 96 (46%) NP2, 25 (12%) NP3 and 75 (36%) NP4 genotype.

The frequency of the genotypes by ARI severity is shown in table 3.21. In the single RSV infection group NP1 and NP3 (both belonging to group B) were associated more with the severe RSV infection with statistical significance (p=0.03).

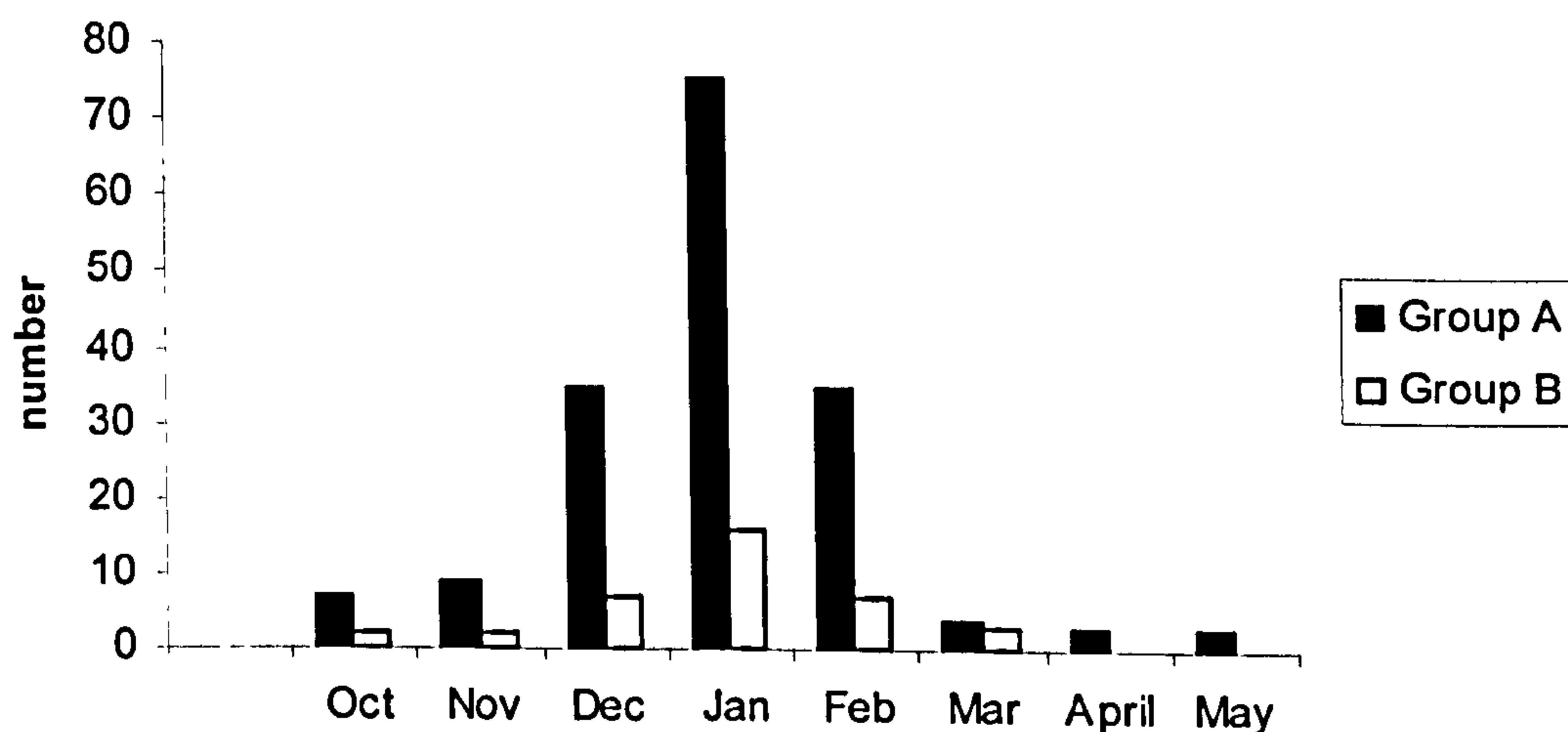
Table 3.21 Frequency of RSV genotypes in children with mild, moderate and severe ARI due to RSV and HMPV/RSV infections

		NP1 N=11	NP2 N=96	NP3 N=26	NP4 N=75
RSV *	mild	2 (18%)	2 (2%)	0 (0%)	8 (11%)
	moderate	1 (9%)	8 (10%)	2 (7%)	5 (7%)
	severe	8 (73%)	73 (76%)	22 (85%)	55 (73%)
RSV/HMPV coinfection	mild	0 (0%)	1 (1%)	0 (0%)	1 (1%)
	moderate	0 (0%)	1 (1%)	0 (0%)	0 (0%)
	severe	0 (0%)	11 (11%)	2 (7%)	6 (8%)

P<0.05

The distribution of the RSV groups per study month is shown in figure 3.13. It appears that although group A predominate during the first part of the study period in comparison to group B that disappeared during the last two months (April and May). Group B seemed to be most common during January.

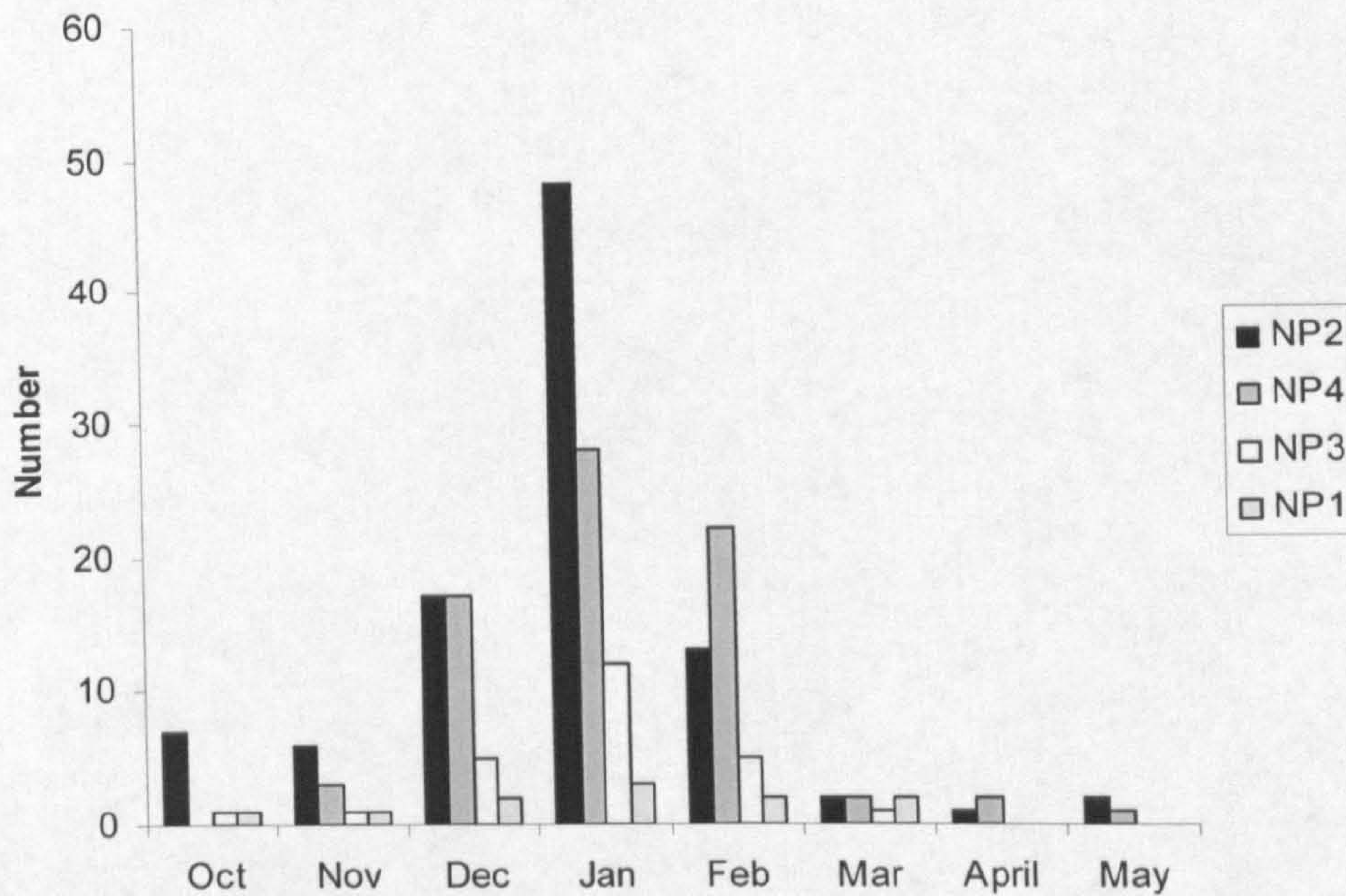
Figure 3.13 Distribution of RSV groups by month (2002-2003)



The distribution of the genotypes per study months was shown in figure 3.14.

NP2, NP3 and NP4 seem to peak during January. NP2 and NP4 were identified in all 8 months of the study but NP1 and NP3 were absent during the last 2 months (April and May).

Figure 3.14 The distribution of RSV genotypes per study month in 2002-2003



A few RSV samples had unusual restriction NP profiles. These samples were sequenced and deposited in European Bioinformatics Institute (EMBL-EBI) as shown table 3.22.

Table 3.22 Sequence accession numbers for RSV

Sample No	Accession No	Type of virus	Ref.
313	BDO81928.1	Group B attenuated strain	(Udem, 2001)
220, 292, 314,243, 360	X00001.1	NP2 point mutation introducing 2nd *Rsa* restriction site	(Elango and Venkatesan, 1983)

Sample No 313 is an attenuated strain of group B (Udem, 2001). This strain was used in the 1960s to vaccinate children (Kim et al., 1971). This specimen was obtained from a child with severe RSV infection who was admitted to the hospital, confirming that this virus can cause severe ARI. The NP amplicon sequences of samples 220, 243, 292, 314 and 360 demonstrated a point mutation introducing a second *Rsa* restriction site in the NP2 genotype.

Discussion

General characteristics of the population

A total of 604 children under 2 years of age who attended with ARI at Alsabeen Hospital for Women and Children, Sana'a were studied prospectively for RSV and HMPV from October 2002 to May 2003. There were 395 (66%) males and 206 (34%) females including 105 (17%) neonates, 186 (31%) children aged from 1-3 months, 104 (17%) from 3-6 months, 129 (22%) from 6-12 months, 55 (9%) from 12-18 months and 22 (4%) from 18 -24 months. A total of 325 (54%) children were recruited from the A&E department, 235 (39%) from the outpatient clinic, 38 (6%) from the wards and 3 from the nursery.

Weight for age and weight for height were taken as the indicators of nutritional status. Malnutrition is quite prevalent in Yemen, the 1996 local multiple cluster survey in Yemen found that 45% of children had <-2 HAZ, 38% had <-2 WAZ and 16% had <-2 WHZ (CSO, 1996). Although the nutritional survey included older children who are more likely to be chronically malnourished than our study population, the high percentage (43% of the 601 children) with age Z scores below - 2 in this study was not surprising. All of them had weight for height Z scores > 0 indicating that most children had suffered acute malnutrition.

Five hundred and thirty two (88%) of the children were being breast fed at time of enrolment and 201 children (33%) had their vaccines up to date at the time of consultation.

Premature birth and immunocompromised status are known risk factors for severe RSV illness. In the current study, there were 21 (4%) premature infants and 21 (4%) immunocompromised children including 8 children with Down's syndrome.

The enrolled children were living in houses with median number of 3 rooms and living with median number of 4 adults and 5 children, indicating that these children were living in crowded conditions. In addition, in 282 (47%) cases there was a family member who smoked (in 80% the father was the smoker). About a third of the enrolled children had indoor animals and in another third there were outdoor animals. For about half (55%) of the families of the enrolled children, gas was the main fuel for cooking and the main source of their water was the government's water project.

A wide spectrum of clinical symptoms involving both the upper and lower respiratory tract has been reported from this group of patients, including cough, tachypnoea, fever, having blue lips, wheezing, being unable to drink and having difficulty to be awaked and signs on physical examination including crackles, cyanosis, chest indrawing and wheezing on auscultation.
The resulting clinical diagnoses ranged from mild upper respiratory infection to bronchiolitis and pneumonia, and some patients were admitted to intensive care unit and required ventilation.

The pO_2 cut off point of ≤ 87% was chosen to classify the study population into mild and severe ARI as Sana'a is situated at a high altitude (2,200 metres above the sea level) (Duke et al., 2002) This is the cut of point of oxygen saturation after which the oxygen dissociation curve is steep (McConnochie et al., 1990). One hundred and seventy eight children (30%) were diagnosed as having mild ARI and 423 (70%) severe ARI. This unusual distribution reflects the characteristics of a tertiary hospital receiving more patients with severe than mild ARI as the health centres treat most children with mild infections and families often come late to the hospital. A pO_2 of 90% is the cut- off point for diagnosing hypoxia in most of regions (Hornsleth et al., 1998), the study population was subdivided into three groups: 423 (70%) with $pO_2 \leq 87\%$, 58 (10%) 88-90%, 120 (20%) >90%. However, 53 (9%) children had pO_2 <70%, 267 (44%) 70-80%, 103 (17%) 80-88%, 104 (17%) 88- 93% and 74 (12%) >93%.

Prevalence of RSV

RSV is responsible of about 50% - 80% of ALRI around the world, (Shay et al., 1999). It is recognized as an important contributor to hospitalization. Despite its importance, there is no information on the prevalence of RSV in Yemen. Our findings thus confirm that RSV is the most frequent pathogen causing ARI in Yemen.

Considering the predominance of children under 1 year enrolled in this study it was not surprising to find RSV as the virus detected most frequently accounting for 40% of infants with ARI mainly severe LRTI (87%) with hospitalisation rate of 31%. This frequency of RSV was higher to that found by other researchers from the same region, including the United Arab Emirates (29%), Saudi Arabia (29%) and Jordan (26%) (Bdour, 2001, Uduman et al., 1996, al-Hajjar et al., 1998). However these are less than some results from the other areas in proximity to Yemen such as, in Saudi Arabia (54%) and North Jordan (50%) (Meqdam et al., 1998, Jamjoom et al., 1993) or reports from other part of the world such as Argentina (41%) (Galiano et al., 2004). Also our results are in agreement to some reports from other part of the world, such as in Sudan (28%), the

Philippines (26%), and Pakistan (33%) (Capeding et al., 1994, Salih et al., 1994, Ghafoor et al., 1990).

Other reports showed far lower frequencies than our results. Frequency rates of 9 % – 19% were reported in Mozambique (9%); Germany (12%); Zambia (14%); Norway (15%) and the Gambia (19%) (Saijo et al., 1998, Roca et al., 2001, Weber et al., 2002, Weigl et al., 2001, Dollner et al., 2004).

The differences in prevalence of RSV in these reports from a round the world might be due to differences in geographical location and climate and the differences in the methods of RSV detection. Some studies used immunological procedures like indirect immunofluorescent assay and direct fluorescent antibody, others used viral culture and some used PCR. Also they varied in their use of one or combination of these methods. The differences in socioeconomic status and the standard of health care and the availability of some preventive measures like immunoglobulin, which is expensive, may also play a very important role in this variability.

Prevalence of RSV groups

In general, Group A predominate RSV outbreaks in different geographical regions. The higher prevalence of group A in Yemen may reflect its higher worldwide predominance (Hall et al., 1990). The present study revealed that group A was the predominating group over the study period; it represented 82% of the RSV samples that were genotyped. Data on the frequency of group A and B in the Middle East and Africa have been scarce. In Jordan, group A predominanted over 3 consecutive years from January 1997 to May 1999 (Bdour, 2001), and in 2001 in South Africa group A predominated in two seasons during 1997-2000 while group A and B co-circulated approximately equally in the other seasons of the study (Venter et al., 2001). Another study from South Africa during the year 2000 showed that 93% of the isolated RSV groups were group A, while during 2001 group A and B nearly equally co-circulated (Madhi et al., 2003b). Group A was also predominant in a study conducted in Vienna, Austria and Zagreb, Croatia (Lukic-Grlic et al., 1998). In Liverpool, group A was predominant for three years over four seasons from 1990 to1992 (Fletcher et al., 1997). This is however not universal, as group B RSV was the most frequent genotype in Mozambique in 1999 (Roca et al., 2001).

Prevalence of RSV genotypes

Only 208 RSV positive specimens could be genotyped, the restriction type of 6 specimens did not fit into the scheme developed by (Cane et al., 1992). These samples after sequencing one was found to be most closely related to an the attenuated group B RSV strain that was used for vaccination of children in the early 1960s (Udem, 2001) and 5 were found to be group A NP2 but with two point mutation introducing a 2nd *Rsa* restriction site (Elango and Venkatesan, 1983). In the present study NP2 genotype was the predominant one (46%) followed by NP4 (36%) then NP3 (13%) and finally NP1 (5%). In comparison to a study from Japan by (Seki et al., 2001) during the period from December 1980-January 1995, in which 125 group A were isolated, NP4 was predominated from the third season to the last one, while the first two seasons were dominated by NP5. NP4 was also predominant in Liverpool, UK in a study by (Greensill et al., 2003).

Genetic variability

Restriction mapping and sequencing of the isolates showed tremendous genetic variability of the N gene. These confirm the genetic variability among group A and B RSV isolates which has been demonstrated previously (Cane and Pringle, 1995a, Roca et al., 2001). Although the present study was done over only one season 2002-2003, i.e. a limited time leading to difficulty to in commenting on the genotype variability over seasons we can show that in the same season there can be variability from one month to another. We found that although NP2 and NP4 were seen throughout the study period, NP4 was not isolated in the beginning of the outbreak i.e. during October, and NP1 and NP3 were not identified during the last 2 months of the study April and May however, they peaked during March. NP2 peaked during December and January, while NP4 peaked during January and February. This change in the same location is in agreement with observations from other regions (Choi and Lee, 2000, Cane et al., 1991, Peret et al., 1998). Although the duration of this study is limited data obtained showed the occurrence of shifts in RSV genotype distribution, which is a consistent feature of the epidemiology of RSV infections.

Prevalence of HMPV

ARI are the leading cause of hospital admission in Yemen. This is the first study of HMPV from the Arabian Peninsula and confirms that it is an important cause of respiratory infection in this geographical area. HMPV seems to be the second most frequent virus causing ALRI in children

(Boivin et al., 2002), causing about 11% of the cases although with milder clinical presentation than RSV.

The prevalence of HMPV is unknown. Infected patients were identified either retrospectively (Peret et al., 2002, van den Hoogen et al., 2001, Boivin et al., 2002, Galiano et al., 2004, Stockton et al., 2002) or by random screening of respiratory infection (Nissen et al., 2002). The reports of HMPV in most of the continents suggest worldwide distribution; however the epidemiology of HMPV remains to be defined. Since the presence of HMPV in Yemen is unknown, this study aimed to determine whether HMPV was circulating in Sana'a during 2002 - 2003.

Our study detected HMPV in 11% of the patients mainly with mild ARI (41.5%). This detection rate was analogous to results of previous studies in the Argentina (11%), Netherlands (10%) and Australia (10%), (Mackay et al., 2003, van den Hoogen et al., 2001, Galiano et al., 2004).

In contrast to our results, the frequency of HMPV was lower in studies conducted in Canada; England and USA (2%, 2% and 6% respectively), which included children as well as young adults and elderly (Boivin et al., 2002, Falsey et al., 2003b, Semple et al., 2005, Stockton et al., 2002)

The variations in the detection rates among these studies may be due to the differences in the study design, the study population's age, season, type of sample, and the detection method. Different HMPV genes such as L, M, N and F have been used as targets for various PCR methods. Variability of these genes may affect the sensitivity of these approaches, especially for this newly described virus for which limited sequence information is available.

Thus it is important to establish the extent of RSV and HMPV disease in Yemen in order to plan cost effective prevention strategies like health awareness and health educational programmes since the other strategies like vaccines, immunoglobulin are expensive.

HMPV as a co-infecting agent with RSV

HMPV and RSV share seasonality and susceptible populations; therefore, it is not surprising that co-infections are detected at a rate of 1 to 3% in various sample sets. In this study the HMPV co-infection detection rate was 4% of children with RSV bronchiolitis. Twenty two children developed severe disease while only three have mild illness.

Group A RSV were the most frequent group that were found as a co-infection with HMPV compared with group B (91% vs. 9%); this is in agreement with others (Greensill et al., 2003).

HMPV co-infection was detectable with each of group A genotypes; nevertheless, NP2 is the predominant co-infecting genotype of RSV with HMPV. There were some group B genotypes associated only with the severe disease and all were NP3 genotype. However, it is uncertain whether co-infections predispose to more severe disease but might raise the possibility of increasing the risk for severity for RSV disease. Some studies have suggested that RSV and HMPV co-infection increases the severity of the ARI episodes Greensill et al. reported 70% co-infection rate of HMPV and RSV and 90% co-infection rate among ventilated infants with HMPV and RSV admitted to the pediatric intensive care unit (Greensill et al., 2003). Although the authors did not include an appropriate control group in their study, this finding suggests that co-infection with both HMPV and RSV is common and that together the two viruses may contribute to increase the severity of the disease. A subsequent study confirmed this observation (Semple et al., 2005). In contrast others have suggested that the co-infection may be protective (Cuevas et al., 2003). Although a higher proportion of the children with co-infections had severe hypoxia than children with RSV alone, this was not statistically significant and whether this is a significant risk factor for severity is still undecided. However, in a study of 23 children with severe RSV ARI and 23 mild RSV ARI, HMPV did not contribute to the severity of RSV as none of the 46 RSV-infected children tested positive for HMPV (Lazar et al., 2004).

Seasonality of RSV and HMPV

Whereas some of the respiratory viruses, such as parainfluenza and rhinoviruses may circulate throughout the year, others such as RSV and HMPV circulate mainly during the winter season in temperate regions and in the late spring-summer season, also called the respiratory season, in the subtropics (Cuevas et al., 2003, Esper et al., 2004).

The results of our study showed that RSV and HMPV circulated widely over the same period. The RSV and HMPV epidemic periods lasted from October to May, even though HMPV and RSV were detected throughout the study period, HMPV peaked during February and March and RSV during December and January. This is in agreement with studies from the Northern hemisphere where HMPV has been reported to peak in the first months of the year (Esper et al., 2004), while in the Southern hemisphere the peaks of HMPV have most often been reported in

April and May (Cuevas et al., 2003, McAdam et al., 2004) , or in September, October and November (Galiano et al., 2004).

Most of these studies however have focused on the months of the year with the highest incidence of ARI and to coincide with the peak incidence of RSV. It is thus necessary to conduct more longitudinal and comprehensive studies to determine the seasonal variation of HMPV around the world. For example, In New York, RSV and HMPV peaked at different times of a two year study (Williams et al., 2004) and in Brazil, RSV and HMPV coincided temporally in 2002 but not in the 2003 peak RSV season (Serafino et al., 2004) and in an Italian study HMPV was detected considerably less frequently in hospitalized children younger than 2 years of age in 2001 than in 2000 and 2002 (7% vs. 37 and 43%) (Maggi et al., 2003).

Clinical Characteristics of children with RSV, HMPV and RSV/HMPV co-infection

The present study identified 244 (40%) children who were infected with RSV; 41 (7%) with HMPV and 25 (4%) had RSV/HMPV co-infection. Two hundred and ninety four (49%) were negative for both viruses.

There was no significant difference in the affected sex, although males were more frequently affected by both viruses. This is in agreement with a studies from Jordan and Gambia (Bdour, 2001, Weber et al., 2002) where more males were affected by RSV than females and study by (Esper et al., 2004) where more males (66%) were affected by HMPV than females. However a South African study showed that 63% of children with HMPV were females (IJpma et al., 2004).

RSV was identified more frequently in children under 6 months of age (74%) and its prevalence decreased after this age. In contrast, HMPV infection occurred more frequently in older children. A similar finding was reported in a study from the Netherlands (van den Hoogen et al., 2003) where HMPV was found significantly less frequently in children < 2 months old than was RSV. Of the 31 HMPV positive children < 2 years old, only 4 (13%) were < 2 months old, whereas 43 (35%) of the 122 RSV positive children < 2 years old were also < 2 months old. Another study from Norway found that median age of children with HMPV was 12 months with of range 1-115 months (Dollner et al., 2004). The age of their target population was older than ours.

Our data showed that 39%, 37%, 32%, and 49% of the children with RSV, HMPV, RSV/HMPV co-infection and negative for both viruses had suffered acute severe malnutrition as their weight for length Z score was > 0. This in contrast to a study by (Adegbola et al., 1994) in Gambia, they

found that RSV was more common in well-nourished children with pneumonia than malnourished children with pneumonia (13% vs. 6%). There were no data about effect of malnutrition and HMPV infection.

Only a third of the children with HMPV in comparison to about half the children with RSV and 36% of children with mixed infection were breast fed exclusively. This may be a result of the older age of children with HMPV. On the other hand, about 59% of children with HMPV had their vaccination up to date in comparison to only 30% of children with RSV and 40% of children with RSV/HMPV co-infection. This may indicate that vaccination might give protection to the HMPV infected children as they have milder disease in comparison to children with RSV or RSV/HMPV infection. However, mild disease in children with HMPV might also be age related.

A history of asthma was recorded more frequently in children with HMPV infection 3 (7%) children, compared to 2 (1%) children with RSV infection and none in the children with RSV/HMPV co-infection. However, HMPV was detected in 23% of cases of exacerbations of asthma (Peiris et al., 2003).

A family history of other members with ARI in the time of enrolment of the child in the study were recorded more in children with RSV and RSV/HMPV 69%, 64% respectively compared to 46% in children with HMPV infection. This may reflect the nature of the mild disease of HMPV in comparison to RSV.

In our study, the socio-economic conditions of children with HMPV were much better than for children with RSV or RSV/HMPV co-infection. This may explain to some extent why severe ARI is more common in RSV or RSV/HMPV co-infection than HMPV infection.

Different viruses can infect the respiratory tract but no single clinical sign or symptom is pathognomic of infection with a specific virus; in fact, most agents can elicit both URTI and LRTI symptoms, making aetiologic diagnosis difficult, if they are not supported by virus- specific laboratory tests (Cane et al., 2003, Boivin et al., 2002).

Our results revealed that there was no significant difference in the complaint of cough or fever between children with RSV, HMPV or RSV/HMPV although fever was more common in the

HMPV infected children. However this finding is in concurrence with a study from Hong Kong where the majority of the children with HMPV infection had a high temperature (Peiris et al., 2003). The present study showed that 98% of children with HMPV had cough which is much higher than results by (Esper et al., 2003), where only 68% of the study population had cough. A study from Norway, reported fever in 86% and cough in 90% of children with HMPV (Dollner et al., 2004).

Tachypnoea, blue lips, wheeze, difficult to awake, inability to drink, and apnoea were more common in children with RSV/HMPV co-infection than with RSV or HMPV alone. In comparison between the single infection with RSV and HMPV they were more common in children with RSV that with HMPV infection, this indicating that HMPV infection mainly causes mild disease and co-infection with RSV increases the risk of severity of the disease. These differences reach statistical significance for the first three symptoms only ($p<0.05$). In this study, number of HMPV infected children who had tachypnoea was lower than results of studies by (Esper et al., 2004) and (Dollner et al., 2004) (63% vs.77 and 80%).

A skin rash was present in our study in only 1 (4%) child with RSV/HMPV co-infection, in 5% of children with RSV and 2% of children with HMPV this compares with results in Hong Kong study where skin rash was found in 13% (higher than our results) and 3% of children with RSV and HMPV respectively (Peiris et al., 2003). Table 3.23 shows some comparison of the clinical presentation of children with HMPV in the present study and others.

Some study compared the symptoms and signs between children with RSV and HMPV table 3.24 compared the results of these studies with our results.

Table 3.23 Comparison of signs and symptoms of children with HMPV in the present study and others

Signs/Symptoms	Current study N=41(%)	(Williams et al., 2004) N=49 (%)	(Esper et al., 2004) N=53 (%)	(Dollner et al., 2004) N= 50 (%)
Cough	40 (98%)	44 (90%)	36 (68%)	45 (90%)
Fever	30 (73%)	25 (52%)	41 (77%)	43 (86%)
Tachypnoea	26 (63%)		41 (77%)	40 (80%)
Wheeze	9 (22%)	25 (52%)	27 (51%)	28 (56%)
Creptation	21 (51%)	10 (20%)		
Chest indrawing	17 (42%)		28 (53%)	
O_2 therapy	13 (32%)		20 (38%)	14 (28%)

Table 3.24 Comparison of signs and symptoms of children with HMPV and RSV in the present study and others

*S/S	Current study		(Boivin et al., 2003)		(Peiris et al., 2003)	
	HMPV N=41	RSV N=241	HMPV N=12	RSV N=118	HMPV N=32	RSV N=32
Cough	40 (98%)	214 (100)	12 (100%)	117 (99%)		
Fever	30 (73%)	182 (76%)	8 (67%)	67 (57%)		
Tachypnoea	26 (63%)	220 (91%)				
Wheeze	9 (22%)	94 (39%)			9 (28%)	12 (38%)
Crepitation	21 (51%)	201 (83%)			8 (56%)	14 (42%)
Chest indrawing	17 (42%)	173 (72%)	11 (92%)	112 (95%)		
O_2 therapy	13 (32%)	134 (56%)				
Skin rash	2 (4%)	11 (5%)			4 (13%)	10 (3%)

*S/S=signs/symptoms

Examination

On examination, there was significant difference between the infecting viruses in the respiratory rate where it was greater in children with RSV than the others (means of 61 breath/ min) ($p<0.001$). Also we found that cyanosis, chest indrawing, crackles and wheeze were significantly more common in the children with co-infection than with single infection and more with RSV infection than HMPV infection. This is in contrast to a study from Brazil, where children with RSV more commonly had these signs than with RSV/HMPV co-infection or HMPV alone (Cuevas et al., 2003) (Table 3.25).

Table 3.25 Comparison between the current study and the Brazilian study (Cuevas et al, 2003)

*S/S	Current study (Yemen)			(Cuevas et al., 2003) (Brazil)		
	HMPV	RSV	RSV/HMPV Co-infection	HMPV	RSV	RSV/HMPV Co-infection
	N=41	N=241	N=25	N=19	N=53	N=8
Cough	40 (98%)	241 (100%)	25 (100%)	19 (100%)	53 (100%)	8 (100%)
Respiratory rate, mean (SD)	40 (15)	61 (13)	57 (15)	44 (10)	51(12)	54 (16)
Temp >37.5%	30 (73%)	182 (76%)	21 (84%)	15 (79%)	43 (81%)	8 (100%)
Cyanosis	17 (41%)	186 (77%)	21 (84%)	0 (0)	4 (8%)	0 (0)
Wheeze	9 (22%)	94 (39%)	9 (36%)	9 (47%)	31 (54%)	2 (25%)
Chest indrawing	17 (42%)	173 (72%)	19 (76%)	4 (21%)	19 (36%)	3 (38%)

*S/S=signs/symptoms

In regard to the radiological findings of disease observed in this study, it is interestingly that chest x-ray findings showed significantly more hyperinflation with RSV infected children and consolidation with RSV/HMPV infected children than HMPV infected. In a study from South Africa, it was found that 4 out of 13 of infants with HMPV (36%) had alveolar consolidation on chest x-ray (Madhi et al., 2003a). However, the findings of our study are in contrast with those in a study from Hong Kong, where the majority of children with HMPV infection were shown to have lobar consolidation visible on chest radiology and abnormal x-rays were found in 68% of HMPV group in comparison to 61% of the RSV group (Peiris et al., 2003). However, the children with HMPV infection in the Hong Kong study were much older than were those with our study (mean age 31.7 vs.7.5). In a study from USA, hyperinflation and consolidation were found in 14% and 28% of infants with HMPV infection respectively (Esper et al., 2004).

The diagnoses of mild upper respiratory tract, pneumonia or bronchiolitis did not differ significantly although the mild upper respiratory tract diagnosis was made more commonly in children with HMPV while pneumonia and bronchiolitis were more common in children with RSV and RSV/HMPV co- infection. In the Hong Kong study HMPV infected children had the diagnosis of bronchiolitis and pneumonia in 9% and 38% respectively, in comparison to the RSV group who receive the diagnosis of bronchiolitis and pneumonia in 31% and 16% respectively (Peiris et al., 2003). In the Norway study 48% and 34% of children with HMPV received the diagnosis of bronchiolitis and pneumonia respectively (Dollner et al., 2004).
The case fatality rate between each group of these admitted cases were 6 (2.5%) in the RSV alone group, 1 (2.4%) in the HMPV group, and zero in the combined group. This means that HMPV may lead to fatality despite the fact that it mainly caused mild disease.

RSV and HMPV seem to have similar clinical presentations, ranging from mild to severe ARI. In our study, we found that HMPV infection resulted in a milder clinical presentation and children with HMPV were less likely to be hypoxic and had lower respiratory rate than those with RSV. Fifty one percent of the HMPV-infected group were diagnosed as mild ARI in comparison to only 15% of the RSV infected group. This finding supports finding of Ebihara et al. who found 37% of their HPMV infected population were diagnosed with wheezy bronchitis, 26% as mild upper respiratory infection, 23% bronchitis and only 14% were diagnosed as pneumonia (Ebihara et al., 2004a). The mild form is more common in the older age group (> 6 months) for both viruses, it reached to statistical significant in the RSV group $p < 0.05$, the means of age in the mild RSV infection is 8 months while for the severe infection it was 6.3 months. A community study,

demonstrated that HMPV is associated with a proportion of mild, community-acquired, self-limiting respiratory illnesses in all age groups (Stockton et al., 2002). This supports our finding that HMPV causes more mild disease than severe one. The milder presentation of HMPV may be partially explained by the older age of the children affected, who have larger respiratory airways and less maternal antibodies, as our younger children with HMPV had a higher frequency of severe ALRI than older children. It is however not possible to differentiate the two infections solely on clinical findings.

The clinical outcome after RSV infection was more severe than that after HMPV infection when looking at the proportion of patients with hypoxaemia, where 59% of the HMPV-infected children had severe hypoxia (pO_2 <88) and needed oxygen supplementation while 87% of RSV infected children had severe hypoxia and also needed oxygen supplementation. These findings are consistent with those of Galiano et al (2004) where 71% of infants with HMPV required oxygen supplementation. This higher proportion is probably due to the very young age of their patients (median age = 5 months) most of them had severe disease and were hospitalised (Galiano et al., 2004) and in the RSV/HMPV co-infected group the percentage of children with severe hypoxia increased to 88%. This supports the finding that HMPV co-infection increase the severity of RSV disease which is in consistent with the findings of a study from UK (Greensill et al., 2003).

HMPV was described as a cause of community- acquired respiratory illnesses (Stockton et al., 2002, Vicente et al., 2003) and in immunocompromised children (Pelletier et al., 2002) and in a healthy child in whom the infection progress from mild upper respiratory infection to acute respiratory failure and advanced fibrotic phase of acute respiratory distress syndrome and culminated in death and in elderly patients (Falsey et al., 2003a).

More studies will be needed to find out the clinical spectrum and the burden of this newly discovered virus and to identify its genotypes and if they have an association with the severity of the infection.

Chapter 4
Risk factors for severe bronchiolitis

Literature Review

Hospitalisations for ALRI have increased steadily since 1980 (Glezen et al., 2000, Shay et al., 1999) and the development of new approaches for the prevention of acute respiratory tract conditions requires studies of the causative agents and quantifying the risk of hospitalisation for vulnerable patients. This chapter investigated the factors associated with severe disease presentation due to RSV and HMPV.

Although much is known about the epidemiologic features of RSV, the wide variation in severity continues to be unexplained. The clinical severity of ARI due to RSV infection has been associated with the presence of underlying medical conditions including premature birth (Cunningham et al., 1991, Groothuis et al., 1988), congenital heart disease (CHD) (MacDonald et al., 1982) and chronic lung diseases (CLD) (Arnold et al., 1999). However, more than half of all hospitalisations for RSV occur in previously healthy children born at term (Boyce et al., 2000). Certain demographic and epidemiological factors described later on in this chapter have been reported to predispose to severe RSV disease (Kaneko et al., 2001), although there is currently insufficient evidence to unequivocally implicate these as risk factors for the development of severe RSV LRI. This chapter aims to elucidate the association between such factors and disease severity in Yemeni children.

Several studies have evaluated differences in clinical severity between groups A and B RSV but have reached conflicting results. In some studies, no differences in clinical severity were detected between the groups involved (Fletcher et al., 1997, Kneyber et al., 1996, Wang et al., 1995a), while in others, group A was reported to be an independent risk factor for severe disease (McConnochie et al., 1990, Walsh et al., 1997).

HMPV seems to account for a significant number of respiratory tract infections in hospitalised children. HMPV causes clinical symptoms similar to those of RSV, ranging from mild respiratory problems to bronchiolitis and pneumonia (Boivin et al., 2002, van den Hoogen et al., 2002) and cannot be differentiated on the basis of their clinical signs. This chapter aims to provide an

understanding of the clinical presentation of HMPV infection and risk factors that may be associated with severe HMPV disease.

Epidemiological factors known to predispose for disease severity due to RSV

Prematurity

Prematurity has been associated with increased disease severity and hospitalisation in RSV. The definitions of premature birth however often differ from one study to another and has been defined as a birth at gestational age (GA) < 36 (Kaneko et al., 2001) or ≤ 37 weeks (Joffe et al., 1999).

There is general agreement that prematurity, even in the absence of chronic pulmonary disease, represents a major risk factor for severe RSV infection and hospitalisation, particularly for children who contract RSV infection during the first 6 months of life (Resch et al., 2005). If it is accompanied by bronchopulmonary dysplasia (BPD) or other underlying CLD this will increase the risk of severe RSV ARI (Navas et al., 1992). In a German cohort of 717 infants with GA < 35 weeks, the risk for rehospitalisation with ARI and RSV was 11% and 5% respectively. Premature infants with CLD had a higher proportion of RSV related rehospitalisations (15%) compared to preterm babies without CLD (4%) (Liese et al., 2003). However, other researchers have found that prematurity itself may only be a marker for disease severity and it is only associated with severe disease in the presence of BPD (Groothuis et al., 1988). The risk of RSV rehospitalisation increases with decreasing GA. The rehospitalisation rate due to RSV in preterm infants with GA ≤ 32 weeks in Germany was significantly lower compared with children with GA ≤ 28 (4% vs. 15% respectively) (Liese et al., 2003). Similarly, in New York, the incidence of RSV hospitalisation increased with decreasing gestational age (14% vs. 4% for infants born at ≤ 26 weeks' gestation versus those born at 30-32 weeks' gestation) (Stevens et al., 2000). RSV hospitalisation rates were higher in preterm compared to full term infants. Thirty six percent of preterm infants in the USA are hospitalised at least once for RSV ARI versus 2.5% of term babies (Cunningham et al., 1991). In a case control study in Alaska, premature birth was associated with six fold increase risk for hospitalisation for RSV infection when compared to term controls (Bulkow et al., 2002), and a study of 1232 children < 2 years old found RSV in 41% of the children. Among RSV positive children, 44 (10%) had a GA <36 weeks, despite the fact that in Italy only 4% of infants are born before the 36th week (Lanari et al., 2002). In children with a diagnosis of bronchiolitis, patients with GA ≤ 35 weeks had a higher prevalence of RSV than

children with bronchiolitis who were ≥ 36 weeks gestation (60% and 47% respectively) (Lanari et al., 2002).

A retrospective cohort of 1721 premature infants in California also found that 3.2% of the children were rehospitalised for RSV ARI. The risk factors for RSV hospitalisation in premature infants were a GA ≤ 32 weeks, duration of oxygen therapy ≥ 28 days at birth and discharge from the neonatal unit within three months before start of the RSV season (Joffe et al., 1999). Premature birth was found in 22% (5/23) of the children with severe RSV ARI admitted to the pediatric intensive care unit in comparison to 13% (3/23) of the children with mild RSV (Lazar et al., 2004). However, the results of a study of 265 infants < 2 years with RSV ARI in New York showed only marginally significant association of prematurity with a greater risk of severe RSV disease (Falsey and Walsh, 1997) and in other studies prematurity has not been associated with severe RSV disease (Kaneko et al., 2001).

Maternal antibodies transfer across the placenta is optimal fairly late in gestation, and around 33 weeks antibody is concentrated on the fetal side of the placenta (de Moraes-Pinto and Hart, 1997). Therefore, premature infants, especially extremely premature infants, have very low levels of neutralizing antibodies against RSV and would be expected to be more susceptible to severe forms of illness. In support of this hypothesis, the hospitalisation rate after subsequent RSV infection has been reduced by approximately 50% after passive administration of neutralizing antibodies against RSV to high risk infants before RSV infection (Groothuis et al., 1993, IM-pact-RSV, 1998). Premature infants have inadequate defense mechanisms against infection and their incomplete airways maturation are the most probable mechanisms that predispose them to an increase in severity of RSV infection.

Underlying medical conditions and severity of RSV ARI

Underlying medical conditions that increase the severity of ARI due to RSV include chronic respiratory disorder such as BPD, cystic fibrosis, a previous diagnosis of asthma, CHD and deficient host defenses such as severe malnutrition and HIV.

Chronic lung disease

PBD has emerged as a leading cause of CLD in early childhood in developed countries. It develops in many premature infants who survive after mechanical ventilation. It affects up to 35% of very low birth weight infants (<1500 gm) (Furman et al., 1996). The definition of BPD,

according to the National Institute of Child Health and Human Development in the United States is based on the need to use supplemental oxygen at 28 to 30 days of life for infants with GA ≥ 32 weeks, and use of supplemental oxygen at 36 weeks postconceptional age for infants with GA < 32 weeks (Jobe and Ikegami, 2001).

At least half of all children with BPD require rehospitalisation within the first year of life. In a study from California, the rehospitalisation rate of 238 premature infants with BPD was 49%, more than twice the rate of rehospitalisation (23%) of children without BPD (Smith et al., 2004).

Pedersen et al. reviewed retrospectively all rehospitalisations during the first 2 years of life of infants born with GA < 28 weeks or birth weight <1,000 gm during 1994 and 1995 in Denmark. They found among 240 eligible infants, 43 (18%) had been rehospitalised 48 times with RSV infections. The proportion of rehospitalisation of RSV in children with CLD (n=30) was higher at 30% compared to 16% for children without CLD (n=210) (Pedersen et al., 2003).

During a study period of 2 years of 30 children with BPD, 27 infants had one or more ARI episodes. RSV infection developed in 59% (16/27) of the children. Children living in houses with 4 or more individuals were significantly more likely to acquire symptomatic RSV infection than were children in smaller households. The presence of an adult smoking in the home was also a risk factor (Groothuis et al., 1988).

Navas et al studied hospitalisation, morbidity and mortality due to RSV infection in 1584 high risk children in Canada, including 200 patients with CLD (139 (70%) with BPD, 20 (10%) with cystic fibrosis, 19 (10%) with recurrent aspiration and 22 (11%) with other conditions. They found that 80% of the children with CLD needed oxygen supplementation, 32% required intensive care and 17% required mechanical ventilation in comparison to other high risk groups. The mortality rate in children with CLD was significantly higher than in the other groups (Navas et al., 1992).

Congenital heart disease

Infants with CHD are at increased risk of severe disease at the time of RSV infection (Navas et al., 1992, Kaneko et al., 2001). The duration of hospitalisation, the need for intensive care and the occurrence of respiratory failure and mortality are increased in children with RSV ALRI and CHD compared to those without CHD (MacDonald et al., 1982). Although no particular congenital heart lesion is associated with RSV infection, death is increased in cyanotic heart

abnormalities and pulmonary hypertension. Navas et al. studied 260 Canadian children with CHD hospitalised with RSV LRTI among 1584 high risk children. Thirty three percent of these children were admitted to the intensive care unit, 19% required mechanical ventilation.The mortality rate in this group was significantly higher than in other groups. The subgroup of patients with pulmonary hypertension had a higher death rate compared to those without pulmonary hypertension (9% vs. 2%). In infants with pulmonary hypertension relatively unoxygenated blood in the pulmonary artery is shunted away from the lung into the systemic circulation and hypoxia is already present in infants with cyanotic heart defects and/or pulmonary hypertension before RSV infections. As a result, these infants are less likely to withstand the increased hypoxia that occurs with RSV ALRI (Navas et al., 1992). Similar to this Canadian study, a case control study from Japan reported that 5 (29%) out of 17 children with severe RSV LRTI had CHD compared to only 1 (6%) out of 137 children with mild RSV ARI (Kaneko et al., 2001).

Age of acquisition of RSV and hospitalisation

A review of recent studies of RSV hospitalisation rates reveals that between 10% and 28% of infants hospitalised with RSV were < 6 weeks of age, 34% to 66% were < 3 months of age, 49% to 70% < 6 months of age, and 66% to 100% <1 year of age (Table 4.1) (Simoes, 2003). Most cases of severe disease occurred in infancy. In a study in the Netherlands of children ventilated for severe RSV disease, most infants had a postconceptual age ≤ 44 weeks (Bont et al., 1999b). This implies that most of the very severe disease in either preterm or term babies occurred when they would have been <6 weeks for their corrected age. In another Italian study of children < 2 years old hospitalised with LRTI the RSV positivity was lower in children 13-14 months old (27%) compared to infants ≤ 3 months (47%) and to children 7-12 months old (39%) ($p<0.01$) (Lanari et al., 2002). In these young infants, the absence of maternal antibodies (Glezen et al., 1981), narrower airways (Martinez et al., 1988), an immature immune system, (Culley et al., 2002) and perhaps a bias toward a Th2-type response to RSV infection may play a role in the causation of severe disease. All of these factors may be responsible either singly or in conjunction for much more severe disease that is seen in very young infants.

Table 4.1 Age at RSV hospitalisation in selected countries

Country	N	Ages (yr)	Cumulative No. (%) of children with age less than (months)				Reference
			1.5	3	6	12	
UK	472	0-1	148 (31%)	228 (48%)	372 (79%)	472 (100%)	(Hart et al., 2000)
Italy	1174	0-2		398 (34%)	672 (57%)	964 (82%)	(Lanari et al., 2002)
Germany	148	0-5		51 (34%)	80 (54%)	111 (75%)	(Weigl et al., 2001)
Holland/Switzerland	151	0-1	42 (28%)			151 (100%)	(Brandenburg et al., 1997a)
Europe/North America	1042	0-3	255 (24%)				(Behrendt et al., 1998)
USA	3553	0-3			2148 (60%)	2940 (83%)	(Boyce et al., 2000)
USA	721	0-1		475 (66%)			(Brooks et al., 1999)
Canada	689	0-2	101 (15%)				(Wang et al., 1995a)
Canada	1516	-	145 (10%)				(Navas et al., 1992)
Alaska	370	0-3			196 (53%)	289 (78%)	(Bulkow et al., 2002)
Taiwan	92	0-6			45 (49%)	67 (73%)	(Tsai et al., 2001)
Argentina	61	0-2			43 (70%)	59 (92%)	(Videla et al., 1998)

Modified from (Simoes, 2003)

Gender

Boys have more severe disease than girls (Bradley et al., 2005, Weber et al., 1998a, Weber et al., 1998b). Studies over the last 30 years indicates that the risk ratio of boys to girls is 1.425:1 (95% CI: 1.40 -1.45; range, 1.2 to 1.7:1) (Brandenburg et al., 1997b, Chan et al., 1999b, Glezen et al., 1981, Hussey et al., 2000, Kaneko et al., 2001, Kristensen et al., 1998, Law and De Carvalho, 1993, Lowther et al., 2000, Lukic-Grlic et al., 1999, Weber et al., 1998a, Weigl et al., 2001). However, not all studies have found that male gender is a risk factor in RSV disease (Madhi et al., 2003a). The reason why males are thought to have more severe disease than females is that they have shorter and narrower airways and therefore are more likely to develop bronchial obstructions (Martinez et al., 1988, Escobar et al., 1988).

Breast feeding

The role of breast feeding in preventing ARI related hospitalisations and death in developing countries is undisputed (Cunningham et al., 1991). In industrialised countries, the protective effect of breast feeding against ARI and bronchiolitis appears to be established (Bulkow et al., 2002), but the specific protective role of breast feeding for RSV ARI appears to be less clear cut. In a large prospective cohort study of 1179 infants found that of these, 460 had ALRI (2.6% were hospitalised) and 148 had RSV (Holberg et al., 1991). In a univariate analysis, breast feeding appeared to be protective against RSV ALRI. However, the multivariate analysis found that breast feeding was not an independent protective factor. In infants <3 months of age, the absence of breast feeding in combination with crowding, smoking, and low maternal education were significant risk factor for development of RSV ALRI (RR, 8.0; 95% CI, 2.8-22.8; $P < 0.001$).

In industrialised countries, breastfeeding has been shown to reduce the severity of ALRI in the first 6 months of life and an inverse relationship between the duration of ALRI symptoms and the length of exclusive breast feeding has been demonstrated (Baker et al., 1998, Cushing et al., 1998). However, other studies have shown no effect of breast feeding on the severity of ALRI (Leventhal et al., 1986, Rubin et al., 1990).

There are many biological reasons why breastfeeding could protect against RSV ALRI. Colostrum contains both anti-RSV IgA and lactoferrin, which may have important antiviral properties (Hanson, 1998). It has also been proposed that breast milk promotes lung maturation, perhaps through prolactin (Hanson, 1998, Martinez et al., 1988). In conclusion, it appears that breast feeding is protective against ALRI and its severe manifestations in developing countries. In industrialised countries, the beneficial effect of breastfeeding is seen

principally in infants living in crowded homes in lower socioeconomic strata (Bulkow et al., 2002, Holberg et al., 1991) when exposed to passive smoking (Bulkow et al., 2002).

Socio-economic factors

The socio-economic characteristics that increase the risk of severe RSV infections include crowding, late birth order, parental smoking, and in particular maternal smoking during pregnancy, indoor pollution and the presence of pets. Infants from lower socio-economic environments tend to be younger when they acquire their first RSV ALRI and are more likely to have severe infections than children from higher socio-economic backgrounds (McConnochie et al., 1988). In a study from Malmö, the bronchiolitis hospitalization rates were determined for the infants living in 10 different administrative residential areas and correlated with socioeconomic factors (percentage of immigrants, per capita income, unemployment rate and households receiving social benefit) in the respective residential areas. The bronchiolitis hospitalization rates varied >4 fold between the 10 residential areas. It varied significantly with per capita income and the percentage of immigrants. Infants living in the area with the highest social burden were hospitalized almost twice as often as those from the rest of the city (Jansson et al., 2002). Low socio-economic status is associated with a higher attack rate in the USA; children from middle income families had a hospitalisation rate of 1/1000 compared to 5-10/1000 in lower-income populations and the former were more likely to have multiple high risk conditions (Wang et al., 1995a)

Crowding/siblings

In a case control study from Atlanta examining the effects of day care on ALRI hospitalisation, a multivariate analysis of variables not related to care outside of the home showed that the number of persons sleeping in the same room with the child was significantly associated with ALRI hospitalisation (RR, 1.93; $P < 0.001$) (Anderson et al., 1988). In a 1983 to 1989 cohort study from Denver of twins and triplets matched with singletons, multiples had more severe RSV ALRI (53% vs. 24%, $P = 0.01$), had more RSV hospitalisations (32% vs. 18%, $P = 0.05$) and ALRI associated with RSV increased with crowding (>1 person/19 m^2, $P = 0.002$ or >1 child/22 m^2, $P = 0.002$) (Simoes et al., 1993). In a cohort studies of 680 premature infants and 999 premature infants ≤ 32 weeks GA conducted between 1998 and 2000, living with siblings in school was a significant risk factor for RSV hospitalisation (RR, 1.86; 95% CI: 1.01, 3.4) (Carbonell-Estrany et al., 2000). In a case control study from Alaska, having 4 or more children <12 years of age in the household was a significant independent risk factor for RSV hospitalisation (OR, 2.13; $P = 0.01$) (Bulkow et al., 2002). There are few papers examining crowding as a risk factor in developing countries.

In a case control study from the Gambia comparing 277 hospitalised children with RSV ALRI compared with 364 control children who had not been admitted to hospital, the main risk factors identified included a larger number of people in the household; having had a sibling who had died; living in a house with a flush toilet rather than a pit latrine or no toilet. The strongest risk factor identified was an increased number of children between 3 and 5 years old in the household (Weber et al., 1999).

Crowding is probably related to a acquisition of higher viral infectious load either through interpersonal transmission or through contact with more infectious viral particles in a more crowded environment and a greater possibility for exposure to RSV in the first year of life through multiple persons in the household (Simoes, 2003).

Passive smoke exposure

A case control study from Rochester examined risk factors for hospitalisation in 53 subjects with bronchiolitis compared with 106 matched control subjects. Maternal smoking was significantly associated with the development of bronchiolitis (OR, 2.33; 95% CI, 1.91-4.57), as well as if any household member smoked (OR 3.21; 95% CI, 1.4-7.3) (McConnochie and Roghmann, 1986). In their multivariate analysis, the OR for passive smoking was 3.9 in children with a family history of asthma and 4 for children without a family history of asthma. Bradley et al. found that those infants exposed to second hand maternal cigarette smoke had significantly severe RSV disease however there were no effect on RSV severity between infants exposed only to smoke while in utero and those infants who were never exposed to cigarette smoke (Simoes, 2003). This indicates that postnatal smoke exposure is more important in terms of RSV severity than intrauterine exposure. In Spain, tobacco smoke exposure increased the rates of RSV hospitalisation of premature infants (RR, 1.63; 95% CI, 1.05-2.56) (Carbonell-Estrany and Quero, 2001). Although in previous studies, (Carbonell-Estrany et al., 2000) and others from Alaska (Bulkow et al., 2002) and Munich (Liese et al., 2003) did not show an independent effect of maternal or parental smoking on the development of RSV hospitalisation. Madhi et al. showed that exposure to cigarette smoke is not significantly different in RSV infected high risk and non high risk infants in South Africa (Madhi et al., 2003a).

Malnutrition

The role of malnutrition as a risk factor for severity of RSV infection is not well defined. Brussow et al., evaluated the titers of serum antibodies to various microbial antigens in 1554 Ecuadorian children < 5 years of age. Underweight children had significantly lower antibody

titres to RSV than control children. Anemia was also significantly associated with lower antibody levels to both T-cell-dependent and –independent antigens. Serum zinc and retinol however were not associated with major differences in antibody levels (Brussow et al., 1995). Similarly, in a Nigerian study, malnourished children did not appear to be at increased risk of contracting RSV infection or suffering more severe disease (Nwankwo et al., 1994). In another study from the Gambia, RSV was found in 13% well nourished children with ALRI compared with only 6% of malnourished children (Adegbola et al., 1994). The nutritional status of children in industrialised countries has not been systematically studied as a risk factor for severe RSV ALRI. Despite the initial suspicion that malnutrition was associated with more severe RSV disease, studies that have examined the causes of pneumonia in well nourished and malnourished children showed that, RSV was less important than bacterial causes (Beaudry et al., 1995) and children with severe malnutrition (marasmus, kwashiorkor and marasmic kwashiorkor), had less severe disease than controls and fewer children died (McConnochie and Roghmann, 1986). These findings are surprising and this chapter has attempted to elucidate this issue.

Immunodeficiency

Immunocompromised children include children with severe combined immunodeficiency, those receiving chemotherapy for malignant diseases or HIV (Chandwani et al., 1990). Children with deficient immunity are at increased risk developing severe disease at the time of RSV infection. The mortality rates of immunocompromised children (during RSV infection) has been reported to be between 15-40% (Hall et al., 1986).

In a study of 10 HIV-infected children and 18 non-HIV infected children hospitalised with RSV ALRI, most RSV infections in HIV-infected children were associated with pneumonia and bronchiolitis and wheezing occurred rarely (one child). In contrast, bronchiolitis occurred in 8 (44%) of the 18 non-HIV infected children admitted concurrently. These differences however were not statistically significant as the number of children was small (Chandwani et al., 1990). In immunocompromised children, there is a profuse and persistent replication of the virus in the lung and histological examination reveals extensive alveolar exudates consistent with pneumonia. In contrast to the picture seen as a result of bronchiolitis, there is predominantly peribronchiolar infiltration with relative sparing of the alveoli (Milner et al., 1985).

Atopy

Children with atopy appear to be more likely to suffer severe RSV infection and to require hospital admission (Sigurs et al., 1995). However, Stein et al. found that there was no association between RSV ALRI and subsequent atopic status (allergy skin prick tests, serum IgE concentrations) after follow up to 13 years age (Stein et al., 1999). Bradley et al. conducted a cross sectional study of 204 hospitalised infants ≤ 12 months of age with their first episode of RSV infection. Severity of RSV bronchiolitis was determined by low O_2 saturation, length of hospital stay and the presence of infiltrates or hyperinflation on chest x-ray. The levels of common allergens (dust mite, cats, dogs and cockroach) within the home environment were significantly elevated in 25% of the homes and there was a strong association between having a pet and the presence of a high level of these allergens. Despite this association, there was no significant association between RSV severity and exposure to allergens in the child's home. Infants with a family history of atopy had a higher O_2 requirements during hospitalisation and the multivariate regression analysis indicated that a history of maternal asthma was protective (Bradley et al., 2005).

In a case control study from the Gambia, mothers of children hospitalised with RSV ALRI complained of asthma more frequently than mothers of controls (4.2 vs. 0.5%, p=0.05) (Weber et al., 1999). The role of atopy in the development of severe RSV infection however is still not clear and further studies are needed.

RSV genotypes

Although much is known about the epidemiologic features of RSV, the wide variation in severity and lifelong recurrences of RSV infections continue to be unexplained. Several studies have evaluated differences in clinical severity between groups A and B. In about half of these studies, no differences in clinical severity were detected between the groups involved (Kneyber et al., 1996, Wang et al., 1995a). For example, Brandenburg et al described RSV group A strain variations of 28 isolates from the Netherlands analyzing the G protein sequences during three consecutive seasons (Brandenburg et al., 2000a). Several lineages circulated repeatedly and simultaneously during the seasons. No relationships were found between lineages and clinical severity or age. Fletcher et al. also reported 114 infants with ARI due to RSV over two seasons (Fletcher et al., 1997). Five of the six known N genotypes were detected. Although NP4 and NP2 were found most frequently, there was no association between the N genotypes and disease severity. Six G (SHL) genotypes were detected and a higher proportion of the infants infected with the SHL2 genotype had severe or moderate disease. Group A seems to be associated with more severe clinical disease (Hall et al., 1990)

and it has been suggested that virus variants within group A are responsible for the discrepancy observed across the studies. Walsh et al. investigated 265 hospitalised infants over a 3 year period (1988-1991) using a severity index of clinical and physiologic parameters to grade illness severity. One hundred and thirty four infants had group A and 131 infants group B strains. In a multistep regression analysis group A was identified to be an independent risk factor for severe disease together with premature birth, age ≤ 3 months and underlying medical conditions among all infants, it was 2.8 times as likely as group B to cause severe disease, independent of other risk factors. They carried an analysis limited to infants without underlying medical conditions. It was striking that group B RSV infected infants without underlying medical conditions required less ventilatory support than children with group A (1/90 vs. 13/107, $p<0.006$) and had significantly lower severity indices. Group B infected infants were six times less likely to have a high severity index (Walsh et al., 1997). Group A infection was reported to be more severe among a study of 157 infants hospitalised in New York during two winters. Mechanical ventilation was required in 13% of the children with group A compared to 2% of those with group B infection. Among infants with underlying conditions or age < 3 months at admission, carbon dioxide tension > 45 mmHg was found in 37% of those with group A compared with 12% of those with group B infection (McConnochie et al., 1990).

Risk factors for severe HMPV ARI

Premature birth and underlying medical conditions

Premature birth and underlying medical condition are known to be high risk factors for severe RSV disease and it is expected that these would be a risk factor for severe HMPV infection. There is however, very scanty information on this subject with only one study that has identified risk factors for severe HMPV. In a USA retrospective review of records of children <18 years old with ARI, 58 out of 1257 (5%) children were infected with HMPV. The following underlying conditions were assessed: asthma, presence of cardiac malformation and premature birth. Thirty eight (63%) of the patients with HMPV had at least 1 of these underlying conditions. Of those patients with underlying conditions, 26% had > 1 risk factor. Cardiac malformation was present in 9 (17%), premature birth in 6 (11%), asthma and premature birth in 6 (11%), asthma was found in 10 (19%) children, cardiac malformation and premature birth in 2 (4%) and asthma plus cardiac malformation in 1 (2%). In addition, 5 patients had BPD, all of them were born prematurely; 3 were diagnosed as bronchiolitis and 2 with an URTI. An additional 4 patients had other chronic respiratory conditions, including pulmonary haemosiderosis, pulmonary hypertension, hyaline membrane disease and previous transplant for pulmonary hypertension (McAdam et al., 2004).

Socio-economic factors

In a study from South Africa, 8 children with HMPV ARI were compared to 19 children with RSV ARI. The percentage of children who lived in families with smokers was 100% and 63% for HMPV and RSV respectively. The mean number of people living in houses of children with HMPV was 4.9 in contrast to 6.2 in RSV infected children. However, these differences were not statistically significant because the sample size of the children with HMPV was small and studies with larger populations are needed (IJpma et al., 2004).

Immunodeficiency

Unfortunately, very scarce information is available on the association of immunodeficiencies and HMPV. Immunodeficiencies were reported in 6 patients with HMPV infection in a study of 28 HMPV infected Canadian patients (Hamelin et al., 2004). Two of these patients were 0 - 4 years old, 2 between 15 and 65 years and 2 had 65 years. Preliminary data suggest that similar to RSV, HMPV infection may have a more fulminant course in severely immunocompromised individuals. Pelletier et al. reported the death of a 17 month old girl with acute lymphoblastic leukaemia due to severe pneumonitis and respiratory failure. NPA obtained before death revealed the presence of HMPV, although no autopsy was performed. Of interest, 1 year earlier, this child had presented with a first episode of bronchiolitis caused by HMPV and the two viral isolates were of different lineages, highlighting the possibility of rapid reinfections in immunocompromised individuals (Pelletier et al., 2002). Similarly, a hematopoietic stem cell transplant recipient died as a result of progressive respiratory failure after an upper respiratory prodrome and HMPV was the sole pathogen identified (Cane et al., 2003).

HIV

HMPV has been reported in children with HIV infection but it is not clear if the disease has a worse evolution in these children. Madhi et al. reported the prevalence of HMPV infection among HIV-1 infected and HIV-1 uninfected South African infants hospitalised for LRTI. A total of 196 NPA samples (81 HIV-1 infected infants, 110 HIV-uninfected infants and 5 infants with unknown HIV-1 infection status) were analysed. Overall, HMPV was identified in 14 (7%) infants, including 10 (9%) and 3 (4%) HIV-1 uninfected and HIV-1 infected infants respectively ($p=0.24$) and one case was found among those with unknown HIV status. Risk factors such as premature birth, CLD and CHD were commonly found in infants with HMPV ALRI (6 (43%) of 14 infants), including 2 infants who were HIV-1 infected and had other underlying risk factors. There were no significant differences between the HIV

uninfected and HIV infected infants possibly as a result of the limited number of infants from whom HMPV was isolated (Madhi et al., 2003a).

IJpma et al. evaluated the viral causal agents in 137 infants referred to Tygerberg Children's Hospital, Stellenbosch University, South Africa from June to August 2002 (IJpma et al., 2004).
The hospital serves a population of poor socio-economic background and children were referred for acute upper or ALRI. HMPV was detected in 8 (6%) and RSV in 21 (15%) of the 137 infants. HIV infection was found in 1 (14%) of the 7 children with HMPV in comparison to none of the 13 children with RSV (IJpma et al., 2004). Nevertheless, these data were insufficient to report on the severity of disease in HIV-infected children.

Gender

Madhi et al reported a slight predominance of male sex among infants with HMPV (Madhi et al., 2003a), while IJpma et al reported a predominance of females (5 of 8 infants) (IJpma et al., 2004). Other studies have not reported significant differences in the incidence of HMPV infection between males and females (McAdam et al., 2004). It is not clear if gender is a risk factor for HMPV infection and severity of disease and further studies are needed.

Scores to assess severity of ARI due to RSV infection

There is no generally accepted clinical score to assess the severity of RSV ALRI in infants and children. McConnochie et al. developed an index for ALRI severity in New York based on six clinical and laboratory observations. These including 1) length of hospital stay >5 days; 2) $pO_2 < 87\%$; 3) $pCO_2 > 45\%$; 4) $pH < 7.35$; 5) apnoea in hospital; and 6) the need for mechanical ventilation. This index had a good correlation with other factors that have been identified as risk factors for severe RSV ALRI (i.e. younger age when infected, premature birth and underlying medical conditions) (McConnochie et al., 1990). The same score was used by Walsh et al. to investigate whether the severity of RSV disease was related to the RSV group, confirming that the score had a strong relationship with disease severity and group A RSV (Walsh et al., 1997). A further clinical score used by Hornsleth et al. used respiratory rate > 60 breaths/min on admission; the demonstration of alveolar or peribronchial infiltration on chest radiographs; a hospital stay > 7 days and the need of respiratory support as continuous positive airway pressure by nasal prongs (Hornsleth et al., 1998). This score however was limited by the poor correlation between oxygen saturation and the respiratory rate (Mulholland et al., 1990) suggesting that the latter is of limited value as indicator of severity of illness. The presence of infiltration in chest x-rays is a marker for lower

respiratory tract infections, but is not associated with a greater clinical severity and although the duration of hospital stay is a rough indicator for severity of illness, the discharge criteria differ in each study setting depending on the policies of the hospitals.

The measurement of the severity of the clinical episodes therefore often differs across studies, according to the availability of resources (e.g. blood gases and ventilatory support). For example in Denmark, continuous positive airway pressure has been used during the last decade as a means of providing respiratory support to infants with severe respiratory distress associated with RSV infection. In developing countries however, respiratory support is often limited to giving oxygen, if available. Most of these severity scores use points to assign relative weights to the characteristics used. The weighting of these characteristics refers to the values each characteristic is allocated during the development of the severity index. These characteristics are often weighted randomly, subjectively or based on clinical experience and availability of resources. Relative weighting has also been assigned to certain characteristics depending on the setting where the index will be used. Severity index scores however should be based on physiological and statistical weights to guarantee its objective development and such scores are not available for developing countries.

Results

General characteristics of children with ARI due to RSV

RSV was identified in 269 (45%) of the 604 < 2 years old children enrolled. The clinical data for three patients were lost and the data of the remaining 266 patients were used for analysis. Thirty four (13%) children with RSV had mild and 232 (87%) severe disease as defined by the pO_2 concentration (pO_2 > and < 88% respectively). The pO_2 however showed a clear bimodal distribution with two peaks above and below a pO_2 of 87% (Figure 4.1). Children with mild or severe RSV ARI had similar gender distributions, with 22 (65%) and 132 (66%) children with mild and severe RSV ARI respectively being male. Children with severe RSV ARI were significantly younger than children with mild presentation. Those with mild RSV ARI had a mean (SD) age of 7.9 (5.6) months (median = 6 months) and range from 1-21 months compared to 4.2 (4.8) months (median = 4 months) and range from <1-24 months for children with severe ARI (p= 0.0001). Children under 3 months of age were more likely to have severe RSV ARI than children above this age (63% vs. 26% respectively, p<0.001). The age distributions of the children with mild and severe RSV ARI are shown in figure 4.2.

Children with severe RSV ARI were more likely to be premature than children with mild RSV ARI (11 (5%) vs. 1 (3%) but the difference was not statistically significant. The gestational age for 8 out of the 11 premature children with severe RSV ARI was known by the parents. One (12%) of these was born at 28 weeks, 3 (38%) at 32 weeks, 1 (12%) at 33 weeks, 2 (25%) at 34 weeks and 1 (12%) at 35 weeks gestation (table 4.2).

Table 4.2 General characteristics of the children with RSV ARI

	RSV		
	Mild ARI N=34	Severe ARI N=232	P value
Male: Female (male %)	22:12 (65%)	154:78 (66%)	0.4
Median age (months)	6	4	<0.001
Range (months)	1-21	<1-24	
Number ≤ 3 months of age	9 (26%)	147 (63%)	<0.001
Premature birth	1 (3%)	11 (5%)	0.5

Figure 4.1 pO_2 distribution of children with RSV

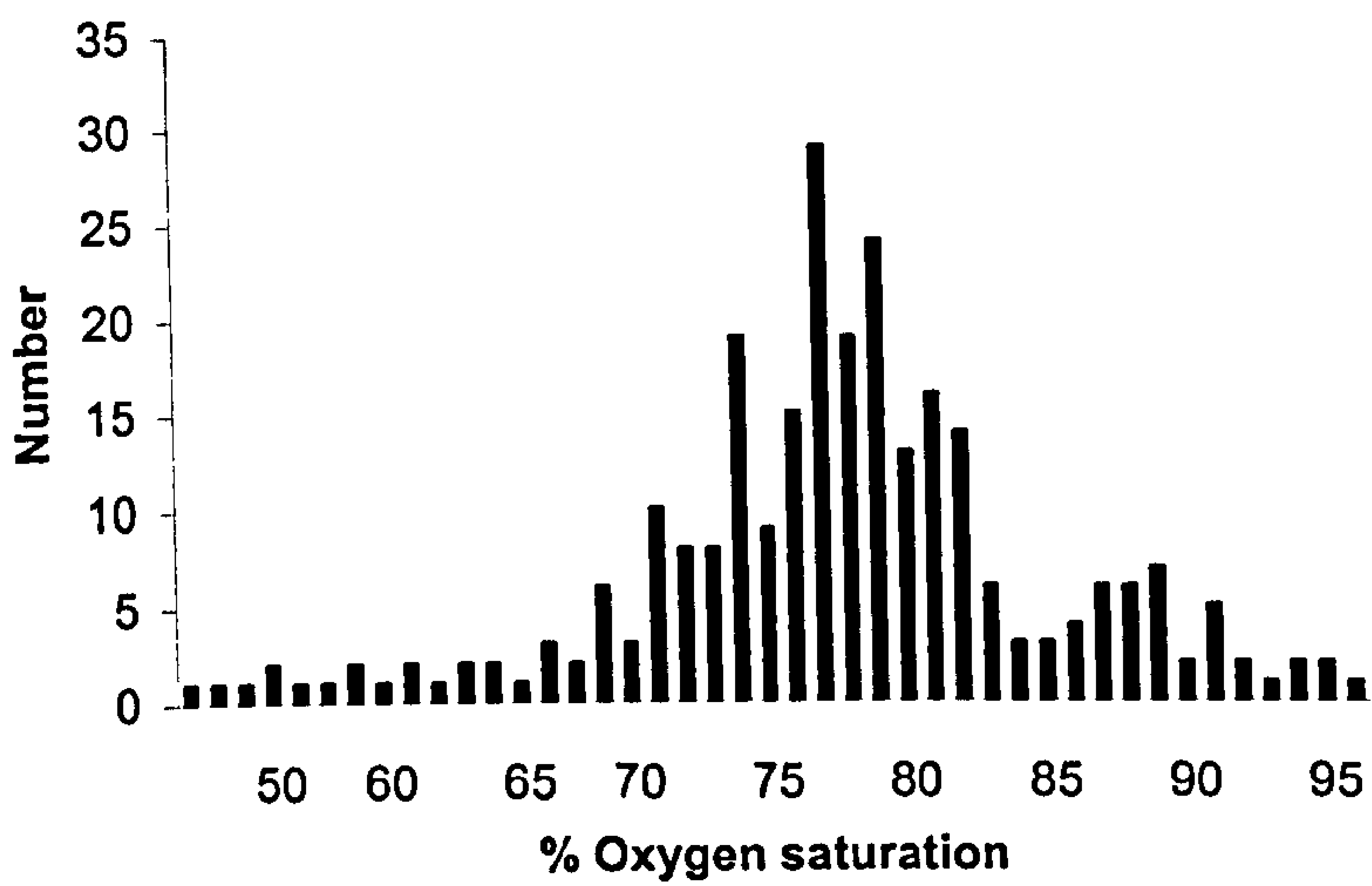
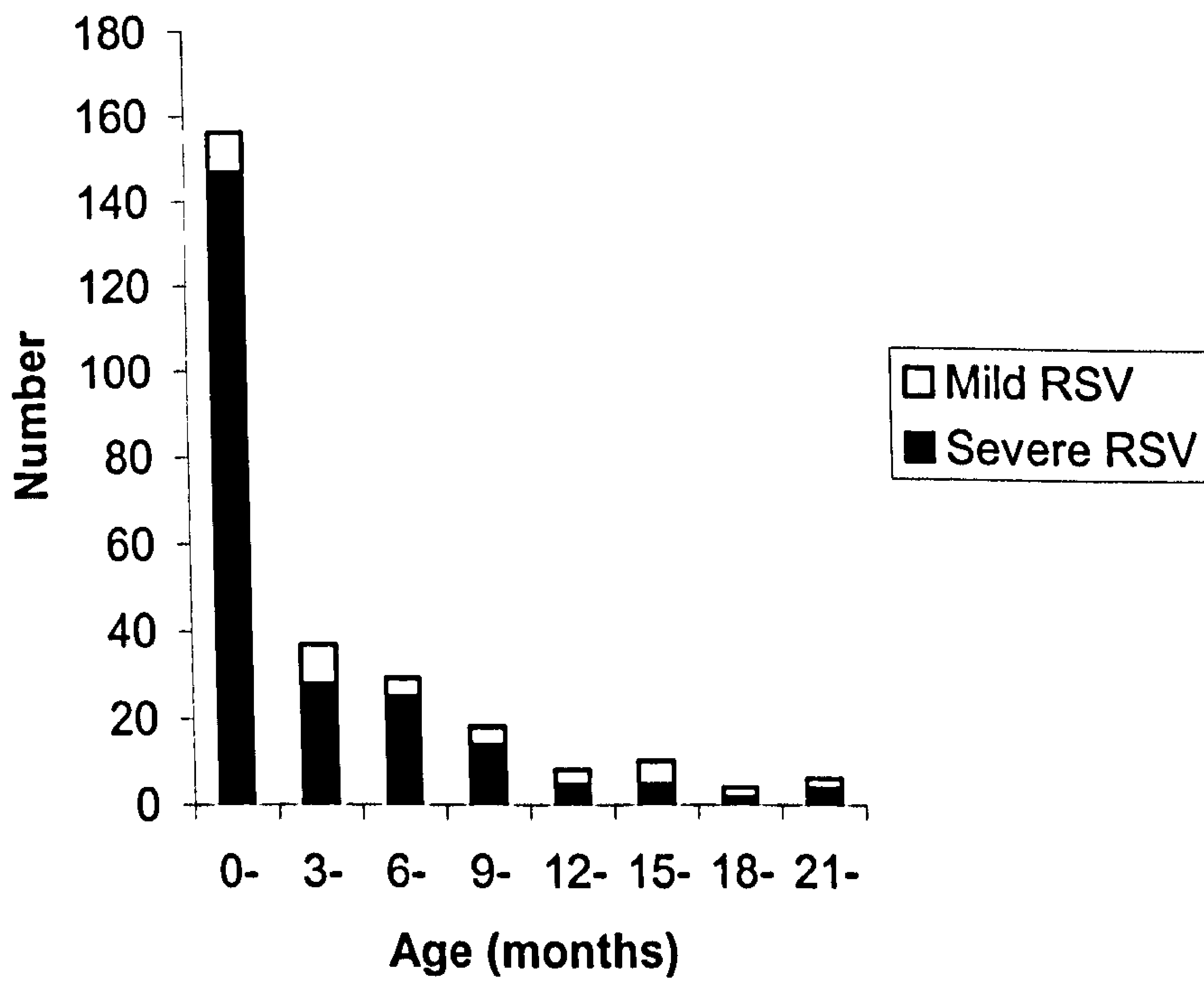
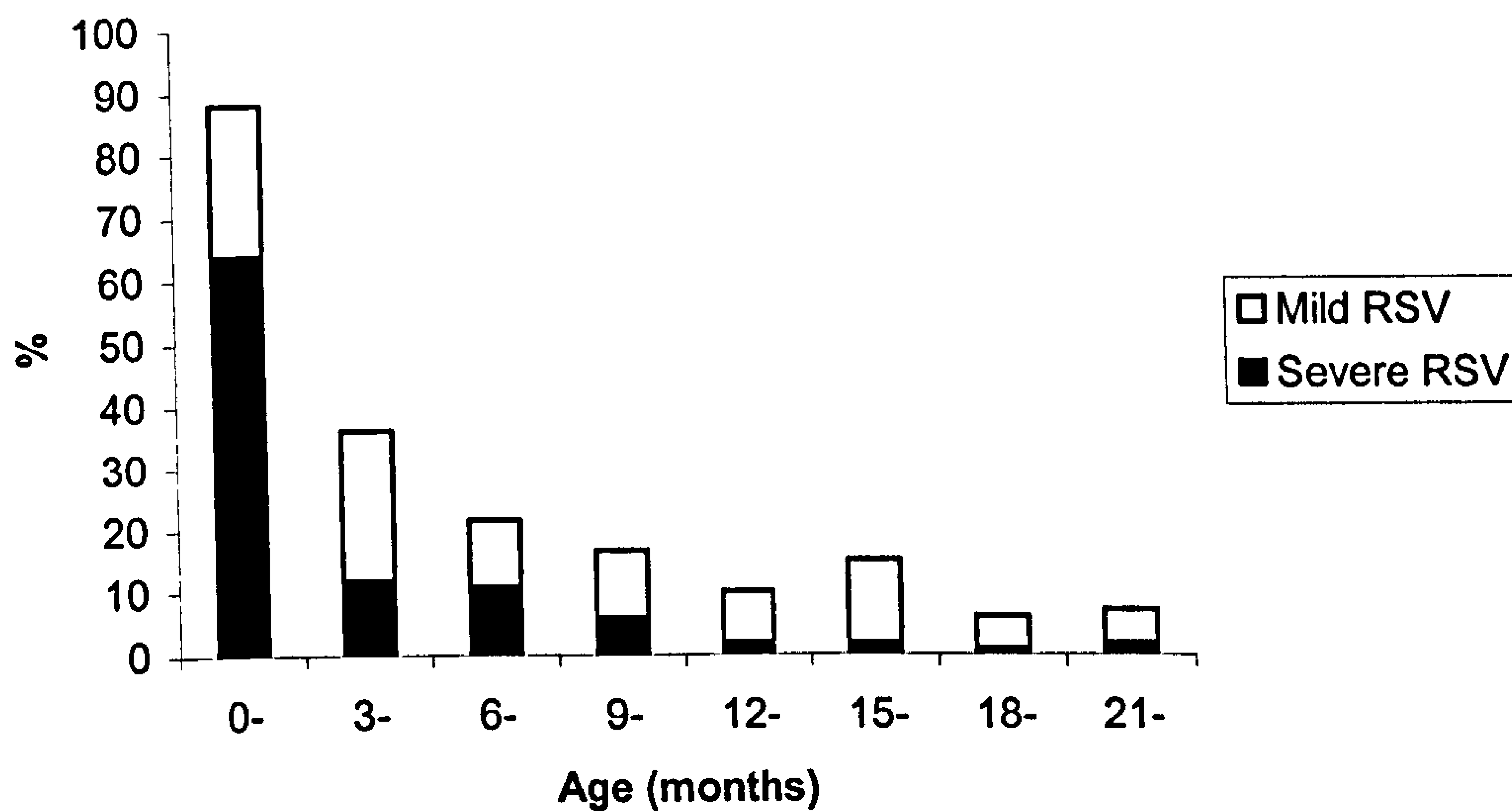


Figure 4.2 Age distributions of children with mild and severe RSV ARI

a. Frequency of children with mild and severe RSV ARI by age groups



b) Proportions of children with mild and severe RSV ARI by age groups



Medical background

There were no statistically significant differences between children with mild and severe RSV ARI regarding their past medical history as shown in table 4.3. Six (18%) children with mild and 40 (17%) with severe RSV ARI had a history of recurrent chest infections. Recurrent wheeze and bronchial asthma diagnosed by a doctor was recorded in only 1 (3%) child with mild presentation and 12 (5%) children with severe RSV ARI had recurrent wheeze. Eczema was recorded in 1 (3%) child with mild ARI and 11 (5%) children with severe ARI. Chronic lung diseases, rickets, severe malnutrition, Down syndrome and congenital heart diseases were reported in very few children.

Table 4.3 Past medical histories of the children with RSV ARI by disease severity

	RSV		
Past history of:	Mild ARI N=34	Severe ARI N=232	P value
Recurrent respiratory infections	6 (18%)	40 (17%)	0.4
Recent wheeze	1 (3%)	13 (6%)	0.2
Recurrent wheeze	1 (3%)	12 (5%)	0.4
Eczema	1 (3%)	11 (5%)	0.5
Asthma	1 (3%)	1 (0.4%)	0.2
Chronic lung diseases	0 (0%)	1 (0.4%)	0.8
Immunodeficiencies	1 (3%)	4 (2%)	0.4
Rickets	0 (0%)	5 (2%)	0.5
CHD with PH*	0 (0%)	2 (1%)	0.7

*CHD with PH= congenital heart disease with pulmonary hypertension

Thirty one (91%) children with mild and 213 (92%) with severe RSV ARI were breast fed at the time of enrolment. Twenty three (74%) of the children with mild RSV ARI were receiving

mixed feeding compared to 97 (46%) of the children with severe presentation. Children with mild infections however were older and this age difference may explain this observation. Similarly, age differences might explain the fact that children with mild RSV ARI were more likely to have their vaccinations up to date at time of recruitment than children with severe RSV ARI: 27 (79%) vs. 54 (23%) respectively (Table 4.4).

Table 4.4 Medical background of the children with RSV ARI

	RSV		
	Mild ARI N=34	Severe ARI N=232	P value
Breast feeding on enrolment	31 (91%)	213 (92%)	0.4
Partial breast feeding	23 (74%)	97 (46%)	<0.001
Ever breast fed	3 (9%)	15 (6%)	0.2
Never breast fed	0 (0%)	4 (2%)	
Mean (SD) age when weaned (months)	3.4 (1.2)	3.5 (1.6)	0.7
Vaccination not up to date	7 (21%)	177 (77%)	<0.001

Housing conditions of the children

There were no significant differences regarding the housing conditions (number of rooms, adults and children per household) between mild and severe RSV ARI, although living in families with a relative who smoked tended to be a more frequent occurrence in children with severe RSV ARI than in children with mild RSV ARI (120 (52%) vs. 9 (26%)). The smoker in the families of children with mild RSV ARI was always the father, while in families of children with severe RSV ARI the smoker was the father in 96 (87%) cases, the uncle in 3 (3%), the father and an uncle in 1 (1%) and the father and the mother in 1 (1%). In only 3 (3%) families the mother was the sole smoker and in 4 families several members of the family smoked.

The mean (SD) number of rooms in the houses of children with mild and severe RSV ARI was 4.3 (5.9) and 3.3 (2) respectively. Twenty one (62%) and 149 (64%) children with mild or severe RSV ARI lived in houses with ≤ 2 rooms respectively. The mean (SD) number of adults per household of children with mild or severe RSV ARI was 4.1 (3.1) and 3.8 (2.6) respectively. Nineteen (56%) and 122 (53%) children with mild and severe RSV ARI lived in houses with ≥ 2 adults per house respectively. The mean (SD) number of children in the home of children with mild and severe RSV ARI was 3.8 (2.9) and 4.8 (4) respectively ($p= 0.1$) and 16 (47%) and 139 (60%) children with mild and severe RSV ARI lived in houses with ≥ 3 children per house respectively ($p= 0.1$).

Nine (26%) children with mild and 97 (42%) with severe RSV ARI had indoor animals (p =0.04) and 2 (6%) and 88 (38%) had outdoor animals (p=<0.001) respectively. Children with severe RSV ARI more frequently report cows and donkeys than children with mild RSV ARI (p=0.003 and p=0.03 respectively). Two (6%) children with mild and 128 (55%) with severe RSV ARI lived in houses where gas was not the main fuel for cooking (p <0.001) and 11 (32%) and 138 (59%) children respectively lived in houses where the main source of water was non- governmental (p = 0.002). A summary of the housing conditions of children with mild and severe RSV ARI is shown in table 4.5.

Table 4.5 Housing conditions of the children with RSV ARI by disease severity

		RSV		
		Mild ARI N= 34	Severe ARI N= 232	P value
Mean (SD) number of	rooms	4.3 (5.9)	3.3 (2)	0.06
	children	3.8 (2.9)	4.8 (4)	0.1
	adults	4.1 (3.1)	3.8 (2.6)	0.5
Smoker at home		9 (26%)	120 (52%)	0.002
≤2 rooms		21 (62%)	149 (64%)	0.4
≥3 children per house		16 (47%)	139 (60%)	0.1
≥2 adults per house		19 (56%)	122 (53%)	0.3
Indoor pets		9 (26%)	97 (42%)	0.04
Indoor	cows	1 (3%)	52 (22%)	0.003
	goats	6 (18%)	69 (30%)	0.07
	chicken	3 (9%)	39 (17%)	0.1
	cats	3 (9%)	26 (11%)	0.4
	dogs	0 (0%)	8 (3%)	0.3
	donkey	0 (0%)	32 (14%)	0.03
Outdoor pets		2 (6%)	88 (38%)	<0.001
Non-governmental source of water		11 (32%)	138 (59%)	0.002
Source of fuel other than gas		2 (6%)	128 (55%)	<0.001

Clinical history on enrolment and severity of RSV ARI

The most frequent complaints at the time of consultation were cough (34 (100%) and 232 (100%) for mild and severe RSV ARI, respectively) and fever (25 (74%) and 172 (74%) respectively) as shown in table 4.6. Parents of children with severe RSV ARI were more likely to describe breathing difficulty, cyanosis and wheezing in their children (p<0.01 for all). Apnoea was reported in 17 of the children with severe ARI but none in those with mild disease.

Table 4.6 Clinical history of children with RSV ARI by disease severity

	RSV		
History of	Mild ARI N=34	Severe ARI N=232	P value
Cough	34 (100%)	232 (100%)	0.1
Duration*	4.7 (2.6)	4.3 (1.8)	0.2
Difficulty in breathing	15 (44%)	227 (98%)	<0.001
Duration	1.5 (2.3)	3.2 (1.7)	0.001
Cyanosis	5 (15%)	191 (82%)	<0.001
Duration	0.4 (1.4)	2.4 (1.8)	<0.001
Fever	25 (74%)	172 (74%)	0.4
Duration	2.9 (3.1)	2.7 (2.5)	0.7
Wheeze	4 (12%)	112 (48%)	<0.001
Duration	0.5 (1.8)	1.4 (1.8)	0.007
Unable to drink	0 (0%)	58 (25%)	<0.001
Duration	0 (0)	0.4 (0.97)	0.009
Difficulty to awake	0 (0%)	26 (11%)	0.01
Duration	0 (0)	1.6 (0.5)	0.1
Apnoea	0 (0%)	17 (7%)	0.09
Duration	0 (0)	0.12 (0.4)	0.1
Skin rash	0 (0%)	12 (5%)	0.1
Duration	0 (0)	0.03 (0.5)	0.6

* Duration prior to consultation, mean (SD) in days for all.

Clinical characteristics of children with mild and severe RSV ARI

On examination children with severe RSV ARI were more likely to have crackles, cyanosis, chest indrawing, wheezing on auscultation and pallor as shown in table 4.7. The mean (SD) respiratory rate among the children with mild RSV ARI was lower (44 (8.7) per minute) than in children with severe ARI (63 (11.9) per minute) and children with respiratory rates ≥ 60 were more likely to have pO_2 < 88% (P=<0.001). Fever (temperature >37.5°C) was more often observed in children with mild than severe RSV ARI (29% vs. 17% respectively), (p=0.04).

Children with severe RSV ARI were more likely to be diagnosed as having pneumonia (54%) or bronchiolitis (42%) than children with mild hypoxia (3% and 9% respectively, p<0.001). Similarly, fewer children with severe disease were diagnosed as having acute upper respiratory tract infections than children with mild hypoxia.

Radiological findings on admission and clinical diagnosis of children with RSV ARI

The staff attending the children was less likely to request chest x rays for children with mild hypoxia than for children with severe hypoxia. Chest x-rays were obtained from 9 (26%) children with mild and 218 (94%) with severe RSV ARI. The x rays of the latter group were

also more likely to be read as abnormal. Patchy change was reported by medical attendant in 4 (44%) of the children with mild ARI and 210 (96%) of the children with severe RSV ARI. Hyperinflation was observed in 5 (56%) children with mild and 176 (81%) children with severe RSV ARI respectively (Table 4.7).

HMPV co-infection

There was no difference in the percentages of children with mild or severe RSV ARI who were coinfected with HMPV (9% vs. 10% respectively) (Table 4.7)

Table 4.7 Clinical signs of the children with RSV ARI

		RSV		
		Mild ARI N=34	Severe ARI N= 232	P value
Crackles		5 (15%)	218 (94%)	<0.001
Cyanosis		2 (6%)	205 (88%)	<0.001
Chest indrawing		2 (6%)	190 (82%)	<0.001
Wheeze		8 (24%)	95 (41%)	0.02
Mean (SD) respiratory rate/minute		44 (8.7)	63 (11.9)	<0.001
Respiratory rate > 60 breaths/minute		2 (6%)	181 (78%)	<0.001
Fever (>37.5°C)		10 (29%)	39 (17%)	0.04
Hepatomegaly		0 (0%)	10 (4%)	0.2
Heart murmurs		0 (0%)	6 (3%)	0.4
Splenomegaly		0 (0%)	4 (2%)	0.5
Pallor		2 (6%)	180 (78%)	<0.001
pO_2 mean (SD)		91% (2.7)	77% (6.8)	<0.001
Clinical diagnosis				
	Pneumonia	1 (3%)	124 (54%)	<0.001
	Bronchiolitis	3 (9%)	98 (42%)	<0.001
	Mild ARI	30 (88%)	10 (4%)	<0.001
Chest x-ray taken (%)		9 (26%)	218 (94%)	<0.001
	Patchy change	4 (44%)	210 (96%)	<0.001
	Hyperinflation	5 (56%)	176 (81%)	0.04
Co-infection with HMPV		3 (9%)	22 (10%)	0.6

Nutritional status of the children with mild and severe ARI due to RSV

The weight for age (WAZ), height for age (HAZ) and weight for height (WHZ) Z scores were calculated for all children. Children with mild and severe RSV ARI had a mean (SD) WAZ of -1.78 (1) and -1.9 (1.6) respectively (p=0.6). Eleven (32%) children with mild and 89 (38%) with severe RSV ARI had WHZ scores below ≤ - 2 (p=0.4). The mean (SD) HAZ for children with mild and severe RSV ARI were -1.15 (1.65) and -1.34 (2.0) respectively (p=0.6). Thirteen (38%) children with mild and 73 (31%) with severe RSV ARI had HAZ scores below ≤ - 2, (p=0.2). The mean (SD) WHZ for children with mild and severe RSV ARI were -1.2 (2.6) and -1.6 (3.4) respectively (p=0.5). None of the children had WHZ ≤ -2 scores. The

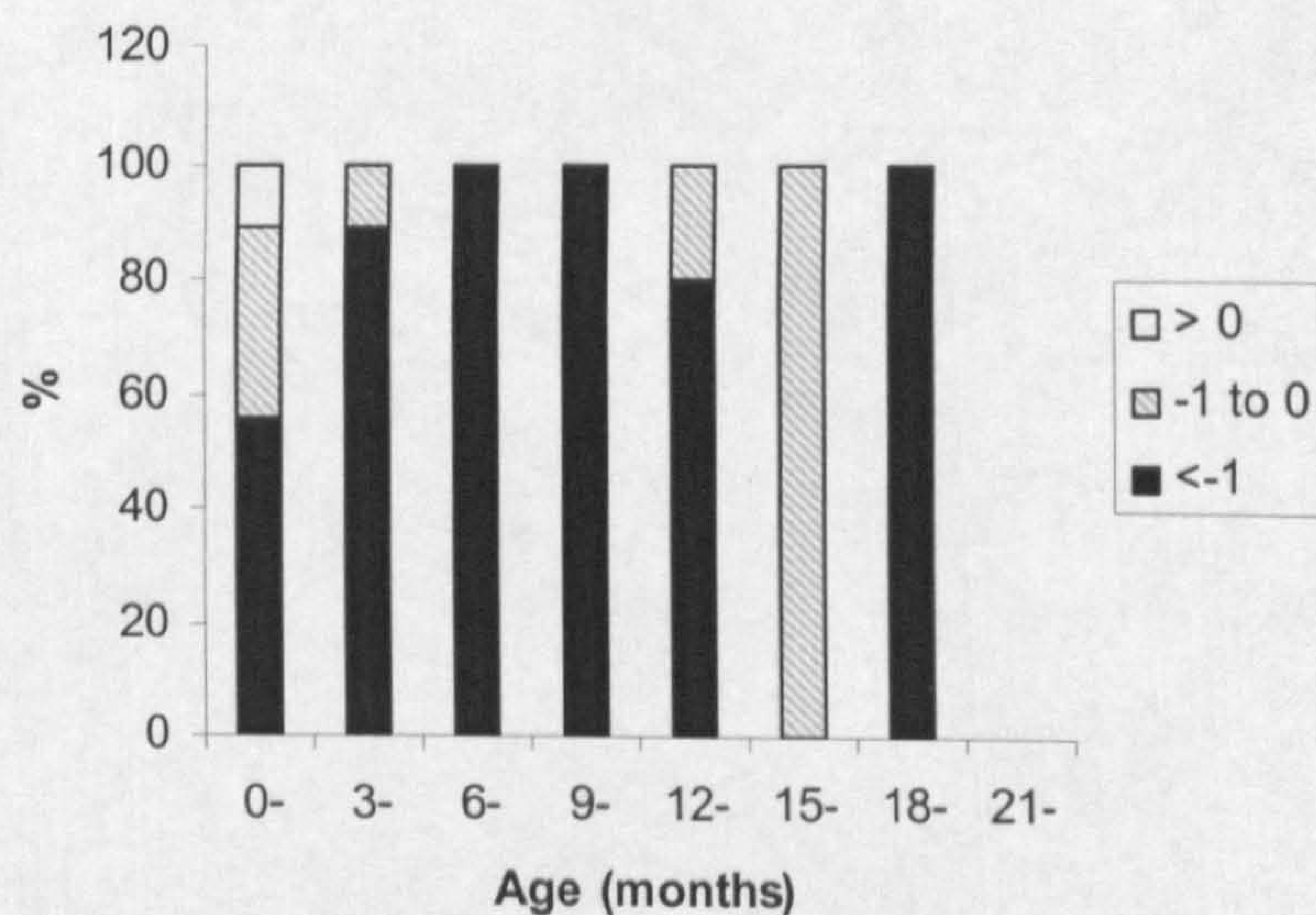
nutritional indicators of the children are shown in table 4.8 and figure 4.3 shows the WAZ scores distribution by age.

Table 4.8 Nutritional indicators of the children with mild and severe RSV ARI

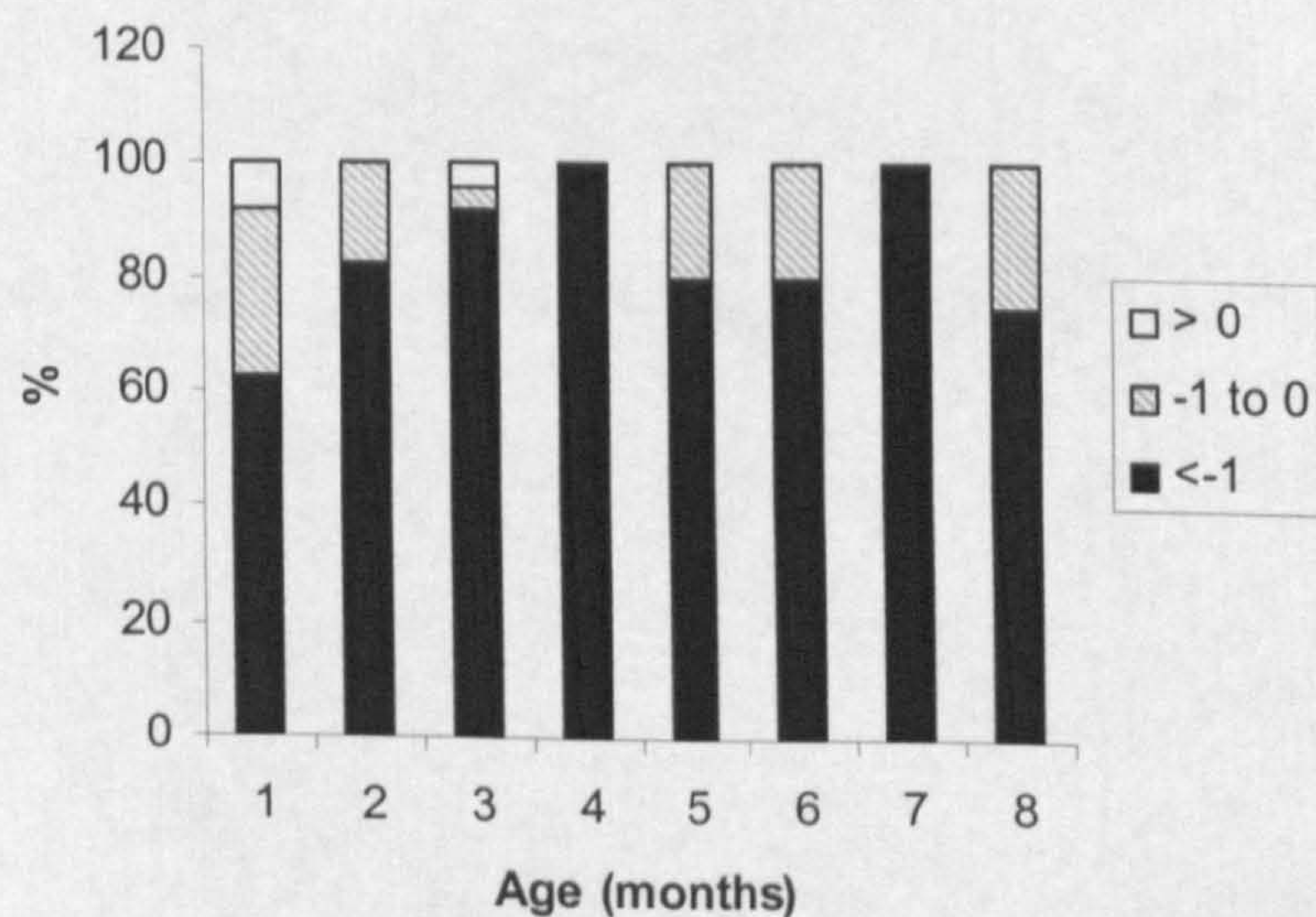
	RSV		
Nutritional indicators	Mild ARI N=34	Severe ARI N=232	P value
Mean (SD) WAZ score	- 1.78 (1)	-1.9 (1.6)	0.6
WAZ ≤-2	11 (32%)	89 (38%)	0.4
Mean (SD) HAZ score	-1.15 (1.65)	-1.34 (2.0)	0.6
HAZ ≤-2	13 (38%)	73 (31%)	0.2
Mean WHZ (SD) score	-1.2 (2.6)	-1.6 (3.4)	0.5
WHZ ≤-2	0 (0%)	0 (0%)	

Figure 4.3 WAZ scores distribution by age in children with (a) mild and (b) severe RSV

a.



b.



Hospital management

The majority of the children with mild RSV ARI were recruited from the outpatient clinic (30, 88%) while the majority of children with severe RSV ARI were recruited from A&E (181, 78%). Two hundred and four (78%) children were admitted and followed up. Two (6%) children with mild RSV ARI and 192 (83%) children with severe RSV ARI were given antibiotics (p <0,001). Oxygen therapy was given to 1 (3%) child with mild RSV ARI and to147 (63%) with severe RSV ARI. None of the children with mild RSV ARI received IVF or bronchodilators while 16 (7%) of the children with severe RSV ARI received IVF and 6 (3%) bronchodilators. Table 4.9 shows the medications given to the children.

Table 4.9 Hospital management of the children with RSV ARI

	RSV		
Children receiving:	Mild ARI N= 34	Severe ARI N= 232	P value
Antibiotics	2 (6%)	192 (83%)	<0.001
Oxygen supplementation	1 (3%)	147 (63%)	<0.001
Anti-cough syrups	19 (56%)	127 (55%)	0.4
Intravenous fluids	0 (0%)	16 (7%)	0.09
Bronchodilators	0 (0%)	6 (3%)	0.4
Steroids	1 (3%)	3 (1%)	0.4

Family histories of the children

Children with severe RSV ARI were more likely to have other family members with chest infections at the time of enrolment than children with mild RSV ARI (165 (71%) and 18 (53%) respectively, P =0.02) as shown in table 4.10. A family history of allergies was found in 3 (9%) and 29 (13%) children with mild and severe RSV ARI respectively (P = 0.3). Five (15%) and 3 (9%) children with mild RSV ARI had a family history of asthma and eczema. In comparison, 24 (10%) and 11 (5%) of the children with severe RSV ARI had a history of asthma and eczema (P= 0.2 for both).

Family histories of tuberculosis and congenital heart diseases were reported in 5 (2%) and 3 (1%) children with severe RSV ARI respectively, but in none of the children with mild RSV. A summary of the family medical history is shown in table 4.10.

Table 4.10 Family history of the children with RSV ARI

Family medical history	RSV Mild ARI N= 34	Severe ARI N= 232	P value
Family member with ARI	18 (53%)	165 (71%)	0.02
Allergies	3 (9%)	29 (13%)	0.3
Asthma	5 (15%)	24 (10%)	0.2
Eczema	3 (9%)	11 (5%)	0.2
Tuberculosis	0 (0%)	5 (2%)	0.5
Congenital heart disease	0 (0%)	3 (1%)	0.7

RSV genotypes and disease severity

The RSV N genotypes were determined for 208 (77%) specimens that belonged to 31 children with mild ARI and 177 with severe ARI. Of these 171 (82%) were group A RSV and 37 (18%) group B as shown in table 4.11. Twenty six (84%) children with mild and 145 (82%) children with severe ARI were infected with group A RSV (P=0.4). Five (16%) children with mild ARI and 32 (18%) children with severe ARI were infected with group B RSV (p= 0.4).

Eight (5%) and 24 (13%) of the children with severe ARI and 3 (10%) and 2 (6%) children with mild ARI were infected with RSV genotype groups NP1 and NP3.

Table 4.11 RSV genotypes and group by disease severity

RSV group	Mild ARI N=31	Severe ARI N=177
A	26 (84%)	145 (82%)
B	5 (16%)	32 (18%)
NP1	3 (10%)	8 (5%)
NP2	12 (39%)	84 (48%)
NP3	2 (6%)	24 (13%)
NP4	14 (45%)	61 (34%)

P=0.4 for RSV groups and 0.3 for RSV genotypes

NP2 was identified in 12 (39%) and 84 (48%) of the children with mild and severe ARI respectively and NP4 was identified in 14 (45%) and 61 (34%) of the children with mild and severe ARI (Table 4.11). None of these differences were statistically significant.

Risk factors for severe RSV ARI

It was assumed that children with RSV ARI with $pO_2 \leq 87\%$ had more severe illness than children with $pO_2 > 88\%$ (see figure 4.1). The factors identified as having an association with severe RSV ARI through the previous section were therefore selected for inclusion into a

multivariate analysis if their P values were < 0.2. To facilitate this analysis, continuous variables were dichotomised. The most suitable cut off points were determined. The age distribution of the children followed a bimodal distribution with a dip just before 3 months (Figure 4.4). Given that children with severe ARI presented more frequently below 3 months (Lanari et al., 2002), this age was selected as a cut off point. The frequency distribution of adults and the number of rooms in the household was slightly skewed to the right. For this reason the median number of adults and the median number of rooms in the household were used as cut off points. Table 4.12 provides a summary of the variables selected for multivariate analysis.

The best fitting conditional logistic regression model for the demographic variables is shown in table 4.13. The variables independently associated with RSV ARI, were age < 3 months (AOR=3.2), the child not having his/her vaccines up to date (AOR= 4.5), the presence of a smoker at home (AOR= 3.5) and using fuel other than gas (AOR=14.0).

Figure 4.4 Age distribution of children with RSV

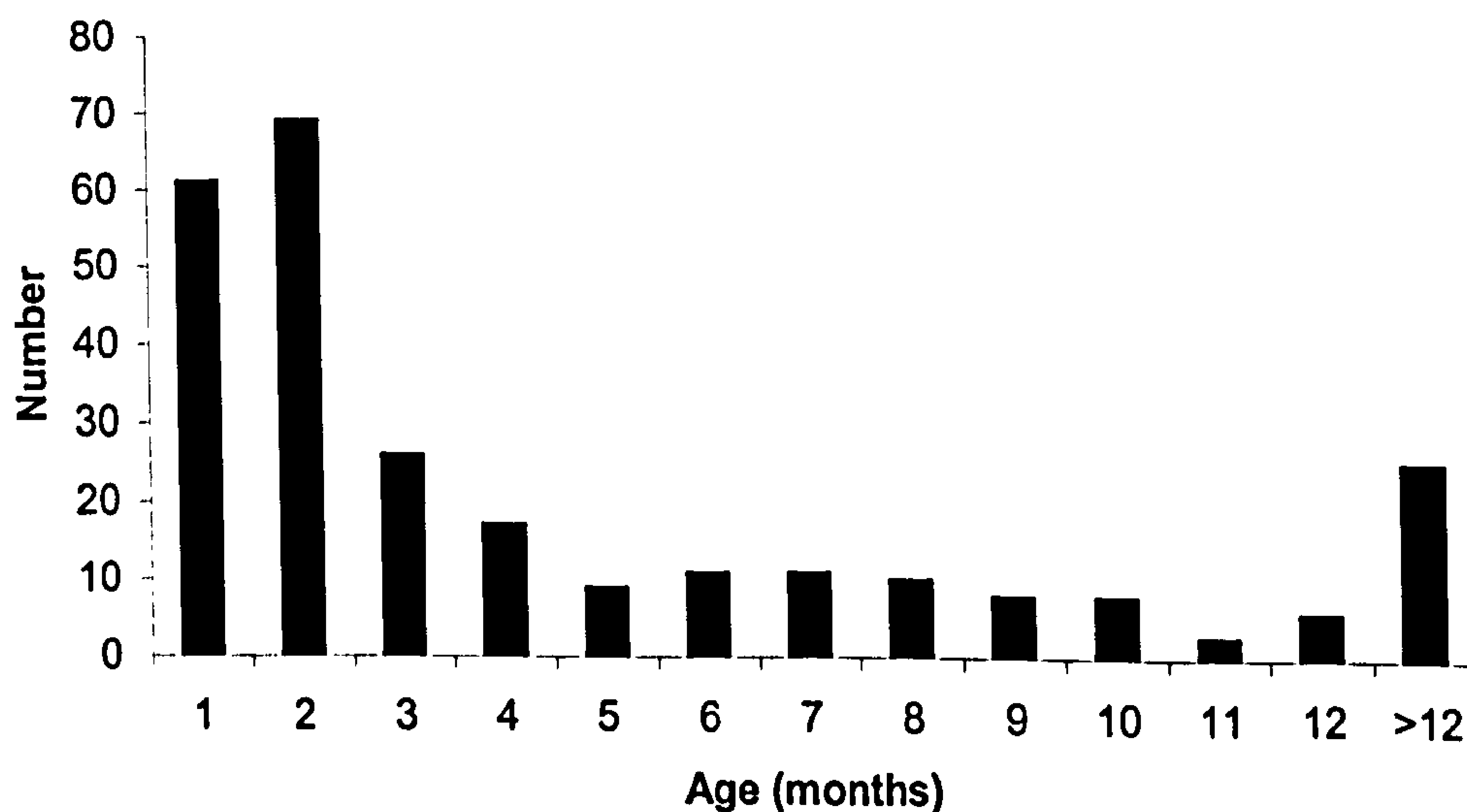


Table 4.12 Characteristics selected for multivariate analysis

	RSV				
	Mild ARI N= 34	Severe ARI N= 232	OR	95% CI	P value
≤ 3 months of age	9 (26%)	147 (63%)	5.10	2.27-11.46	<0.001
>3 children in the house	16 (47%)	139 (60%)	1.60	0.77-3.33	0.1
Smoker at home	9 (26%)	120 (52%)	2.97	1.33-6.65	0.001
Indoor pets	9 (26%)	97 (42%)	1.99	0.88-4.46	0.04
Non-governmental water	11 (32%)	138 (59%)	3.1	1.43-6.8	0.01
Source of fuel other than gas	2 (6%)	128 (55%)	19.5	5.3-123.5	<0.01
Family member with ARI	18 (53%)	165 (71%)	2.18	1.05-4.5	0.02
Mixed breast feeding	12 (35%)	136 (59%)	2.59	1.22-5.5	0.01
Vaccines not up to date	7 (20%)	178 (77%)	12.3	5.12-30.06	<0.001
Rural residency	3 (9%)	93 (40%)	6.7	2.3-29	<0.001

Table 4.13 Logistic regression of demographic and socio-economic criteria to identify of children with severe RSV ARI

	OR	AOR	95% C.I.	P value
Use of fuel other than gas	19.5	14.0	3.1 - 64.0	<0.001
Vaccines not up to date	12.3	4.5	1.7 - 12.0	0.002
Smoker at home	3.0	3.5	1.4 – 8.9	0.007
≤ 3 months of age	5.1	3.2	1.2 - 8.2	0.01

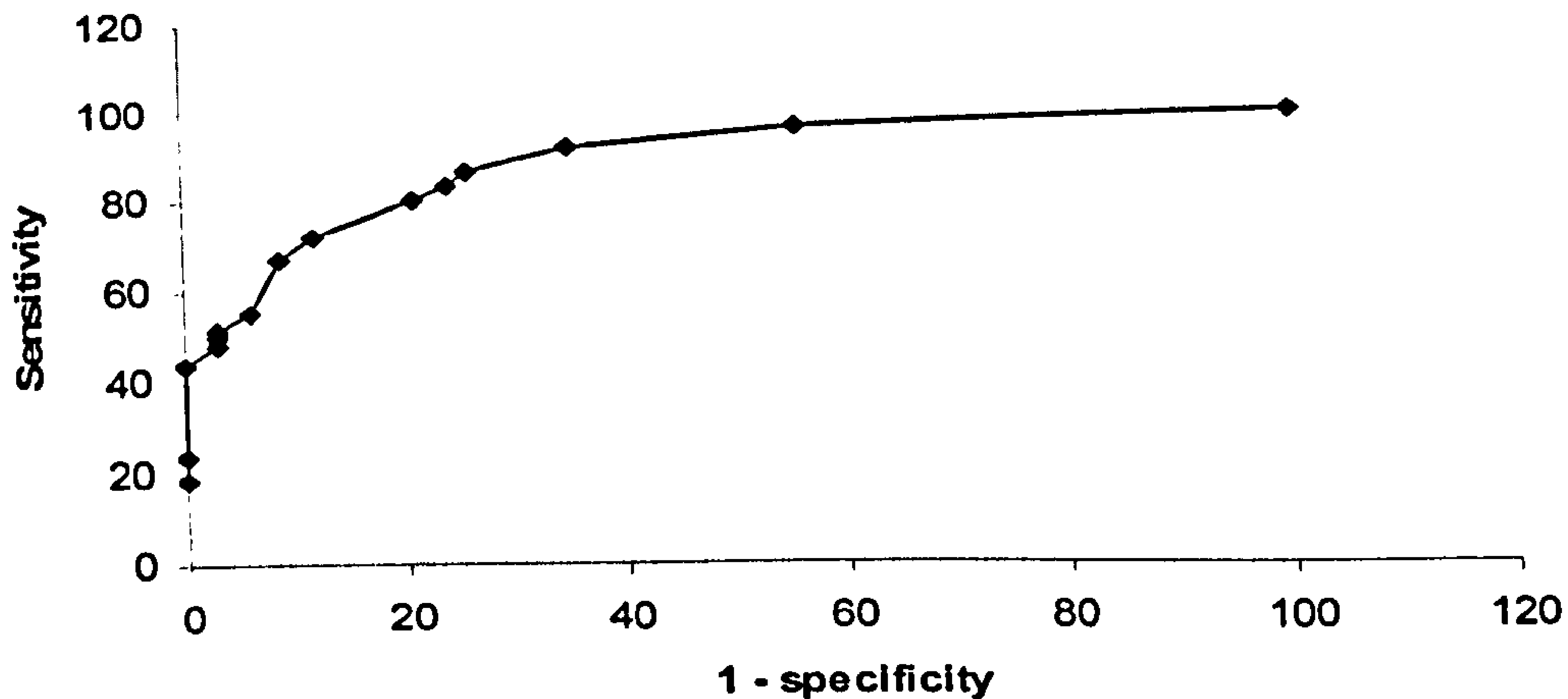
Severity score for children with RSV

Each variable was given a weighted score that corresponded to the value of the AOR and the AOR for each child was added to produce a sum score. This process was repeated for all cases and controls. The sensitivity and specificity was calculated for each cut off point as illustrated in table 4.13.and a ROC curve was drawn to find the optimal cut off point that gives the highest sensitivity and specificity for the score (Figure 4.5) a sum score of 7 has a sensitivity of 83% but a specificity of 76%. Given the low predictive value of the curve however this approach did not merit further analysis.

Table 4.14 Sensitivity and specificity of the RSV severity score

Cut off points	Cases n=227	Controls n=34	Sensitivity	Specificity	1- specificity
0	8	15	100	0	100
3	10	4	96	44	56
4	13	6	92	65	35
5	7	1	86	74	26
7	7	1	83	76	24
8	18	3	80	79	21
9	12	1	72	88	12
12	28	1	67	91	9
14	8	1	55	94	6
17	3	0	51	97	3
18	5	0	50	97	3
19	9	1	48	97	3
22	45	0	44	100	0
23	18	0	24	100	0
26	36	0	19	100	0

Figure 4.5 ROC curve of the severity score to identify children with severe RSV ARI



General characteristics of children with mild and severe HMPV ARI

HMPV was identified in 66 (11%) of the 604 children enrolled. Twenty (30%) children with HMPV had mild and 46 (70%) severe disease. Seventeen (85%) and 30 (65%) of the children with mild and severe disease were male respectively (p=0.08). Children with severe HMPV ARI were younger than children with mild presentation (p= 0.02), with a mean age (SD) of 5.7 (5.4) months (median 3 months) and 9.1 (5.9) months (median 7 months) respectively (p= 0.02). More children ≤ 3 months of age had severe HMPV ARI than children above this age (20% vs. 52% respectively) as shown in Figure 4.6. Children with severe HMPV ARI were

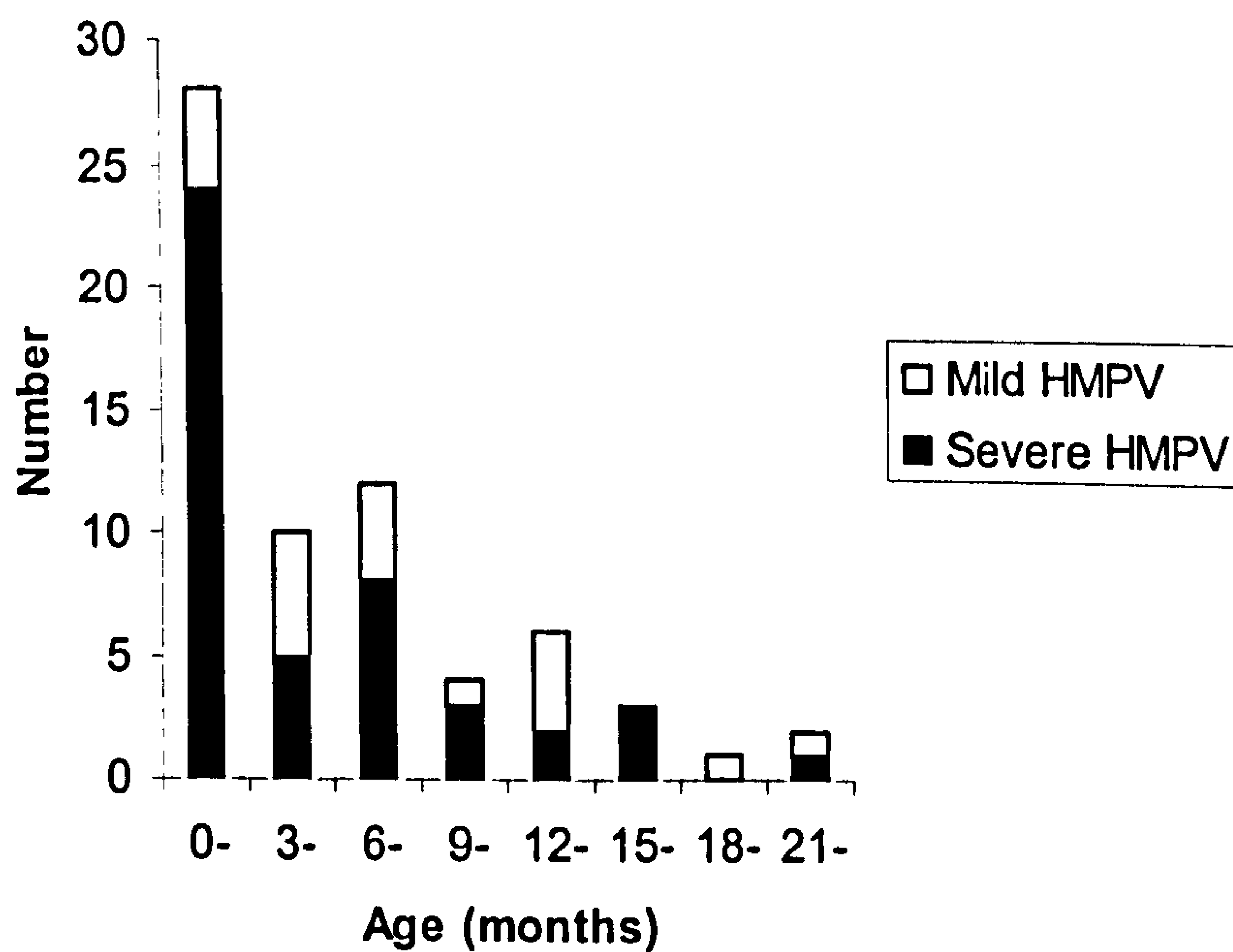
more likely to be premature than children with mild HMPV ARI (3 (7%) vs. 1 (5%)) but the difference was not statistically significant as shown in table 4.15.

Table 4.15 General characteristics of the children with mild and severe HMPV ARI

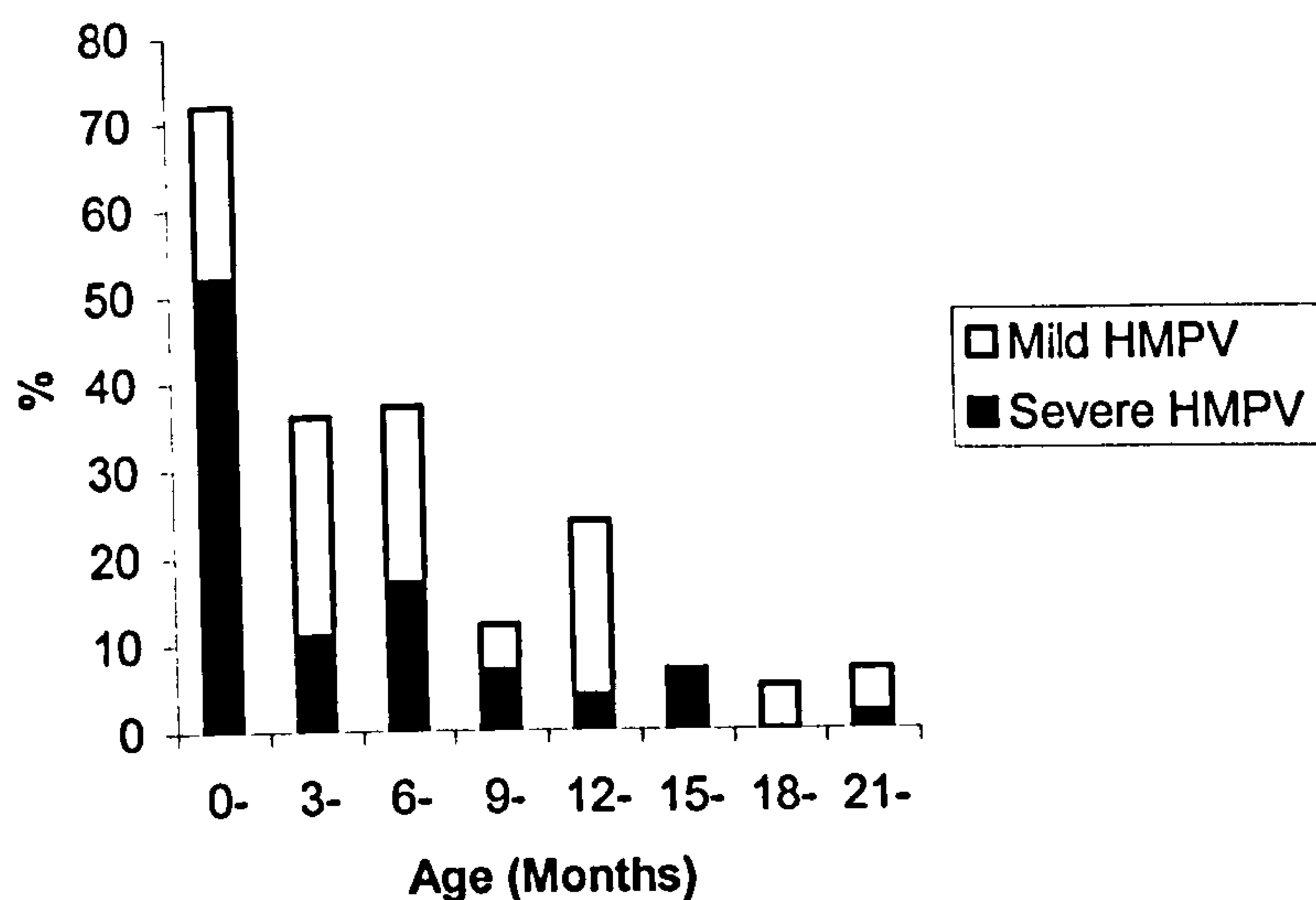
	HMPV		
	Mild ARI N= 20	Severe ARI N= 46	P value
Male: Female (% male)	17:3 (85%)	30:16 (65%)	0.08
Median age (months)	7	3	0.02
Range (months)	1-24	1-24	
≤ 3 months of age	4 (20%)	24 (52%)	0.01
Premature birth	1 (5%)	3 (7%)	0.6

Figure 4.6 Age distribution of children with mild and severe HMPV ARI

a. Frequency of children with mild and severe HMPV ARI by age groups



b) **Proportions of children with mild and severe HMPV ARI by age groups**



Medical background

There were no statistically significant differences between children with mild and severe HMPV infection regarding their past medical history with the exception of recurrent ARI. Two (10%) children with mild and 15 (33%) with severe HMPV ARI had a history of recurrent chest infections (p=0.04). None of the children with mild HMPV had recent or recurrent wheeze or asthma. Recurrent wheeze was reported in 5 (11%) children with severe HMPV ARI (p>0.1). None of the children with HMPV reported histories of congenital heart or chronic lung diseases, immunodeficiencies or rickets. Eczema was recorded in 1 child each with mild and severe ARI. A summary of the past medical history of the patients is shown in table 4.16.

Table 4.16 Past medical history of the children with HMPV ARI

	HMPV		
Past history of:	Mild ARI N= 20 (30%)	Severe ARI N= 46 (70%)	P value
Recurrent respiratory infection	2 (10%)	15 (33%)	0.04
Recent wheeze	0 (0%)	6 (13%)	0.1
Recurrent wheeze	0 (0%)	5 (11%)	0.1
Eczema	1 (5%)	1 (2%)	0.5
Asthma	0 (0%)	3 (7%)	0.3
Congenital heart diseases	0 (0%)	0 (0%)	0.3
Chronic lung diseases	0 (0%)	0 (0%)	0.3
Immunodeficiencis	0 (0%)	0 (0%)	0.3
Rickets	0 (0%)	0 (0%)	0.3

All 19 (95%) children with mild and 39 (85%) children with severe HMPV ARI were being breast fed at time of enrolment. Of these 15 (79%) with mild and 23 (59%) with severe

HMPV ARI were having partial breast feeding, which was related to the older age of children with the mild presentation. A higher proportion of children with mild HMPV ARI had their vaccinations up to date than children with severe HMPV ARI (16 (80%) vs. 16 (35%) respectively; p=<0.001) (table 4.17).

Table 4.17 Medical background of the children with HMPV ARI

	HMPV		
	Mild ARI N= 20 (30%)	Severe ARI N= 46 (70%)	P value
Previously breastfed	1 (5%)	6 (13%)	0.6
Breast feeding on enrolment	19 (95%)	39 (85%)	0.2
Partial breast feeding	15 (79%)	23 (59%)	0.1
Never breastfed	0 (0%)	1 (5%)	0.6
Mean (SD) age when weaned (months)	3.8 (1.4)	3.3 (1.4)	0.3
Vaccines not up to date	4 (20%)	30 (65%)	<0.001

Housing conditions of the children

There were no significant differences regarding the housing conditions (number of rooms, adults and children per household and the presence of a smoker at home) between mild and severe HMPV ARI.

One (5%) child with mild HMPV ARI and 14 (30%) with severe HMPV ARI were living in houses with outdoor animals (p=0.01) and 4 (20%) and 15 (33%) children with mild and severe HMPV ARI were living with indoor animals respectively. Of these, cows were significantly more frequent inside the houses of children with severe HMPV ARI (p=0.02).

Two (10%) and 23 (50%) children with mild and severe HMPV ARI lived in houses where gas was not the main fuel for cooking (p=0.001) and 5 (25%) and 22 (48%) children with mild and severe HMPV ARI lived in houses where the main source of water was from a non-governmental water project (p=0.04). A summary of the housing conditions of children is shown in table 4.18.

Table 4.18 Housing condition of the children with mild and severe HMPV ARI

		HMPV		
		Mild ARI N= 20	Severe ARI N= 46	P value
Mean (SD) number of	rooms	3.1 (1.5)	3.1 (1.6)	0.9
	children	5 (2.8)	4.6 (3.2)	0.5
	adults	3.6 (3.2)	3.7 (2.2)	0.8
≤ 2 rooms		9 (45%)	19 (41%)	0.3
> 3 children per house		14 (70%)	29 (63%)	0.3
>2 adults per house		9 (45%)	28 (61%)	0.1
Smoker at home		10 (50%)	28 (61%)	0.2
Indoor pets		4 (20%)	15 (33%)	0.2
Indoor:	cows	0 (0%)	9 (20%)	0.02
	goats	1 (5%)	7 (15%)	0.2
	chicken	2 (10%)	7 (15%)	0.4
	cats	3 (15 %)	7 (15%)	0.6
	dogs	0 (0%)	0 (0%)	0.2
	donkey	0 (0%)	4 (9%)	0.2
Outdoor pets		1 (5%)	14 (30%)	0.01
Non-governmental source of water		5 (25%)	22 (48%)	0.04
Non-gas source of fuel		2 (10%)	23 (50%)	0.001

Clinical history on enrolment and severity of HMPV ARI

The most frequent complaint on presentation of the children with mild and severe HMPV ARI was cough (19 (95%) and 46 (100%) respectively) followed by fever (14 (70%) and 38 (83%) respectively) without significant differences between the groups as shown in table 4.19.

Children with severe HMPV ARI were more likely to have difficulty of breathing, cyanosis, wheezing and to be unable to drink ($p<0.01$ for all). Apnoea was only seen in children with severe HMPV ARI, although this was not statistically significant ($p=0.4$).

Table 4.19 Clinical history of the children with mild and severe HMPV ARI

	HMPV		
History of	Mild ARI N= 20	Severe ARI N= 46	P value
Cough	19 (95%)	46 (100)	0.3
Duration*	4.3 (2.8)	4.2 (2.1)	0.9
Difficulty of breathing	6 (30%)	42 (91%)	<0.001
Duration	1.35 (2.9)	2.6 (1.6)	0.01
Fever	14 (70%)	38 (83%)	0.1
Duration	2.1 (2.8)	3.2 (2.3)	0.1
Cyanosis	6 (30%)	36 (78%)	<0.001
Duration	0.9 (1.9)	2.2 (1.8)	0.01
Wheeze	1 (5%)	19 (41%)	0.002
Duration	0.5 (2.2)	1.3 (2)	0.01
Difficulty to awake	0 (0%)	5 (11%)	0.1
Duration	0 (0)	0.24 (0.88)	0.2
Unable to drink	0 (0%)	10 (22%)	0.01
Duration	0 (0)	0.35 (0.88)	0.07
Apnoea	0 (0%)	2 (4%)	0.4
Duration	0 (0)	0.07 (0.33)	0.3
Skin rash	1 (5%)	1 (2%)	0.5

* Duration, mean (SD) in days for all.

Clinical characteristics of children with mild and severe HMPV ARI

There were statistical significant differences between mild and severe HMPV ARI regarding the presence to crackles, cyanosis, chest indrawing, wheezing on auscultation and pallor (10% vs. 89%, 5% vs. 80%, 5% vs. 76%, 10% vs. 35% and 0% vs. 74% respectively) as shown in table 4.21.The mean (SD) respiratory rate of children with mild HMPV ARI was lower (38.7 (8.3) per minute) than in children with severe ARI (58.4 (13.6) per minute ($p<0.001$)). Children with mild and severe HMPV ARI however had similar frequencies of fever (temperature >37.5°C) which, interestingly was more often observed in children with mild presentation (25% vs. 17% respectively, p=0.2).

Children with severe HMPV ARI were more likely to be diagnosed as having pneumonia (48%) and bronchiolitis (41%) than children with mild clinical presentations (0% and 5% respectively, p=<0.05 for both). Similarly, most children with mild hypoxia were diagnosed as having mild acute upper respiratory tract infections.

Radiological findings and clinical diagnosis of children with HMPV ARI on admission

The staff attending the children was less likely to request chest radiographies for children with mild HMPV ARI. Chest x-rays were obtained from 3 (15%) children with mild and 42 (91%) children with severe HMPV ARI (p=0.001). Chest x-rays were more likely to be read

as abnormal in children with severe ARI. Patchy change was found in 1 (33%) child with mild ARI and 40 (95%) with severe HMPV ARI ($p < 0.01$). Hyperinflation was observed in 1 (33%) and 33 (79%) children with mild and severe HMPV ARI (p=0.1).

RSV coinfection

There were statistical significant differences in the proportion of children who had RSV co-infections in the mild and severe HMPV ARI group. RSV was coinfecting 3 (15%) of the children with mild clinical presentation and 22 (48%) of those with severe hypoxia ($p < 0.01$) (Table 4.21).

Nutritional status of the children with mild and severe HMPV ARI

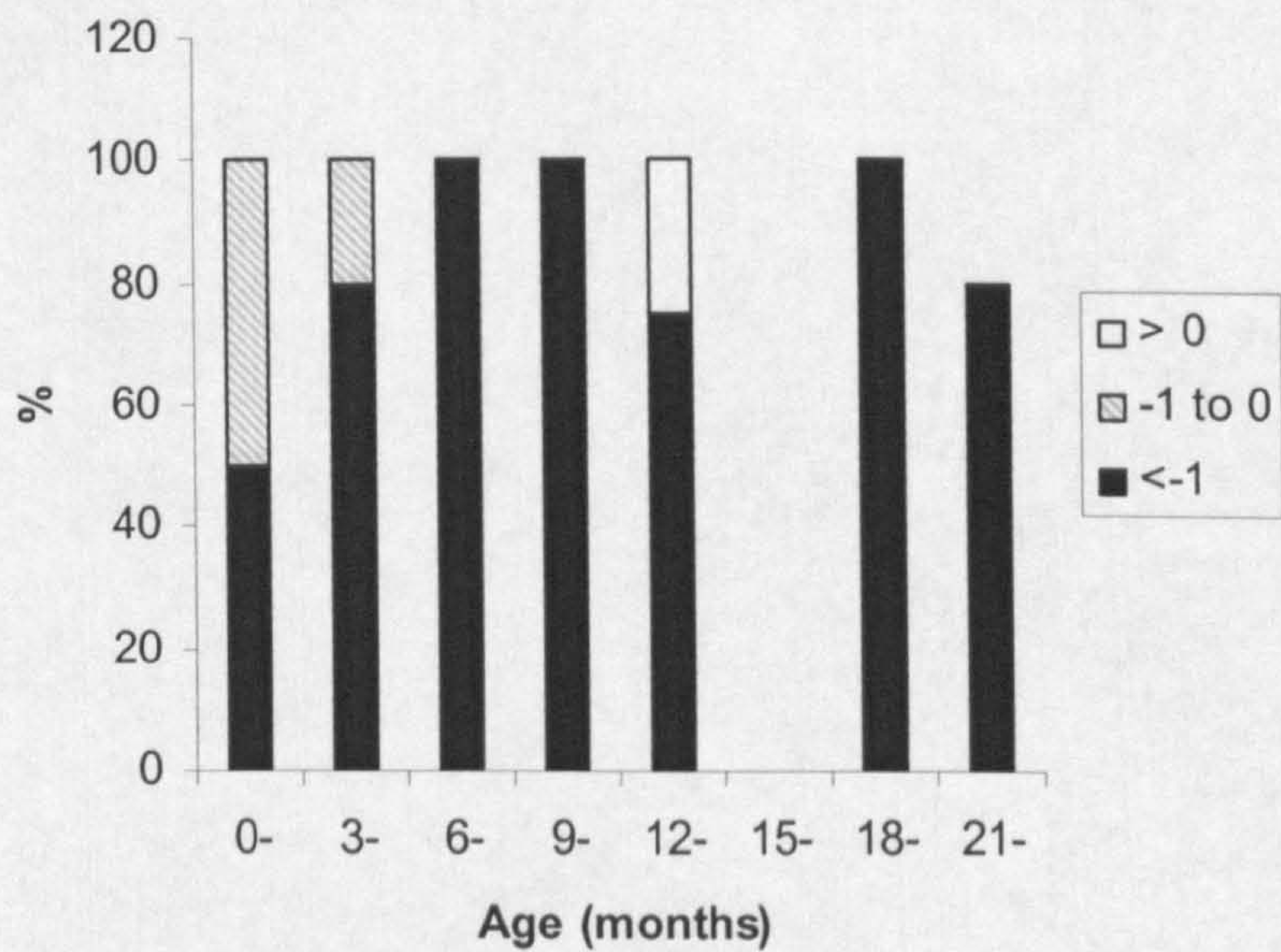
The mean (SD) WAZ scores for children with mild and severe HMPV ARI were -2.5 (2.7) and -1.6 (1.1) respectively (p=0.06). Seven (35%) children with mild and 16 (35%) with severe HMPV ARI had WAZ scores below < - 2 (p=0.4). The mean (SD) HAZ for children with mild and severe HMPV ARI were -2.59 (3.27) and -1.34 (2.5) respectively (p=0.09). Twelve (60%) children with mild and 19 (41%) with severe HMPV ARI had HAZ scores below < - 2, (p=0.08). The mean (SD) WHZ for children with mild and severe HMPV ARI were -0.6 (2.6) and -0.9 (3.2) respectively (p=0.6). None of the children had WHZ score ≤ -2. The nutritional indicators of the children are shown in Table.4.20. Figure 4.7 show the WAZ scores distribution by age.

Table 4.20 Nutritional indicators of the children with mild and severe MPV ARI

	HMPV		
Nutritional indicators	Mild ARI N= 20 (30%)	Severe ARI N= 46 (70%)	P value
Mean (SD) WAZ score	-2.5 (2.7)	-1.6 (1.1)	0.06
WAZ ≤ -2	7 (35%)	16 (35%)	0.4
Mean (SD) HAZ score	-2.59 (3.27)	-1.34 (2.5)	0.09
HAZ ≤ -2	12 (60%)	19 (41%)	0.08
Mean (SD) WHZ score	-0.6 (2.6)	-0.9 (3.2)	0.6
WHZ ≤ -2	0 (0%)	0 (0%)	

Figure 4.7 WAZ scores distribution by age in children with (a) mild and (b) severe HMPV

a.



b.

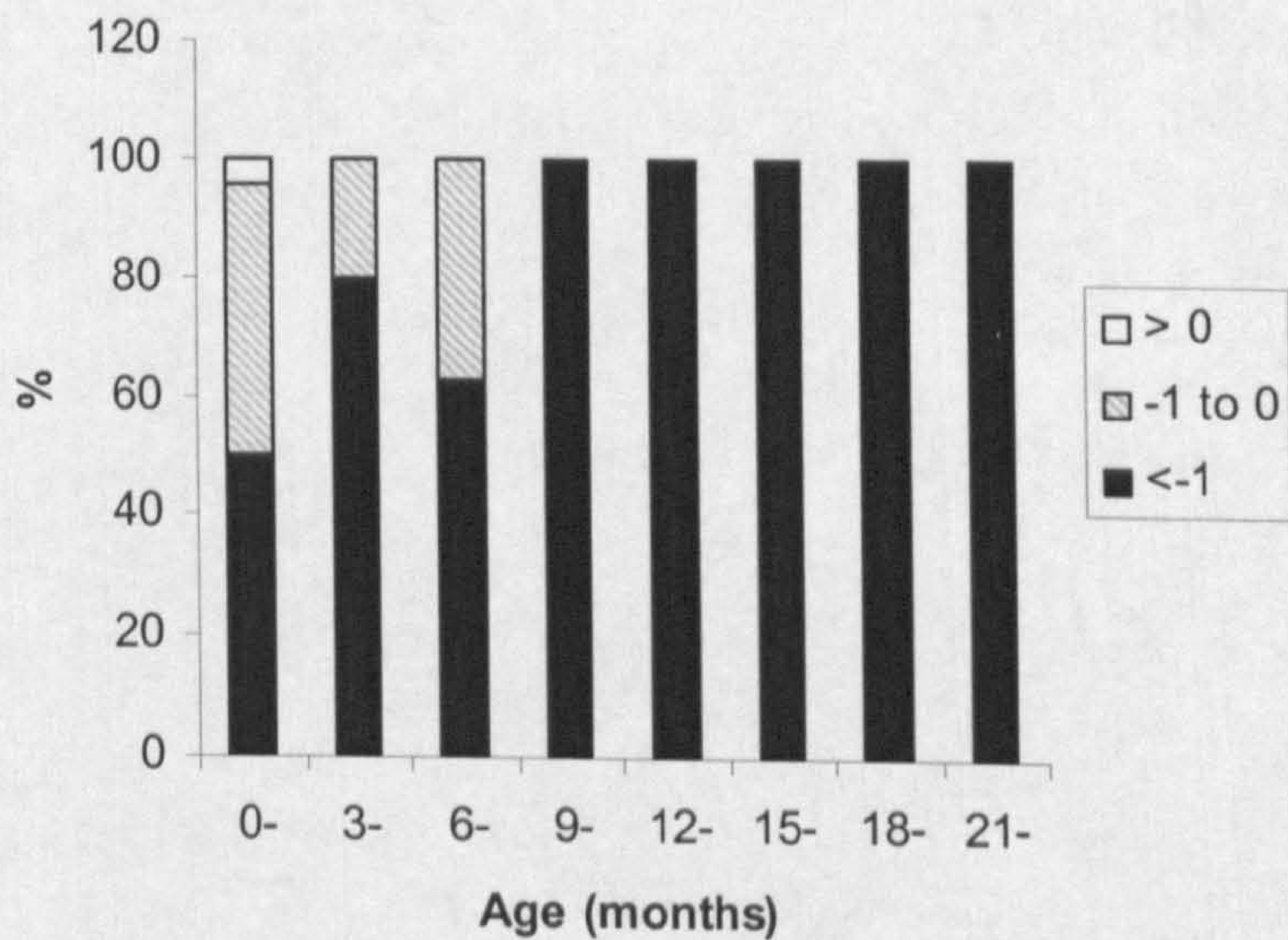


Table 4.21 Clinical characteristics of the children with HMPV ARI

		HMPV		
		Mild ARI N= 20 (30%)	Severe ARI N= 46 (70%)	P value
Crackles		2 (10%)	41 (89%)	<0.001
Cyanosis		1 (5%)	37 (80%)	<0.001
Chest indrawing		1 (5%)	35 (76%)	<0.001
Wheeze		2 (10%)	16 (35%)	0.03
Fever (>37.5°C)		5 (25%)	8 (17%)	0.2
Mean (SD) respiratory rate/minute		38.7 (8.3)	58.4 (13.6)	<0.001
Hepatomegaly		1 (5%)	3 (7%)	0.6
Splenomegaly		0 (0%)	1 (2%)	0.6
Heart murmur		1 (5%)	3 (7%)	0.6
Pallor		0 (0%)	34 (74%)	<0.001
Mean (SD) pO_2		92% (3.1)	76% (6.4)	<0.001
Clinical diagnosis				
	Bronchiolitis	1 (5%)	19 (41%)	0.002
	Pneumonia	0 (0%)	22 (48%)	<0.001
	Mild ARI	19 (95%)	5 (11%)	<0.001
Chest x-ray taken (%)		3 (15%)	42 (91%)	<0.001
	Hyperinflation	1 (33%)	33 (79%)	0.1
	Patchy change	1 (33%)	40 (95%)	0.01
Co-infection with RSV		3 (15%)	22 (48%)	0.01

Hospital management

As expected, the majority of children with mild HMPV ARI were recruited from the outpatient clinic (19, 95%), while majority of children with severe HMPV ARI were recruited from the A&E department (31, 67%).

Only one (5%) child with a mild presentation in contrast to 37 (80%) children with severe HMPV presentation were given antibiotics (<0.001). None of the children with mild HMPV ARI received oxygen supplementation, IV fluids, bronchodilators or steroids while 27 (59%) of the children with severe ARI received oxygen and 42 (91%) were given IV fluids. Table 4.22 shows the medications received by the children.

Table 4.22 Hospital management of the children with HMPV ARI

	HMPV		
	Mild ARI N=20 (30%)	Severe ARI N= 46 (70%)	P value
Antibiotics	1 (5%)	37 (80%)	<0.001
Anti-cough syrup	6 (30%)	25 (54%)	0.03
Oxygen supplementation	0 (0%)	27 (59%)	<0.001
Intravenous fluids	0 (0%)	42 (91%)	0.6
Bronchodilators	0 (0%)	1 (2%)	0.6
Steroids	0 (0%)	1 (2%)	0.6

Family history of the children

Twenty eight (61%) children with severe HMPV ARI had other members of the family with chest infections in comparison to only 7 (35%) with mild HMPV ARI (p=0.03). A family history of allergies was in found with similar frequencies (4 each) in children with mild and severe HMPV ARI. Similarly, there were no significant differences regarding a family history of asthma, eczema, tuberculosis or congenital heart diseases as shown in table 4.23.

Table 4.23 Family history of children with HMPV ARI

	HMPV		
Family history	Mild ARI N= 20 (30%)	Severe ARI N= 46 (70%)	P value
ARI	7 (35%)	28 (61%)	0.03
Allergies	4 (20%)	4 (9%)	0.1
Asthma	1 (5%)	5 (11%)	0.4
Eczema	1 (5%)	5 (11%)	0.4
Tuberculosis	0 (0%)	0 (0%)	0.6
Congenital heart disease	0 (0%)	1 (2%)	0.6

Risk factors for severe HMPV ARI

Similar to the approach used for RSV, it was assumed that children with $pO_2 \leq 87\%$ had severe illnesses and that those with $pO_2 > 88\%$ had mild illnesses. Again, similar to RSV, continuous variables were dichotomised using the same cut off points used for RSV. Figure 4.8 shows the age distribution of children with HMPV.

Table 4.24 lists the demographic and socio-economic variables that selected for multivariate analysis.

Figure 4.8 Age distribution of children with HMPV

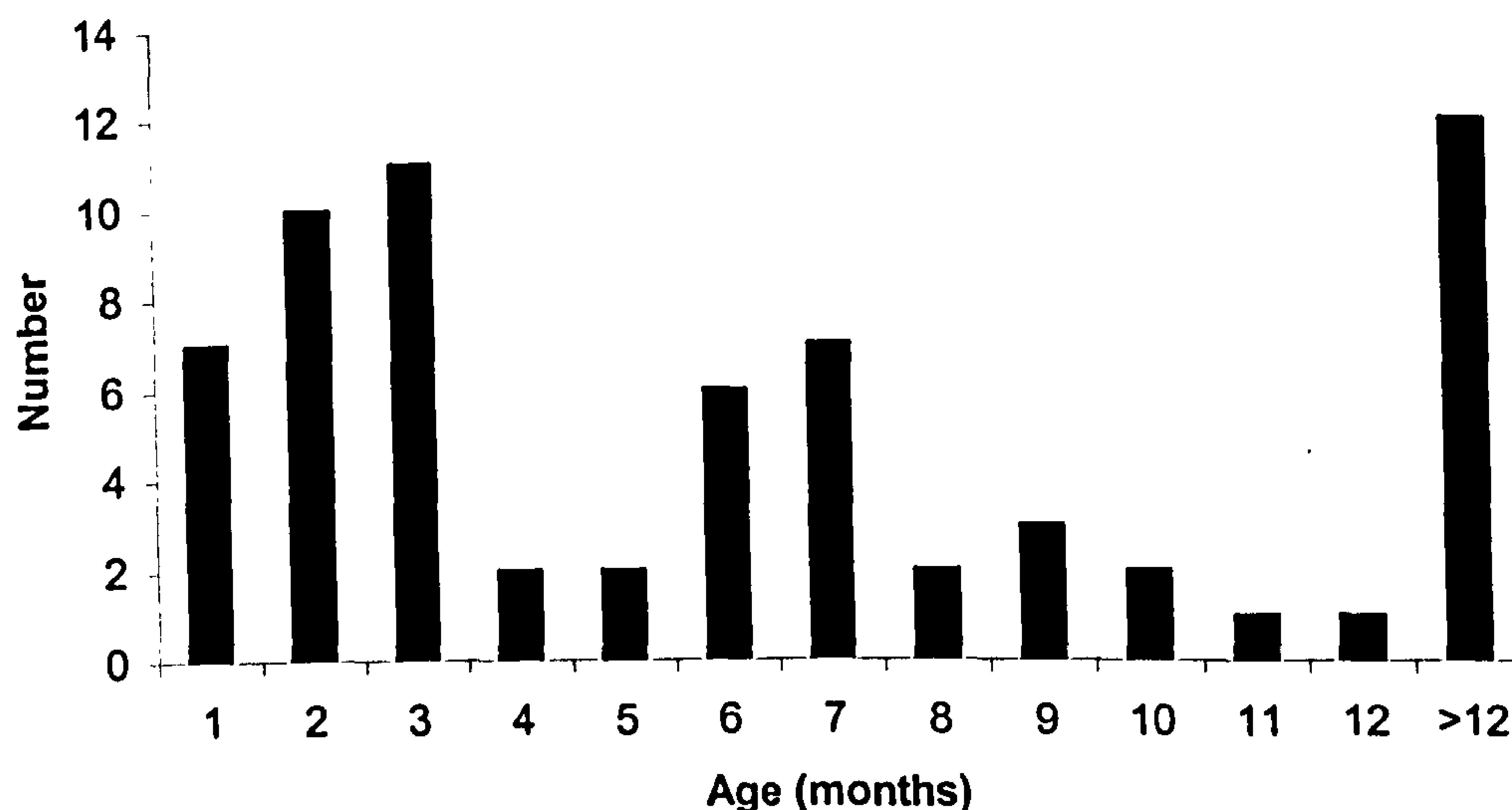


Table 4.24 General, characteristic selected for multivariate analysis

	HMPV				
	Mild ARI N= 20	Severe ARI N= 46	OR	95% CI	P value
Male: Female (%Male)	17:3 (85%)	30:16 (65%)	3.02	0.76-11.88	0.08
Age ≤ 3 months	4 (20%)	24 (52%)	4.36	1.26-15.06	0.01
Smoker at home	10 (50%)	28 (61%)	1.55	0.54-4.48	0.2
> 2 adults per household	9 (45%)	28 (61%)	1.99	0.65-5.49	0.1
Indoor pets	4 (20%)	15 (33%)	1.93	0.55-6.8	0.2
Non-governmental source of water	5 (25%)	22 (48%)	2.75	0.86-8.8	0.04
Non-gas source of fuel	2 (10%)	23 (50%)	9.0	1.87-43.3	0.001
Family member with ARI	7 (35%)	28 (61%)	2.88	0.96-8.61	0.03
Allergies	4 (20%)	4 (9%)	2.52	0.58-11.77	0.1
Recurrent respiratory infections	2 (10%)	15 (33%)	4.35	0.89-21.26	0.04
Mixed breast feeding	5 (25%)	23 (50%)	3.0	0.93-9.6	0.03
Vaccines not up to date	4 (20%)	30 (65%)	7.5	2.14-26.24	<0.001
Rural residency	2 (10%)	15 (33%)	4.3	0.96-30.4	0.05

Logistic regression analysis

The best fitting conditional logistic regression model for the demographic variable is shown in table 4.25. The variables independently associated with an increase risk of severe HMPV ARI included an age ≤ 3 months (AOR= 10.3), using fuel other than gas (AOR = 13.1) and a history of recurrent ARI (AOR= 13.0).

Table 4.25 Logistic regression of demographic and socio-economic criteria of children with HMPV infection

Term	OR	AOR	95% C.I.	P value
Fuel other than gas	9.0	13.1	2.2-77.9	0.005
≤ 3 months age	4.36	10.3	2.2–47.7	0.002
Past history of recurrent ARI	4.35	3.0	2.0-84.0	0.007

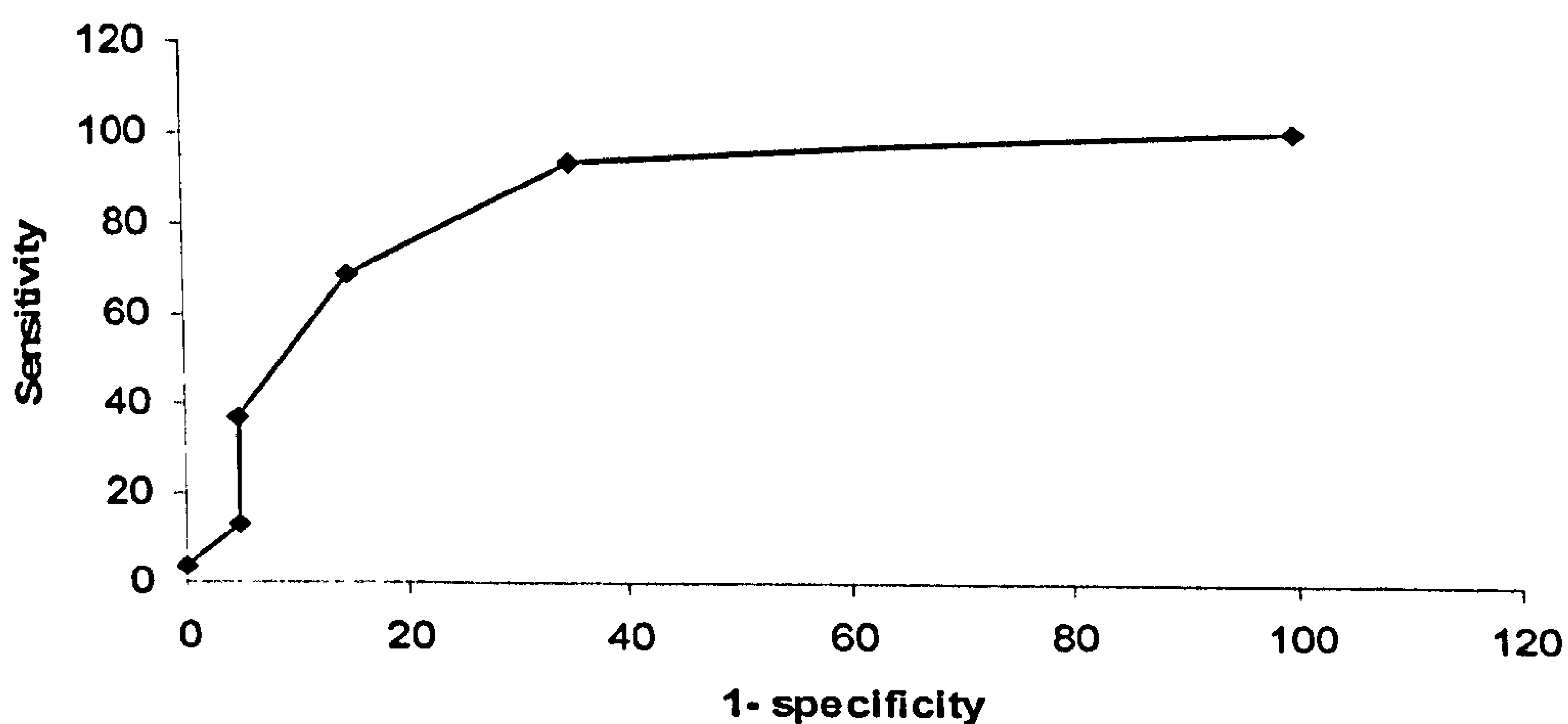
Severity Score for children with HMPV

The sum score was determined for cases and controls in the same way as explained for RSV and the sensitivity and specificity was calculated for each cut off point as illustrated in table 4.26. The ROC curve was drawn to find the optimal cut off point that resulted in the highest sensitivity and specificity for the score (Figure 4.9). Similarly to RSV, the low predictive values of the curve do not merit further analysis.

Table 4.26 Sensitivity and specificity of the RSV severity score

Cut off points	Cases n=46	Controls n=20	Sensitivity	Specificity	1-specificity
0	3	13	100	0	100
10	11	4	93	65	35
13	15	2	69	85	15
23	11	0	37	95	5
26	4	1	13	95	5
36	2	0	4	100	0

Figure 4.9 ROC curve of the severity score to identify children with severe HMPV



Discussion

Risk factors for severe clinical presentation of RSV–related bronchiolitis

Patients younger than 3 months of age frequently develop severe RSV infection and our findings confirm that in Yemen, an age less than 3 months is one of the most important independent variables for severe RSV ARI (OR = 5.1). This is in agreement with other reports from industrialized countries, where young children have an OR for hospitalization of 5.9 compared to older children (Holberg et al., 1991, Kaneko et al., 2001).

Previous reports have defined children at high risk for severe RSV ALRI as children who have CLD, CHD and infants who are born prematurely (Boyce et al., 2000, Navas et al., 1992, Bulkow et al., 2002, Kaneko et al., 2001). In our study however, these factors were not associated to severe RSV ALRI. Several factors may explain these findings. Most children in Yemen are born outside hospitals and have very limited access to health services. Children who are very premature or have severe congenital malformation have a significantly higher mortality than in industrialised countries. This may result in a cohort effect and children at high risk being under represented among those attending the hospitals (Kaneko et al., 2001). This effect is illustrated by the small number of preterm infants and infants with CLD in our study. In addition, this selection bias would result in an inadequate power to identify prematurity and CLD as risk factors in hospitalized children in Yemen. A prospective cohort study of preterm and term babies could be conducted to re-examine these associations.

Case control studies from the United Kingdom and North America have suggested that breast feeding protects against RSV hospitalisation (Bulkow et al., 2002, Downham et al., 1976, Pisacane et al., 1994) although studies from Atlanta and Rochester have not confirmed this protective effect (Anderson et al., 1988, McConnochie and Roghmann, 1986) and in the Gambia Weber et al have not shown a protective role of breast feeding in developing countries (Weber et al., 1999). These latter studies however did not report how they defined exclusive breast-feeding. Maternal antibodies passively acquired by the fetus is present through the first few months of life, when the prevalence of the more severe forms of RSV illness is greatest, casting doubt on a major protective role for antibody-mediated immunity (Kaneko et al., 2001). Our study did not find that breast feeding was a protective factor for disease severity in Yemeni children. Breast feeding however is a very common practice in Yemen and nearly all the study population had been breast fed for a certain period of their life and this might also explain the discrepancy between the studies in UK and USA and The Gambia.

Children who did not have their routine vaccines up to date at the time of admission were found to be at risk of severe RSV. No comparable results have been published. However this may act as an indirect marker for poverty and low health awareness. In other words, low vaccine compliance may be an indicator for low socioeconomic status which in turn is a risk factor for severe disease.

Low socio-economic status has been associated with a higher attack rate of RSV infection and children in low socio-economic strata are more likely to have multiple high risk conditions (Jansson et al., 2002). Our study observed that some social and economic characteristics also increased the risk for severe RSV infections in Yemen. These included using a source of fuel other than gas, using a non-governmental source of water, the presence of outdoor and indoor pets, especially the presence of cows and donkeys, the presence of a smoker in the household and having a family member with an ARI. Of these factors, only the presence of a smoker in the household and using a source of fuel other than gas were independent risk factors once the confounding effect between the variables had been considered. These results are in agreement with a case control study from Rochester that examined these associations and reported that parental smoking, and in particular maternal smoking during pregnancy, indoor pollution and the presence of pets increase the risk of severe RSV infections (McConnochie et al., 1988). Supporting our results, Lanari et al, also found that environmental smoke exposure was an independent risk factor for severe RSV ARI (Lanari et al., 2002). In contrast, Madhi et al. reported that exposure to cigarette smoke was not significantly different in high risk infants with and without RSV or between high risk infants with RSV and healthy infants, but that low socio-economic status was an important risk factor for severe RSV ARI (Madhi et al., 2003b).

Male gender has been found to be a significant risk factor for an increased incidence of RSV ALRI in the first year of life (Holberg et al., 1991) and previous studies have reported that gender is also a risk factor for severe RSV ALRI, with males having more severe disease than females (Simoes, 2003). Our results however did not demonstrate that gender was a risk factor for acquiring severe RSV infection in Yemen. This is in agreement with other reports from Africa which did not identify gender as a risk factor for severe infection (Madhi et al., 2003b, Weber et al., 1999). These conflicting results may be due to differences in the study location, gender culture of the study population, study design and circulating RSV strains which may affect differently males and females.

No significant differences were observed in the nutritional parameters of children with mild and severe RSV ARI. This may be due to the fact that most of our population was malnourished. There is currently a debate about the role of malnutrition as a risk factor for severity in RSV infection. In agreement with our results, a Nigerian study reported that RSV

was less frequently isolated from malnourished than from well-nourished children, and that malnourished children, had milder diseases than well nourished children (Nwankwo et al., 1994). Interestingly, in the Gambia, RSV was found in 13% of well-nourished children with ALRI compared to 6% of malnourished children (Adegbola et al., 1994). Other studies from the Gambia (Weber et al., 1998b) and South Africa (Hussey et al., 2000) however have reported that malnutrition is not a risk factor for the development of RSV LRI.

Our study showed no significant differences in the family history of asthma, eczema and allergies between children with mild and severe RSV ARI. This is in agreement with Bradley et al who found that levels of common allergens within the home environment had no effect of RSV severity (Bradley et al., 2005). However, Weber et al reported that Gambian mothers of children hospitalised with RSV complained of asthma more frequently than mothers of non-hospitalised children with RSV (4.2% versus 0.5%, $P = 0.05$) (Weber et al., 1999). Thus, there is still controversy about the role of atopy in the development of severe RSV infection in developing countries and further research is needed.

There was no significant difference in the clinical severity of children with group A or group B RSV ARI as represented by others (Wang et al., 1995b, Kneyber et al., 1996). There is no agreement in the literature on whether a predominant group causes more or less severe clinical spectrum than others. About 50% of the reports have indicated that group A infection results in more severe disease than group B (Hall et al., 1990, Walsh et al., 1997, McConnochie et al., 1990, Taylor et al., 1989). It has been suggested that virus variants within group A are responsible for the discrepancy observed across the studies. Similarly, our study did not find a relation between the N genotypes of RSV groups and clinical severity of the disease, which is similar to reports from elsewhere in the Netherlands and the UK (Brandenburg et al., 2000c, Fletcher et al., 1997).

Few studies have evaluated the role of HMPV coinfection with RSV in inducing more severe disease (Semple et al., 2005). Our results suggest that coinfection did not represent a risk factor for disease severity although the number of children with co-infections was too small for analysis. In agreement with this results, coinfection with HMPV did not appear to increase the severity of RSV illness in studies from Italy and Greece (Maggi et al., 2003, Xepapadaki et al., 2004). However, a study in an intensive care unit in the UK, 21 (70%) of 30 patients ventilated for RSV had HMPV coinfections (Greensill et al., 2003), and 9 (60%) of 15 children admitted with HMPV infections in Germany also had RSV infection (Konig et al., 2004). This raises the possibility that HMPV strains may vary with location and time. In addition, the studies of intensive care children lack a control group and are difficult to interpret. In a recent case control study from the UK, dual infections with RSV and HMPV

resulted in a 10 fold increase in the risk of admission to a pediatric intensive care unit for mechanical ventilation. This study however also observed that if the number of children with dual infection were compared by disease severity there were no significant differences (Semple et al., 2005) and further studies are needed.

Risk factors for severe ARI associated with HMPV hospitalisation

Risk factors for severe ALRI due to HMPV have not been established. In the current study the risk factors identified included young age, being male, a child not having his/her routine immunisations to date, being partially breast fed, having a history of recurrent chest infections or allergies, the presence of more than 2 adults in the household, the presence of smokers, indoor pets, using a source of fuel for cooking other than gas and using water that did not belong to the government water pipe network. However, on multivariate analysis, only an age < 3 months, a history of recurrent chest infections and using non-gas fuel were independent risk factors for severe HMPV ARI. These factors were also reported as potential risk factors in a study by Robinson et al (2005) who reported that possible risk factors for HMPV hospitalisation in Northern Alberta were an age < 2 years, being male or born preterm, having gastro-oesophageal reflux or aspiration, CHD or global developmental delay (Robinson et al., 2005). Similar to our study, the Robinson study was too small to identify factors with smaller OR and larger case control and prospective studies will be required to elucidate risk factors for HMPV. Most of the studies to date (including this one) were done in tertiary care hospitals where there is a selection bias towards children with underlying medical problems and included a limited number of patients.

In conclusion, the factors independently associated with an increase risk of severe RSV are age less than 3 months, the child not having his/her vaccines up to date, the presence of a smoker at home and using a source of fuel for cooking other than gas. In contrast, only age less than 3 months, having a history of recurrent ARI and using a source of fuel other than gas were the only risk factors found to be independently associated with an increase risk of severe HMPV.

Chapter 5

Immunopathogenesis of RSV, HMPV and RSV/HMPV ARI

Introduction

Although there are well established clinical risk factors for developing severe RSV infection such as prematurity (Navas et al., 1992) and bronchopulmonary dysplasia (Groothuis et al., 1988), most children admitted with severe RSV bronchiolitis do not have any of these risk factors. In addition, the experience of the immunisation program with the F1-RSV vaccine in the 1960s that resulted in RSV disease enhancement instead of protection (Kim et al., 1969), suggests that immunological mechanisms may be key to the severity of RSV bronchiolitis in infancy. Given the exponential increase in understanding of the immunology of infections in recent years, this chapter focus in the immunopathogenesis of RSV.

Beside RSV, the pathogenesis of HMPV has not yet been explored. Most reports to date have examined the incidence or prevalence of HMPV in different populations and children and adults differ in both type and severity of disease manifestations. It is also unclear whether similarities in signs and symptoms of infants infected with HMPV and RSV are associated with similar immunological mechanisms.

This study therefore was conducted to improve our understanding of the immunopathogenesis of RSV and HMPV ARI and its association with disease severity in children.

Literature review

Innate immunity

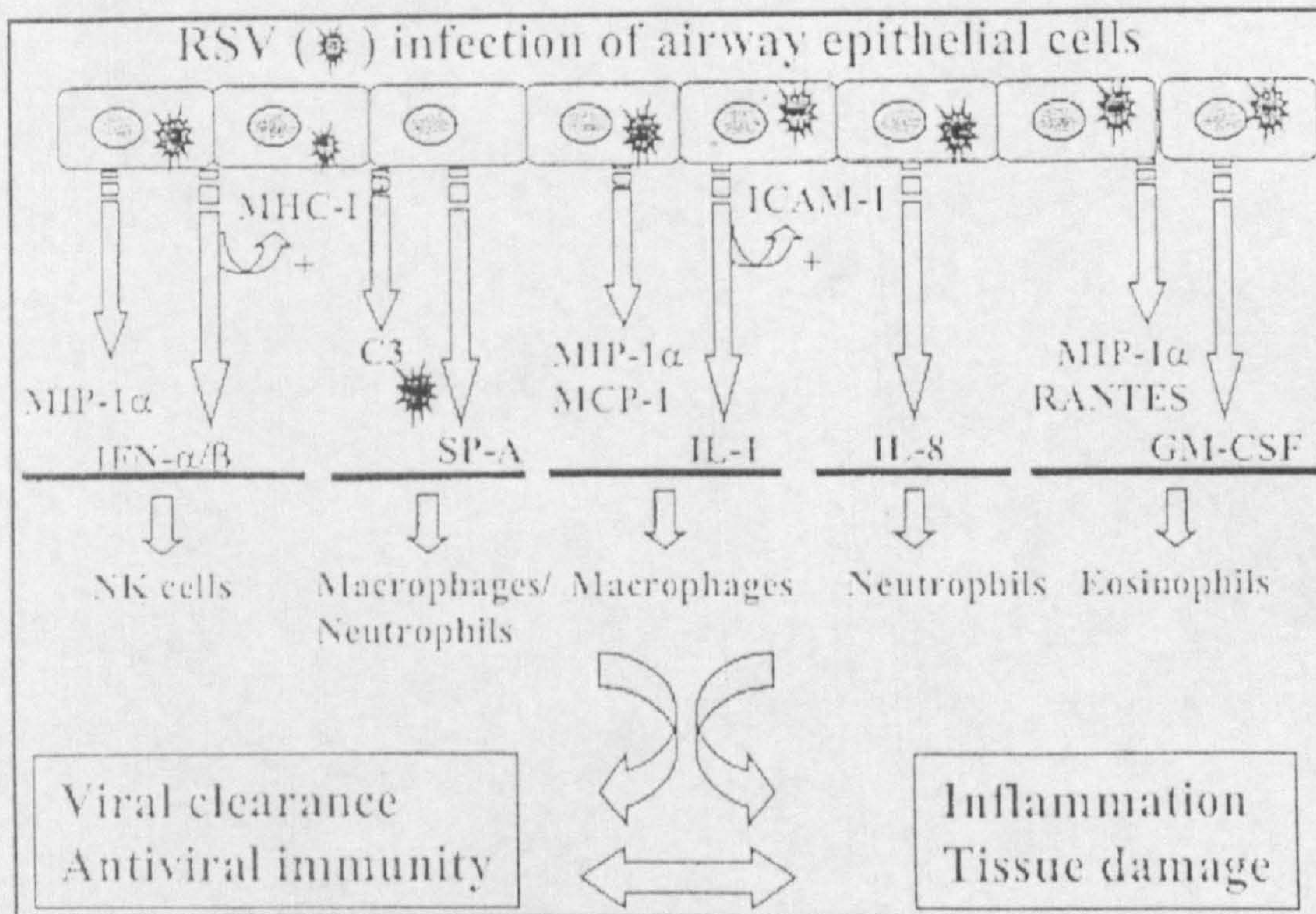
Innate immunity contributes to the earliest phase of the immunological host defence against RSV by recruiting effector molecules and phagocytic cells to the site of infection through the release of cytokines and chemokines (Noah et al., 1995).

The epithelial regulation of innate immunity to respiratory syncytial virus

Infection of the respiratory epithelial cells, the main function of which is to provide a protective physical barrier against injurious inhaled stimuli, is the first event occurring after RSV inhalation or inoculation into the nasal mucosa (Garofalo and Haeberle, 2000). Following inoculation of the virus into the nasal mucosa and infection of the local respiratory epithelium, RSV spreads along the respiratory tract mainly by cell-to-cell transfer of the virus

along the intracytoplasmic bridges. RSV targets ciliated columnar airway epithelial cells via their apical surfaces (Zhang et al., 2002). RSV induces profound effects on the ciliated cells: ciliostasis, clumping and loss of cilia from live cells and sloughing of cells (Tristram et al., 1998). This is rapidly followed by the induction of a network of epithelial cell gene products that have profound immune and inflammatory regulatory functions (Garofalo and Haeberle, 2000). The surge of subsequent events involves mediators and cellular elements that belong to the innate immune system (Figure 5.1).

Figure 5.1 Innate immunological response following RSV infection of airway epithelial cells. Source (Garofalo and Haeberle, 2000)



MHC-1= major histocomatability complex; MIP-1α = macrophage inflammatory protein-1α; MCP-1= monocyte chemotactic protein-1; SP-A= surfactant protein-A; RANTES= Regulated upon activation, normal T-cell expressed and secreted; INF-α/β = interferon – α/β; C3= complement 3, NK=natural killer cells; IL1=interleukin-1; IL8= interleukin 8. ICAM-1=intercellular adhesion molecule (ICAM-1) and GM-CSF= granulocyte-macrophages colony stimulating factors

Recognition of RSV products by host cells

Recognition of RSV products by host cells is mediated by pattern recognition receptors. RSV F protein activates the innate immune response via Toll-like receptors 4 (TLR4) and CD14, which are expressed primarily on monocyte TLR 4 receptor cells (Gagro et al., 2004, Kurt-Jones et al., 2000). It was found that RSV infection increases both the amounts of TLR4 and

its membrane localisation on airway epithelia through MD2, a small secreted glycoprotein that binds to the extracellular portion of TLR4. RSV increases concentrations of both TLR4 and MD2 leading to activation and thus inflammatory cytokine production (Monick et al., 2003).

The RSV G glycoprotein has structural similarities to the CX3C chemokine fractalkine. RSV G glycoprotein binds the human CX3CR1 receptor. The interaction of the CX3C motif on the G glycoprotein with CX3CR1 on cells appears to facilitate infection and to be capable of modulating the immune response, as suggested by the ability of G glycoprotein to induce migration of leukocytes (Tripp et al., 2001).

Some of the components of the innate immune system known to be involved in RSV infection will be reviewed in this section, in particular alveolar macrophages, surfactant protein-A (SP-A), neutrophils, NK cells and eosinophils that have a prominent role in the immunomodulation, all of which may be significantly affected by the infected respiratory mucosae epithelium.

Alveolar macrophages and surfactant protein –A

Alveolar macrophages are formed when circulating monocytes migrate into lung tissue. They are a major component of the innate-effector immune system in the lung. Both *in vitro* and *in vivo* studies have demonstrated that alveolar macrophages co-express RSV-specific proteins and human leukocyte-associated antigen-DR (HLA-DR) molecules, thus may serve as effective antigen presenting cells (Midulla et al., 1993) and secrete protent inflammatory and immunomodulatory cytokines (e.g. IL6, TNF-α, IL10) in response to RSV infection (Panuska et al., 1995). These cytokines further up-regulate the immune response increasing vascular permeability and causing the activation and recruitment of lymphocytes, neutrophils, NK cells and possibly eosinophils to the site of the infection. When apoptosis of RSV-infected neonatal and adult mononuclear cells was compared, a decreased cellular apoptosis from both sources was observed although the decrease was more pronounced in neonatal cells (Krilov et al., 2000). These observations may help to explain the mechanism behind the more severe disease seen in young infants.

Pulmonary surfactant is the first line of innate lung defence. It consists of a layer of phospholipids (primarily lecithin and sphingomyelin) together with several surfactant proteins. Broncho-alveolar lavage fluid (BAL) from RSV–infected ventilated infants shows a decrease in surfactant protein A, B and D concentrations (Kerr and Paton, 1999).

In *vitro* SP-A has shown to neutralize RSV by binding by the F protein but not the G protein (Ghildyal et al., 1999). However, Hickling et al. showed that SP-A binds to the G glycoprotein to enhance RSV infection of HepC-2G cells (Hickling et al., 2000). Thus the role of collectins in innate immunity is not clear.

Barr et al., presented the results of a study *in vitro* that examined the effect of SP-A on RSV uptake by peripheral blood mononuclear cells (PBMC) and the macrophage-like cell line U937. The authors showed that SP-A enhances uptake of fluorescein isothiocyanate (FITC) - labelled RSV by both cell types. As a result of this event, the production of TNF-α and IL10 is altered. In both cells, opsonisation of RSV by SP-A increases TNF-α and decreases IL10 production compared to RSV alone (Barr et al., 2000).

Neutrophils

Early after RSV infection, neutrophils are the predominate component of the cellular inflammatory response in the respiratory airway mucosa (Noah et al., 1995). In a study of RSV infection in infants, the neutrophils were found to represent a median of 93% of cells in the upper airway and 76% in the lower airway (Everard et al., 1994). Epithelial-derived IL8 is the main neutrophil chemoattractant secreted after RSV infection (Jaovisidha et al., 1999). As a result of the interaction between RSV and neutrophils, effector functions of neutrophils are activated, resulting in cytotoxicity directed against epithelial cells (Shaw et al., 2003). A direct stimulatory effect of RSV on neutrophils has also been reported. Following *in vitro* exposure to RSV, peripheral blood neutrophils secrete the chemokines IL8, MIP-1α, MIP-1β, and granule-associated myeloperoxidase (MPO) (Jaovisidha et al., 1999). This observation is supported by studies in children with RSV infection, where higher levels of MPO and neutrophil elastase have been demonstrated in nasopharyngeal secretions in subjects with bronchiolitis compared with those with milder forms of the disease (Abu-Harb et al., 1999).

Natural Killer Cells

Natural killer cells (NK) are important effectors in the innate immune response and play a key role in the first line of host defence against viral infections. Studies in mice have shown that the majority of lymphocytes recovered from BAL fluids during the first days of primary RSV infection have phenotypic characteristics of NK cells and are responsible for much of the early production of IFN-γ and NK activity peaks around this time. They also demonstrated that NK cells can be stimulated by IL12- independent mechanisms to produce INF-γ and enhance the activity of cytotoxic CD8 lymphocytes (Hussell and Openshaw, 1998). The role of NK cells in the context of RSV infection is not fully known. However, NK cells were

found to be an important contributor to the expansion of CD8+ T cells and the amplification of antiviral immune responses to RSV (Johnson et al., 2002).

An investigation was performed using the mouse model and the cold-adapted temperature-sensitive strain CP52 of RSV, which was being evaluated as an RSV vaccine candidate. This strain has a deletion of both the viral SH and G genes. The absence of the G and SH protein in the CP52 strain markedly increased the number of BAL NK cells after both primary and secondary infections, (Tripp et al., 1999). Therefore, it appears that the G protein and/or the SH protein inhibits trafficking of NK cells to the lung.

Eosinophils

Several groups have shown that during RSV infection eosinophils are recruited and degranulate into the airway mucosa (Olszewska-Pazdrak et al., 1998, Harrison et al., 1999). RSV infected respiratory epithelial cells appear to regulate the multistep process of eosinophil transendothelial migration, chemotaxis, and degranulation by secreting a number of immuno-mediators such as epithelial-derived RANTES, which is the main chemokine that displays strong chemotactic activity for eosinophils *in vitro* (Becker and Soukup, 1999, Saito et al., 1997). RSV-infected epithelial cells can also activate eosinophils and uninfected epithelial cells, thus triggering further eosinophil degranulation (Olszewska-Pazdrak et al., 1998). Indeed, the killing and eradication of infected cells by cytotoxic eosinophil products may positively impact the outcome of RSV infection. On the other hand, eosinophil-mediated cytotoxicity against bystander uninfected epithelial cells may significantly contribute to the enhanced pathology observed in certain RSV infections.

The direct interaction of the virus with eosinophils is another important aspect of the relationship between RSV infection and eosinophils to be considered. A study by Olszewska-Pazdrak et al. described that culture of highly purified preparations of blood eosinophils with RSV (A strain) results in the destruction of RSV virions. This effect was directly dependent on the action of the secreted ribonuclease, eosinophil-derived neurotoxin (EDN) and expression of RSV F glycoprotein in the cells, as detected by typical intracytoplasmic granular fluorescence immunoreactivity (Olszewska-Pazdrak et al., 1998). Moreover, RSV viral particles were seen by transmission electron microscopy in phagocytic vacuoles at the periphery of eosinophil cytoplasm (Kimpen et al., 1996). These studies suggest that RSV is able to infect human eosinophils. By infecting eosinophils, RSV is able to induce the production of oxygen radicals, to prime eosinophils for enhanced superoxide generation and leukotriene C4 release, and to trigger intracellular signalling and transcriptional events that

lead to the production of the chemokines RANTES and MIP-1α (Kimpen et al., 1992, Olszewska-Pazdrak et al., 1998).

Eosinophils have the ability to inactivate RSV released by infected epithelial cells by the release of eosinophil cationic protein (ECP), an eosinophil-derived enzyme that belongs to a family of RNases, thus limiting the spread of the infection (Soukup and Becker, 2003). Significantly higher concentration of ECP has been shown to be present in nasopharyngeal aspirate obtained from subjects affected by bronchiolitis compared to those with localised upper respiratory tract illness. ECP is thus currently used as a marker of RSV disease severity (Domachowske and Rosenberg, 1999).

Adaptive immunity

Adaptive immunity has two components: cell-mediated and humoral. In RSV infection, the cell-mediated response promotes viral clearance whilst the humoral response is primarily involved in protective immunity. The adaptive immune response features immunological memory and is based on the clonal selection of lymphocytes bearing antigen specific receptors.

Cell-mediated immunity

The cell-mediated immune response is characterised by lymphocyte surface antigen expression onto CD8+ cytotoxic lymphocytes (CTL) and CD4+ T helper cells. Both of these cell types have antiviral as well as immunopathogenic capabilities (Hussell and Openshaw, 2000). CD4+ T helper cells are subdivided into T helper1 (Th1) and Th2 lymphocytes on the basis of cytokine secretion. The term Th1 cytokines (also referred to as Type-1 cytokines) and Th2 cytokines (or Type-2 cytokines) refers to the patterns of cytokines secreted by two different subpopulations, CD4 (+) T cells that determine the outcome of an antigenic response toward humoral (Th2) or cell-mediated (Th1) immunity. Type -1 cytokines include: IL12, IFN-γ, and TNF-α &β, while type-2 cytokines include IL4, IL5, IL6, IL10 and IL13 (Tripp et al., 2002).

Both types of T helper cells influence each other by the cytokines they secret. For example INF-γ , a Th1 cytokine, can downregulate Th2 function while Th2 cytokines, such as IL10, can suppress Th1 function (Mobbs et al., 2002). It thus appears that these functional subsets are mutually antagonistic such that the decision of which subset predominates within an infection may determine also its outcome.

In murine models of RSV infection, the induction of different CD4+ T-cell responses may be dependent on the sensitising RSV antigen. Immunising mice with a vaccine expressing F protein induces a Th1 CD4+ T-cell response and strong CTL response, while immunisation by a vaccine expressing the G protein promotes activation of Th2 CD4+ T-cell responses and induces eosinophilic infiltrates in the lung following subsequent RSV infection (Graham et al., 2000).

Cytokine responses and severity of RSV infection in children

Many groups have tried to identify a dominant Th1 or Th2 cytokine profile in blood, NPAs and BAL taken from infants with RSV disease (Anderson et al., 1994, Bendelja et al., 2000, Brandenburg et al., 2001, Roman et al., 1997).

The idea that RSV bronchiolitis might be a predominately Th2 type disease comes from the vaccine experience in the early 1960s (Kim et al., 1971). However, only limited evidence for the role of Th2 cytokines in the pathogenesis in bronchiolitis has been found to date. Renzi et al. have shown INF- γ expression by PBMCs to be lowered in severe compared to mild RSV disease (Renzi et al., 1999). Similarly, van der Sande et al showed that severe RSV infection in early life is associated with Th2 cytokine production (van der Sande et al., 2002) and Legg et al demonstrated that severe RSV disease expresses excessive type 2 or deficient type 1 response compared with mild disease (Legg et al., 2003).

On the other hand, other groups have demonstrated that in severe RSV disease Th1 cytokine responses characterised by INF-γ production predominate when compared with mild disease. Bendelja et al found that INF-γ protein concentrations are directly associated with RSV disease severity consistent with a Th1 response (Bendelja et al., 2000).

Some studies provided the clinical evidence in infants that in RSV bronchiolitis there are divergent T cell responses and suggested that more than one mechanism may be responsible for immune-mediated disease enhancement. Mobbs et al. found that either IL4 mRNA or INF-γ was present in BAL fluid in large amounts. The reciprocal cytokine was not detected suggesting that counter inhibitory mechanisms regulated type1 and 2 cytokines in the lung of these infants with bronchiolitis (Mobbs et al., 2002). Also, de Waal et al, detected mixed Th1 (INF-γ) and Th2 (IL4) responses in three groups of children, 13 with RSV-URTI, 9 with non RSV-URTI and 11 with severe RSV bronchiolitis and did not detect significant differences (de Waal et al., 2003). Table 5.1 summarises the results of selected studies, INF-γ was selected to represent Th1 and IL4 to represent Th2. Hence still there is a large controversy about whether Th1 or Th2 cytokine responses predominate in severe RSV disease.

Table 5.1 Th1/Th2 and RSV infection

Country	RSV infection and Population	Level of cytokines measured	Type of samples studied	Main results	Reference
The Nether-lands	95 RSV positive and 16 RSV negative. 111 children < 6 m	Low IL4 and IL10 Increased INF- γ	Plasma ELISA; Intercellular Cytokine Immunoflouresance	Th1 predominance irrespective of the clinical severity.	(Brandenburg et al., 2000b)
UK	11 RSV positive, 7 RSV negative. 1-1.5 years old, 3 adults with no history of allergies, 7 asthmatics.	Predominance of IL 2 and INF- γ	Cytokine mRNA in PMNCs	Th1 predominance	(Anderson et al., 1994)
Chile	15 RSV infected children <13 months of age 17 healthy controls.	Low IL4 and IL5 High IL4, low INF- γ, high ratio IL4/INF- γ in RSV	PMNCs supernatant using ELISA	Th2 predominance	(Roman et al., 1997)
UK	24 ventilated infants	Either high IL4 or INF-γ	Cytokine mRNA in NPA and BAL	Polarization of cytokines production. In some high IL4, in others high INF-γ	(Mobbs et al., 2002)
Croatia	20 RSV infected infants; 10 healthy controls. 3 weeks to 24 months	More IL4 in peripheral T cells and less INF- γ in RSV than in controls. INF- γ increase in severe disease, IL4 increase in mild disease	Intracellular cytokines in PMNCs	Th2 predominance in RSV Th1 predominate in severe disease	(Bendelja et al., 2000)
Korea	18 asthmatic infants 22 bronchiolitics (16 with eosinophila & 6 without) and 14 controls	High IL5 and IL5/INF-γ in asthmatics and bronchiolitics with eosinophilia	Cytokines in BAL	Th2 in subgroup of bronchiolitis	(Kim et al., 2003)

Role of selected cytokines and chemokines in RSV infection

The putative role of selected cytokines and chemokines in RSV infection are described below:

TNF α and IL6

TNF-α and IL6 are important proinflammatory cytokines with a broad range of biological activities. Human TNF-α is a non-glycosylated protein of 17 kDa and a length of 157 amino acids. IL6 is a protein of 185 amino acids glycosylated at position 73 and 172 (Ibelgaufts, 2003). Alveolar macrophages and respiratory epithelial cells are important sources of both TNF-α and IL6 (Panuska et al., 1995).

IL6 concentration was reported to be related to nasal symptoms and signs of a viral URI. Children in a day care centre underwent serial nasal lavages by Noah et al. in order to assess nasal cytokine expression during acute URI. IL6 and TNF-α were markedly elevated in nasal lavage fluid (NLF) during acute URI compared to baseline and all, except TNF- α, decreased significantly by 2-4 weeks later. Cytokine patterns in RSV-positive and –negative illnesses did not differ significantly. In a subgroup of children who also underwent superficial mucosal biopsy their cells (90%-95% epithelial) showed increased levels of transcripts for IL6 in 7 of the 9 subjects, suggesting that epithelial cells may be one source of the cytokines during acute URI (Noah et al., 1995). IL6 has been detected in respiratory secretions obtained through nasal wash and tracheal aspirates of children with RSV infection. It has been detected in samples obtained from children with bronchiolitis with RSV infection at significantly greater concentrations than in samples from controls. Its concentration in nasal wash is correlated with its concentration in tracheal aspirates (Sheeran et al., 1999).

The release of IL8, IL6 and the soluble forms of the soluble TNF receptor (sTNF-R) from Human pulmonary type II-like epithelial cell (A549) after RSV infection was analysed by Arnold et al. They found that RSV infection alone induced a time- and RSV dose-dependent IL8 and IL6 release from A549 cells (Arnold et al., 1994). IL6 may drive nasal disease but protect from systemic and lower respiratory disease expression. This was supported by the results of Gentile et al, which demonstrated that intranasal challenge with recombinant IL6 protein caused increased nasal secretions but had no effect on pulmonary functions (Gentile et al., 2001).

Interestingly, Scholma et al. found that there was an association between high BAL IL6 concentrations and the risk of developing bronchiolitis obliterans (BO) as they did a prospective cohort study of 60 lung transplanted patients, followed for 2-8 years with either histological evidence of BO within 1 year of lung transplantation (n=19) or no pathology, good outcome for at least 24 months and well-preserved lung functions (n=41). They found significantly higher levels of IL6 in patients with BO than others (Scholma et al., 2000). On the other hand Magnan et al. reported results on *in vitro* cytokine production by alveolar macrophages isolated from BO patients, showing that the production of IL6 was not elevated in BO patients and they suggested that during BO tissue repair dominated over tissue injury and that Scholma's study only monitored the patients for a median of 41 days after lung transplantation and none of the patients at that time was classified as having BO (Magnan et al., 1996). As IL6 is associated with tissue injury Scholma et al (2000) hypothesised that patients with elevated IL6 were at high risk of developing BO. This theory was supported by Magnan's study where IL6 concentrations were elevated during the acute rejection of the lung transplant (Magnan et al., 1996). Thus IL6 seems to have a role in the early stages of developing BO.

Comparative studies on mononuclear cells from neonatal cord blood and adult peripheral blood have shown that the production of IL6 and TNF- α in response to RSV infection is less efficient in neonates compared with adults (Matsuda et al., 1996).

Oh et al. assessed the contributions of RSV versus influenza A virus (IFAV) in the pathogenesis of upper airway inflammation in wheezy young children. They compared IL6 levels in NPA from non-asthmatic children with respiratory virus infection (RSV in 17 children and IFAV in 13 children), asthmatic children (RSV in 9 and IFAV in 10) and 22 atopic healthy controls. Levels of IL6 did not differ between non-asthmatics and athmatics children with IFAV, however IL6 was found significantly higher in asthmatic and non-asthmatic children with RSV than unaffected healthy control children. They also found that the nasopharyngeal epithelium from children with RSV infection secreted more IL6 than that of children with IFAV infection (Oh et al., 2002).

TNF-α is a critical mediator of host defence against infection but may cause severe pathology when produced in excess. Fujita et al. using a murine model of over-expression of TNF-α under control of the human surfactant protein C promoter (SP-C/TNF-α transgenic mice) demonstrated that overproduction of TNF-α produces an increase in lung volume and pulmonary hypertension. Histologically it produces pulmonary emphysema through chronic lung inflammation and activation of elastolytic enzymes. They found that there was decrease

in the elastic recoil, although pulmonary fibrosis was very limited and only seen in subpulmonary, peribronchial, and perivascular regions (Fujita et al., 2001).

Individuals vary in the amount of TNF-α produced when their PBMC are stimulated *in vitro*, and family studies indicate that much of this variability is genetically determined (Knight and Kwiatkowski, 1999, Knight et al., 1999).

TNF-α has been claimed to have antiviral activity against RSV (Merolla et al., 1995). However, viral clearance is impaired in SP-A –/ – mice compared to SP-A+/+ animals, despite increased levels of TNF-α in the lung tissues of SP-A –/ – mice (LeVine et al., 1999). This finding is partially in contrast with the increased production of TNF-α by RSV/SP-A-exposed monocytes and U937 cells compared to those exposed to nonopsonized RSV reported by Barr et al (Barr et al., 2000). An explanation for this discrepancy is that the airway epithelium may represent, alone or in combination with macrophages, the source of lung TNF-α and IL6, all of which were found increased in SP-A deficient mice (Barr et al., 2000). If this is the case, the mechanism(s) for enhanced cytokine/chemokine production by epithelial cells in absence of SP-A remains to be defined. Nevertheless, the SP-A –/ – mouse model provides convincing evidence against the hypothesis of an antiviral activity of TNF-α in RSV infection.

In a study by Sung et al. the concentrations of TNF- α were significantly lower in the NPA of infants infected with RSV than in children infected with IFAV (Sung et al., 2001).

Concentrations of TNF-α increased in the acute stage and up to 3- 4 weeks after the onset of bronchiolitis. A recent study measured messenger ribonucleic acid (mRNA) expression and protein concentrations of TNF-α and IL6 in the lungs of 24 term and 23 preterm infants with RSV and 10 control infants intubated but without infective or respiratory disease. TNF-α and IL6 mRNA concentrations in infants with bronchiolitis were significantly higher than in control infants. There were no significant differences between term and preterm infants. TNF-α and IL6 protein was higher in term than preterm babies. On the day of intubation term infants with bronchiolitis had significantly higher concentrations of sTNFR1 (TNF-α – receptors 1) than preterm infants but their level did not change between the day of intubation and extubation (McNamara et al., 2004).

The ratio of IL6/TNF-α is determined by measuring the level of IL6 and TNF-α in a sample of NPA and has been related to the severity of the disease. A high ratio IL6/TNF- α has been associated with low disease severity (Hornsleth et al., 1998). Relatively high concentrations of IL6 may decrease the extent of airway inflammation produced by RSV.

It seems that IL6 is associated with mild forms of disease while an excess of TNF-α has destructive activity to lung tissues after RSV infection.

Interleukin 7

Human IL7 is a glycoprotein of 17.4 kDa (152 amino acids) (Ibelgaufts, 2003). IL7 is a requisite and potent growth and survival factor for early T-cell development (Bhatia et al., 1995). Regulation of T-cell number occurs in large part by modulation of T- cell populations in the periphery. The precise factors and mechanisms by which peripheral T-cell homeostasis is regulated remain ill defined. However, Mackall et al, (2001) found that supraphysiologic doses of IL7 after bone marrow transplantation, potently modulates the contribution of the thymic –independent peripherally expanded progeny to T-cell regeneration in thymus-bearing mice. Concurrently, IL7 also potently up-regulates thymic-dependent T-cell regeneration. The net result is that animals provided with hyperphysiologic doses of IL7 during the process of T-cell regeneration regenerated significantly larger peripheral T-cell pools (Mackall et al., 2001).

A case control study of the effect of IL7 in improving reconstitution of antiviral CD4 T- cells was conducted by Lu et al. They tested the effect of IL7 in severely lymphopenic baboons who had received total body irradiation and antithymocyte globulin (ATG). A group of baboons was treated with recombinant baboon IL7 for 2 months and another group was treated with placebo. The median CD4 Tcells count was significantly higher in the IL7 treated group compared to placebo. The animals were pretransplant cytomegalovirus (CMV)-seropositive, and the median CMV- specific INF-γ producing CD4 T cell count at the end of IL7/placebo treatment was significantly higher in the IL7-treated animals. These results led to the conclusion that IL7 stimulates the expansion of CD4 T cells, including functional antiviral cells (Lu et al., 2005).

In another study, the role of IL7 was also assessed in mice infected with herpes simplex virus type-1 (HSV-1). Two hundred IU of IL7 or IL2 were administered twice daily as an optimum therapeutic dose in mice. They compared the ability of the two interleukins to enhance the ability of T cells to clear HSV-1 infection. The enhanced immune protection was measured as a reduction in the amount of virus recovered from the pinna. The administration of HSV-1 immune T cells to naïve mice significantly increased their ability to clear the virus. IL7 treatment provided an additional 20 fold reduction in virus load, compared with T cell therapy alone. Combination of IL2 and T cell therapy provided about seven fold reduction compared to T cell therapy alone. To find out the mechanism of IL7 action the HSV-1 sensitised secondary immune T cells were depleted using either anti-CD4 or anti-CD8 antibodies in the

presence of complement to determine the role of CD4+ helper and CD8+ cytotoxic immune T cells in providing the enhanced immune response to HSV-1. They found that the depletion of CD4 T-cells had no effect on the ability of the IL7 and immune T cells to clear the viral infections. On the hand, on depletion of CD8+ cells, the ability of the adoptively transferred immune T cells to clear vial infection was abolished. These results confirm the role of CD8+ T cells in clearing viral infection and show that IL7 is likely to be involved in enhancing the CTL function, clonal expansion, or both. Addition studies are needed to distinguish between these two mechanisms (Wiryana et al., 1997).

Moreover, IL7 was found to be produced in the skin in response to *Schistosoma mansoni* infection. Since the induction and the modulation of the granuloma formation, in Schistosomiasis, is under the control of several cellular populations in which CD4 and CD8 T cells play a key role, IL7 seems to play a critical role in further the development of immunity against the *Schistosoma* (Auriault et al., 1996).

The observations of the potent effects of IL7 on mature T cells raise the possibility that this cytokine may act as a modulator of immune reconstitution and could be a potential vaccine adjuvant. However, the role of IL7 in the immunopathogenesis of RSV or HMPV as single or dual infections has not been described.

Interleukin 10

IL10 is a homodimeric protein with subunits having a length of 160 amino acids (Ibelgaufts, 2003). Macrophages are the main source of IL10 production (Bartz et al., 2002). Its role in primary RSV infection is controversial. Studies in progress indicate that IL10 may function as a potent suppressor of early inflammatory events in RSV-infected mice, without exhibiting antiviral activity (Panuska et al., 1995). In an *in vitro* infection study with alveolar macrophages RSV induced higher concentrations of IL10 than influenza (Panuska et al., 1995), suggesting that RSV could suppress the production of early immunoregulatory cytokines through the induction of IL10, which may be related to the induction of serious RSV infection. The hypothesis is that the incomplete immunity may result, in part, from RSV-infected alveolar macrophage production of IL10, which can interfere with the production of immunoregulatory cytokines (Panuska et al., 1995). IL10 is also induced by RSV but not by influenza or parainfluenza viruses in another study (Bartz et al., 2002).

An evaluation of the role of IL10 during RSV infection in a C57BL/6 transgenic (TG) mouse model in which the expression of murine IL10 cDNA was regulated by a human salivary amylase promoter (IL10 TG mice), showed that mice expressed a large amount of IL10 in the

nasal mucosa and salivary glands (Ruan et al., 2001). In IL10-TG mice, RSV replication was significantly lower, both in lungs and nasal mucosa, than in control-non-TG mice after intranasal inoculation. This suppression of virus recovery was no longer observed in anti-IL10-treated TG-mice. These results suggest that increased levels of IL10 in TG-mice suppressed RSV replication in the respiratory tract. IL10-stimulated T cells also displayed cytotoxic activity against infected murine nasal epithelial cells. In the nasal mucosa of RSV-infected TG-mice apoptotic epithelial cells were observed more frequently than in control mice.

In another study, concentrations of IL10 in respiratory secretions of children with RSV were greater than in samples obtained from control children, suggesting a Th2 response during RSV infections (Sheeran et al., 1999).

The immunoregulatory properties of this cytokine should also be considered as IL10 is also associated with recurrent wheezing in RSV-infected children (Bont et al., 2000a). A report indicates that monocyte IL10 production during RSV bronchiolitis is associated with recurrent wheezing in the following years. IL10 response during the acute phase of RSV bronchiolitis was comparable to the response of healthy control subjects. During the convalescent phase, IL10 responses were significantly increased in patients as compared with healthy subjects. At follow up, 27 children (58%) had recurrent episodes of wheezing. IL10 levels, measured during the convalescent phase, were significantly higher in patients who developed recurrent wheezing during the year after the infection than in patients without recurrent episodes of wheezing. No association was found between INF-γ responses, IL4 responses or INF- γ /IL4 ratios and recurrent wheezing (Bont et al., 2000a).

A recent study by van Benten et al, studied the amount of immune stimulation and nasal immune responses in infants with URTI due to RSV and Rhinovirus (57% of them from atopic families) and a control group of children matched for family history of allergy, age and gender but without symptoms. The number of nasal brush cell positive for Th1, Th2, regulatory and proinflammatory cytokines were measured. Fewer regulatory cytokine IL10 positive cells were found during rhinovirus and RSV URTI compared to non- positive cells and to non-infected children. This decrease in IL10 positive cells was accompanied by an increase in the Th1 cytokine TNF-α. IL10 responses were inversely related to TNF- α responses. No enhanced responses were observed for INF-γ, IL12 and IL18. The study showed that cytokine responses did not depend on the age of the child or atopy in the family. These results concluded that a reduction in nasal IL10 responses during URTI in infants could facilitate the induction of a TNF- α response (van Benten et al., 2005).

Hoebee et al. found a significant association between RSV bronchiolitis and the IL10 592C allele in 207 children with RSV that were compared to 447 adults. Children who were homozygous for the IL10-592C or 592A allele had a higher risk of hospitalisation for RSV bronchiolitis than the heterozygous carriers (Hoebee et al., 2004). The low production IL10 genotype has been associated with more frequent diagnosis of RSV pneumonia (Gentile et al., 2003).

Interleukin 12

IL12 is a heterodimeric 70 kDa glycoprotein consisting of a 40 kDa subunit (306 amino acids; 10 percent carbohydrates) and a 35 kDa subunit (197 amino acids; 20 percent carbohydrate) linked by disulfide bonds that are essential for the biological activity of IL12 (Ibelgaufts, 2003). IL12 is produced primarily by antigen-presenting cells and strongly promotes the differentiation of naïve CD4+ T cells to the Th1 phenotype. This characteristics suggest that it has a critical role as a regulator of Th1-derived immune responses inducing the lytic functions of CTL and NK cells (Bont et al., 2000b).

IL12 induce IFN-γ production by either NK or CD8+. CD4+ T cells also produce IFN-γ but do not appear to contribute to enhanced disease. This is supported by the observation that IL12 treated mice do not experience weight loss when CD8+ T cells and/or NK cells are absent. The role of NK and CD8+ cells is, however, quite different with respect to disease augmentation, although IL12 stimulated CD8+ T cells enhance the severity of weight loss during RSV challenge, IL12 stimulated NK cells are purely protective. These results suggest that IL12 may be beneficial in selected patients with RSV lung disease and that toxicity develop depending on the patient's immune status. Indeed IL12 might be safe and effective in patients with T cell dysfunction who suffer prolonged infection, while stimulating active natural immunity (Hussell and Openshaw, 2000).

Garofalo et al. however demonstrated in a study of 41 children less than 14 months of age, 10 had URI and 31 with bronchiolitis that INF-γ production is independent of IL12 as INF-γ was detected in 77% of the NPA samples but IL12 was detected in only 15%. The presence and levels of INF-γ did not correlate with those of IL12 (Garofalo et al., 2004).

To determine the effect of IL12 on eosinophil, airway hyper-responsiveness, and late asthmatic response, Bryan et al., conducted a controlled clinical trial of 39 adults with allergic asthma. Of these, 19 were randomly assigned to receive recombinant human IL12 and 20 to receive placebo. IL12 caused a decrease in blood and sputum eosinophils, but only minor

effects on airway hyper-responsiveness to histamine and no effect on airway responsiveness to inhaled allergens (Bryan et al., 2000).

The role of IL12 in the immune response to RSV infection was examined in a mouse model using C57BL/6 mice. These mice respond to RSV infection with low pathophysiologic abnormalities compared to BALB/c and DBA-2 mice and were chosen to confirm if IL12 was the cytokine responsible for the mild responses to RSV (Tekkanat et al., 2001). Antibodies specific for IL12 were used to neutralise the cytokine *in vivo.* Neutralising anti-IL12 or control antibodies were given to C57BL/6 mice intra-peritonealy 1 hour before intra-tracheal RSV and every other day thereafter until day 12 of infection. The mice were then examined for changes in airway hyper-reactivity at specific time points after infection. The mice treated with neutralising antibodies against IL12 had a significant increase in airway hyper-reactivity, whereas untreated mice and mice treated with control antibodies had minimal airway hyper-reactivity, comparable to previous experiments (Tekkanat et al., 2001). Binding of the cytokine to its receptor results in activation of its associated signal transduction pathways resulting in a unique response. The signal transducer and activator for transcription (Stat) family of proteins are involved in one of these intracellular signal transduction pathways. IL12 activates T helper cells via a Stat-4-induced signalling pathway. To confirm the importance of IL12 in suppressing severe airway hyper-reactivity responses in RSV infection, a similar RSV infection experiment in Stat-4 deficient mice was done. Stat-4-/- mice on a C57BL/6 background had significantly higher airway hyper-reactivity responses compared to Stat-4+/+ mice. Histologicaly, Stat-4-/- mice had increased numbers of eosinophils and goblet cell hypertrophy when compared to RSV controls but not to the extent observed in anti-IL12 treated C57BL/6 mice (Tekkanat et al., 2001).

The association between monocyte IL12 production and disease severity was investigated in 30 children ventilated for RSV bronchiolitis (Bont et al., 2000b). There was an inverse relationship between the duration of the ventilation and IL12 production suggesting that a low monocytes IL12 response during initial RSV infection might adversely affect the clinical outcome of patients with severe RSV bronchiolitis. Thus monocyte production of IL12, which is an antiviral cytokine, may influence in the type and duration of clinical RSV disease. Although cell-mediated immunity may be involved in induction of RSV bronchiolitis, this study indicated that cell-mediated immunity is also needed for recovery from respiratory insufficiency.

Blanco-Quiros et al found that there were alterations in the Thl/Th2 balance at the time of delivery in neonates who develop acute bronchiolitis later in life (Blanco-Quiros et al., 1999). Cord blood was obtained from all children born in a Spanish hospital and several months later, 11 of these children were admitted to the hospital with acute bronchiolitis. These cases were compared with another 12 randomly selected infants without bronchiolitis and who had been born consecutively under similar conditions. The control group was followed for 48 months to ensure they did not develop bronchiolitis. Neonates who later developed bronchiolitis had a significantly lower IL12 in cord blood (295 pg/ml vs. 507, P=0.001).

Interferon –gamma

IFN- γ is a dimeric protein with subunits of 146 amino acids (Ibelgaufts, 2003). The production of INF-γ is part of a cascade of events in the first line of defence during virus infection. Although it is known that both NK and T cells make this cytokine, NK cells are thought to be an important early source, where it is produced in abundant amounts during the first 4 days of the local response to RSV infections (Hussell and Openshaw, 1998). This cytokine has a direct antiviral effect and is particularly important in stimulating the cytolytic activity of NK cells and CD8+ CTL. Apparently, it is important that CTL effectively clears the virus before the antigen load becomes too high (Graham, 1995). It is therefore conceivable that the level of IFN-γ produced early in the course of RSV infection may affect this clinical expression. Severe RSV disease has been associated with reduced levels of IFN-γ expression and lower T-lymphocyte counts in the acute phase of illness (Aberle et al., 1999). These findings suggest that weak IFN-γ production by PBMC early in the course of RSV infection may be associated with the development of severe RSV disease, and may also help explain at least some of the unique features seen in infants with RSV bronchiolitis and its sequelae. IFN-γ levels are severely decreased locally in NPA of mechanically ventilated infants with RSV LRTI compared with hospitalised infants with RSV LRTI who did not required mechanical ventilation (Bont et al., 2001). In a study of 20 infants aged 3-44 weeks in Austria, moderate RSV disease ($O_2 \geq 93\%$) was associated with higher INF- γ mRNA expression in the acute phase of illness than in children with severe RSV disease. There were however no differences in the levels between the two groups in the convalescence stage (Aberle et al., 1999). The low level of INF-γ expression found in infants with severe disease may be due to an inhibitory effect of RSV which is capable of suppressing both non-specific and RSV-specific lymphocyte proliferation or to immaturity of the infant's immune system.

RSV is associated with lower INF-γ production in young babies at high risk of atopy, regardless of their atopic status. In these children the mean INF-γ/IL10 ratio was significantly lower and INF-γ is significantly lower during RSV infection, suggesting a suppression of Th1 cytokine at the respiratory tract level during RSV infections. There are no differences in cytokine responses between atopic and non-atopic babies, suggesting that the cytokine response to RSV is not due to the babies' pre-existing atopic status (Joshi et al., 2003).

Renzi et al. reported that among children hospitalised with acute RSV infection, those who developed asthma had significantly lower INF-γ levels produced by their PBMC at the time of the acute RSV infection than those without asthma. This suggests that children who will go on to develop asthma after bronchiolitis either have an existing deficiency in INF-γ production or that they respond to the virus in a different way to children who do not develop asthma (Renzi et al., 1999).

The INF-γ responses in PBMC of 50 infants hospitalised with primary RSV infection or their first episode of ALRI due to adenovirus, parainfluenza or rhinovirus were compared by Aberle et al. The early production of INF-γ was either weak or absent in infants experiencing their first RSV infection. In contrast, children with infections due to other viruses had strong INF-γ response, indicating that infants are able to respond with competent INF-γ production to a first respiratory viral infection in the first months of life. This response however was not observed during primary RSV infection (Aberle et al., 2004). In contrast a study by Tripp et al. comparing INF-γ levels of 21 children, aged 1-21 months, hospitalised for RSV ALRI and 26 controls with non RSV-illness or elective surgery found that RSV induced both Th1 and Th2 responses that were higher than in non-RSV infections. In this study, IL2, INF-γ and IL4 levels were significantly higher in children with RSV with relative increase of INF-γ than IL4 consistent with bias towards Th1 type response(Tripp et al., 2002).

RSV-infected children produce less INF-γ and more IL4 than healthy children and children with severe RSV have higher INF-γ than children with mild disease (Bendelja et al., 2000). However, the non-structural proteins NS1 and NS2 of bovine and human RSV were reported to inactivate INF- γ in RSV infected human and bovine epithelial cells and macrophages (Schlender et al., 2000, Spann et al., 2004a). The INF-γ genotype (A874T) however is associated with ALRI severity and length of staying an intensive care unit infant with RSV (Gentile et al., 2003).These conflicting results call for more research to clarify the role of INF-γ in the pathogenesis of RSV infection.

Chemokine responses and severity of RSV infection

The pathology of RSV infection is partly due to excessive proinflammatory leukocyte influx mediated by chemokines (Everard et al., 1994). Chemokines are essential mediators of normal leukocyte trafficking and are multipotent cytokines that localise and enhance inflammation by inducing chemotaxis and cell activation of different types of inflammatory cells typically present at inflammatory sites. There are four clearly defined subgroups of chemokines on the basis of their structural and functional properties. The alpha-chemokines, have the first two cysteine residues of members separated by single amino acids and these proteins, are therefore also called C-X-C chemokines. In the Beta-chemokines, the first two cysteine residues are adjacent, and they are also called C-C chemokines. Gamma-chemokines or C-chemokines on the other hand differ from the other chemokines by the absence of a second cysteine residue. A fourth group of members of the small group of chemokines with CX (3) C cysteine signature motif, are called Delta chemokines or CX3C or C-X-X-X-C (Ibelgaufts, 2003). Two of these chemokines, interleukin- 8 (IL8) and Regulated Upon Activation, Normal T cell Expressed and Secreted (RANTES) are described in more detail below.

Interleukin 8 and RANTES

IL8 is a member of the C-X-C family of chemokines. It is an important neutrophil chemoattractant and activator has possesses chemotactic activity for primed eosinophils (Jaovisidha et al., 1999).

Epithelial cells of the respiratory tract may be one source of IL8 during acute URI (Noah et al., 1995). *In vitro* studies with human cell lines have shown that RSV increases the production of IL8 by airway epithelial cells (Harrison et al., 1999), alveolar macrophage (Becker et al., 1991), PBMC (Arnold et al., 1995) and neutrophils (Jaovisidha et al., 1999). It has been suggested that IL8 is responsible for the accumulation of neutrophils in the alveolar space in RSV bronchiolitis (Jaovisidha et al., 1999) and induce a marked but transient reduction in neutrophil deformability (Drost and MacNee, 2002).

The IL8 concentrations in the airways of infants with bronchiolitis correlate with blood cell counts (Sheeran et al., 1999) and myeloperoxidase concentrations (Harrison et al., 1999).

Plasma IL8 is also elevated in infants with RSV bronchiolitis, and levels are higher in ventilated infants than to those who were not ventilated (Bont et al., 1999a). Although the production of IL8 in the lung has not been investigated extensively in RSV bronchiolitis,

increased IL8 concentrations are found in the BAL of patients with bronchiolitis (Harrison et al., 1999). A high IL8 mRNA / HGPRT mRNA (hypoxanthine guanine phosphoribosyl transferase gene) ratio in NPA and BAL of ventilated infants with RSV bronchiolitis are associated with disease severity (Smyth et al., 2002). Further evidence for the importance of IL8 in the pathogenesis of RSV bronchiolitis was provided by studies of infants with RSV bronchiolitis and their families, which investigated a genetic variant associated with increased IL8 production. A greater likehood of transmission of this variant was found in infants with RSV bronchiolitis, particularly in those with severe disease (Hull et al., 2000).

RANTES is a member of CC family of chemokins. It is an important monocyte, eosinophil and basophil chemoattractant (Ibelgaufts, 2003).

There is a time dependent increases in RANTES mRNA and protein levels when primary-culture airway epithelial cells are infected with RSV (Koga et al., 1999). The specificity of this response was demonstrated by the findings that RSV, but not adenovirus infection was effective in stimulating RANTES expression and that the expression of RANTES mRNA, but not of other β-chemokines was induced by infection. Similarly Sung et al. compared the serum concentrations of some cytokines and chemokines including RANTES in children with RSV infection with those of children infected with IFAV and found that RANTES concentrations were significantly higher in the former group (Sung et al., 2001). TNF-α and INF-γ synergistically induce RANTES protein secretion and gene expression in alveolar epithelial cells through NF-KB and interferon regulatory factor 1(Casola et al., 2002). In murine models RANTES is an important mediator of airway hyperreactivity during RSV infection and its effects can be modified by inhibition of RANTES (Tekkanat et al., 2002). Treatment with anti-RANTES downregulates IL4 and IL5 and upregulates IL12. This alteration of IL12 may provide an environment that is susceptible to hyperreactive responses inducing altered lung function. These data suggest that RANTES –mediated activation plays a significant role in the exacerbation of pathophysiological responses to pulmonary RSV infections.

The presence of histamine and ECP in nasopharyngeal secretions of infants with RSV bronchiolitis implies the activation of basophil and eosinophil leukocytes, but the specific mechanism of their recruitment has not been elucidated. Therefore the pattern of chemokines produced in response to RSV infection was investigated in primary cultures of human nose- and adenoid-derived epithelial cells (Saito et al., 1997). RANTES, which was present in negligible concentrations in uninfected cultures, was strongly induced by RSV infection, in a dose- and time-dependent manner. Through the release of RANTES, epithelial cells may

control the selective concentration and activation of basophils and eosinophils in RSV-infected airway mucosa (Saito et al., 1997). To determine whether RANTES will increase with infection *in vivo*, RANTES was measured in NLF from children with RSV positive and RSV negative URI and children when they were well. RANTES was increased significantly during RSV infection compared with non-RSV infection and with asymptomatic baseline in the same children (Becker et al., 1997).

To address the question of whether the increase in RANTES concentration was a consequence or a cause of severe disease, 580 infants admitted to 10 hospitals in southeast England with severe RSV and 580 infants born consecutively at John Radcliffe Hospital in Oxford during 1999-2000 were investigated (Hull et al., 2003). The study demonstrated an association between CC chemokine receptor 5 (the key receptor for RANTES and MIP-1α) and severity of RSV disease. Both the -2554T and the -2459G alleles were found more commonly in cases of severe RSV than in control subjects and the effect seemed to be strongest in subjects who were homozygous for these alleles.

Hornsleth et al. (2001) tested 21 inflammatory mediators ratios as possible indicators of the severity of RSV disease in a study of 46 infants aged 0-9 months. Of these 25 patients had acute severe RSV infection and 21 mild disease (Hornsleth et al., 2001). The concentration of seven of these mediators was measured by ELISA in samples of NPA, four cytokines: IL1, IL6, IL10 and TNF-α; the cytokine receptor TNF-R1 and the chemokines; IL8 and RANTES. The following inflammatory mediator ratios were related to severity of disease: TNF-R1/RANTES, IL8/RANTES and RANTES/IL10 (Hornsleth et al., 2001). RANTES and IL8 were detected in respiratory secretions, NW and TA of children with RSV infection. They were found to be significantly greater in children with RSV than in samples from a control group of children. Their concentrations in (NW and TA) were significantly correlated to each other (Sheeran et al., 1999). TA RANTES, and IL8 concentrations inversely correlated with clinical markers of RSV disease severity (Sheeran et al., 1999). However, Noah et al. compared IL8 and RANTES levels in NLF and nasal epithelial cells of 47 RSV-positive (26 hospitalised children with bronchiolitis and 15 without bronchiolitis) and 51 RSV-negative infants (Noah et al., 2002). Both IL8 and RANTES, expressed as a ratio to total protein in NLF, were increased in RSV-positive compared to RSV-negative children. Among RSV-positive illnesses, neither chemokine differed significantly between bronchiolitic and non bronchiolitic children. However, bronchiolitic infants had significantly increased RANTES/IL8 ratios than non-bronchiolitic infants in NLF and nasal epithelial cells. These findings suggest that the clinical manifestations of RSV infection are related to chemokine balance at the respiratory mucosal surface.

Results

Cytokines and chemokine concentrations

A total of 152 (49%) out of the 310 children with RSV and/or HMPV infections were randomly selected to measure the NPA concentrations of cytokines IL6, TNF-α, IL7, IL10, IL12 and INF-γ and the chemokines IL8 and RANTES. Ninety six (63%) of the children were infected with RSV, 33 (22%) with HMPV and 23 (15%) had RSV/HMPV coinfections.

Forty one (27%) children had pO_2 > 88% and 111 (73%) ≤ 88%. One hundred (66%) of the children were male and 52 (34%) female. The mean age of the children was 6 months with a range of 0 to 24 months and a median of 3.8 months. Seventy five children (49%) were ≤ 3 and 77 (51%) > 3 months old. Some NPA samples had only a small amount of material left for analysis and these were diluted before measuring the cytokines and chemokines. This procedure however, resulted in unreliable readings and these readings were excluded. The numbers of samples measured for each of the cytokines and chemokines are summarized in table 5.2.

Table 5.2 Number of samples tested for cytokine and chemokine concentrations by virus

Cytokine / chemokine	RSV n (%)	HMPV n (%)	RSV/HMPV n (%)	All n (%)
IL6	94 (63)	33 (22)	23 (15)	150 (98)
TNF-α	81 (69)	19 (16)	17 (15)	117 (76)
IL7	83 (62)	31 (23)	20 (15)	134 (88)
IL10	86 (63)	33 (24)	17 (13)	136 (89)
IL12	96 (67)	27 (19)	20 (14)	143 (93)
INF-γ	83 (63)	30 (23)	18 (14)	131 (86)
IL8	64 (58)	29 (26)	18 (16)	111 (73)
RANTES	96 (66)	29 (20)	20 (14)	145 (94)

% = % of samples selected for anaylsis

Cytokine and chemokine concentrations in all specimens

IL6 and TNF-α concentrations were evaluated in 150 and 117 NPA specimens respectively. Their geometrical mean (GM) concentrations and standard errors (SE) were 37.5 (1.11) pg/ml and 158.7 (1.13) pg/ml respectively, with a range from 5.7 pg/ml to 2713.4 pg/ml for IL6 and from 8.3 pg/ml to 4196.6 pg/ml for TNF-α. IL7 was measured in 134 specimens with a GM (SE) concentration of 10.3 (1) pg/ml and a range from 1.9 pg/ml to 61.8 pg/ml. IL10 is the signature cytokine of type 2 cytokines. Its GM (SE) concentration was 20.6 (1.06) pg/ml with a range from 5 pg/ml to 97 pg/ml.

IL12 and INF-γ are molecules representative of type 1 cytokines. Their GM (SE) concentrations were 15.3 (1.06) pg/ml and 73.6 (1.05) pg/ml respectively. IL12 concentrations ranged from 3.3 pg/ml to 92.4 pg/ml while INF-γ concentrations ranged from 18.4 pg/ml to 710.8 pg/ml.

The IL8 GM (SE) concentration was 3316.7 (1.07) pg/ml with a range from 48.5 pg/ml to 9894.9 pg/ml and RANTES had a GM (SE) of 98.2 (1.15) pg/ml, ranging from 3.8 pg/ml to 4916.3 pg/ml.

Cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV coinfections

The cytokine and chemokine concentrations of children with RSV, HMPV and RSV/HMPV co-infections were analyzed to describe the immune responses to single and combined infections with either virus. Their GM cytokine and chemokine concentrations and ranges are shown in table 5.3.

The GM IL6 concentrations were higher in children with RSV (46.5 pg/ml) than in children with HMPV (20.3 pg/ml; $p=0.002$) and were at an intermediate level in children with RSV/HMPV (35.9 pg/ml). The GM IL6 in children with coinfections was higher than in children with HMPV alone ($p=0.01$). In contrast, TNF-α, concentrations were lower in children with RSV and RSV/HMPV than in children with HMPV, however, only the differences between children with RSV and HMPV were statistically significant ($p < 0.01$). Children with RSV had a wider range of values of both IL6 and TNF-α than those observed in HMPV or RSV/HMPV.

IL7 GM concentrations were significantly higher in children with RSV than in children with HMPV. IL7 GM concentrations in children with RSV or RSV/HMPV however were similar. Children with RSV/HMPV coinfections had the highest IL7 concentrations detected.

IL10 concentrations were similar in children with RSV, HMPV and RSV/HMPV ($p > 0.05$ for all), although children with HMPV had a wider range of values.

IL12 concentrations were borderline statistically significantly lower in children with RSV than in children with HMPV. Children with RSV/HMPV coinfections had the widest range of IL12 values among the three groups.

INF- γ concentrations were lower in children with RSV and HMPV than in children with RSV/HMPV but this difference was only statistically significant between RSV and RSV/HMPV ($p = 0.02$).

The GM concentrations, standard errors, 25th and 75th centiles and p values for IL6, TNF-α, IL7, IL10, IL12 and INF-γ of the children are shown in figure 5.2.

IL8 concentrations were lower in children with RSV and HMPV infections than in children with RSV/HMPV. However, only the differences between HMPV and RSV/HMPV coinfections were borderline statistically significant. RSV infection resulted in a wider range of values than HMPV or RSV/HMPV.

RANTES concentrations were lower in children infected with RSV and RSV/HMPV than in children with HMPV. The differences between children with RSV and HMPV were borderline statistically significant. Children with RSV/HMPV had a wider range of values than children with single infections. IL8 and RANTES by RSV, HMPV and RSV/HMPV are shown in figure 5.3.

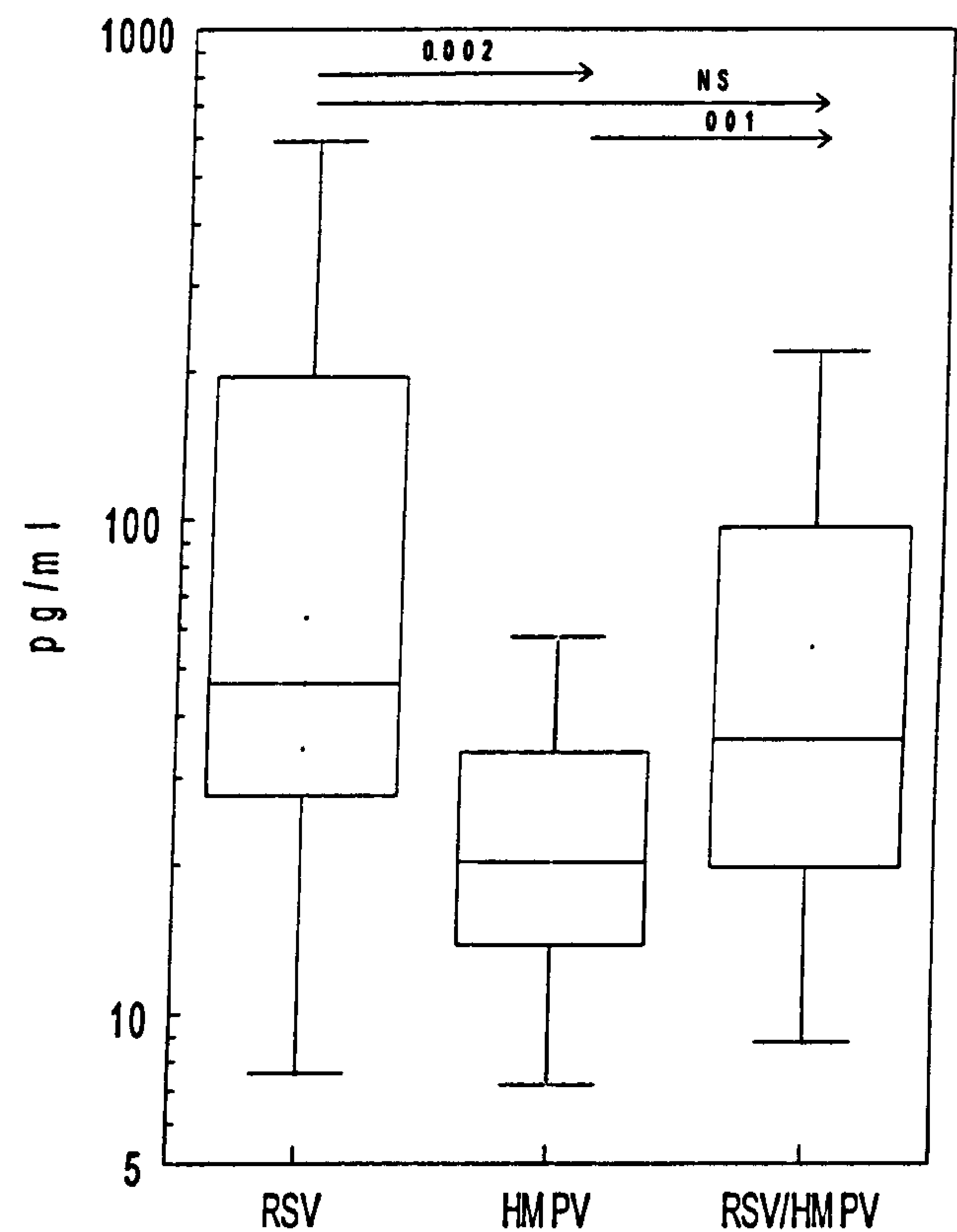
Table 5.3 Cytokine and chemokine NPA concentrations in children with RSV, HMPV and RSV/HMPV

Cytokine/chemokine GM (SE) and range	RSV	HMPV	RSV/HMPV	P^1 value	P^2 value	P^3 value
IL6	46.5 (1.17)	20.3 (1.12)	35.9 (1.24)	**0.002**	0.4	**0.01**
	5.7 - 2713.4	6.7 - 92.9	7 - 299			
TNF-α	133.8 (1.15)	313.6 (1.23)	167.2 (1.36)	**0.009**	0.5	0.09
	8.3 - 4196.6	95 - 1477.3	27 - 3269.6			
IL7	11.3 (1.08)	7.3 (1.14)	11.5 (1.18)	**0.002**	0.8	**0.03**
	1.9 - 61.8	2.3 - 39.5	2.4 - 49.6			
IL10	20.3 (1.07)	24 (1.14)	16.5 (1.17)	0.2	0.2	0.09
	5.8 - 90.4	5 - 97	5.7 - 47.3			
IL12	14.5 (1.07)	19.2 (1.13)	14.3 (1.22)	**0.05**	0.9	0.2
	3.3 - 92.4	9.5 - 82.3	3.8 - 84.4			
INF-γ	69.7 (1.04)	74.4 (1.14)	93.2 (1.18)	0.5	**0.02**	0.3
	31.2 -173.9	18.4 - 450.6	31.1 - 710.8			
IL8	3480.5 (1.08)	2586.1 (1.17)	4057.9 (1.12)	0.06	0.3	**0.05**
	884 - 9894.9	48.5 - 6431.9	1652.6 - 7925			
RANTES	79.8 (1.19)	169.5 (1.33)	120.2 (1.45)	**0.03**	0.3	0.4
	3.8 - 3569.1	16 - 3083.4	6 - 4916.3			

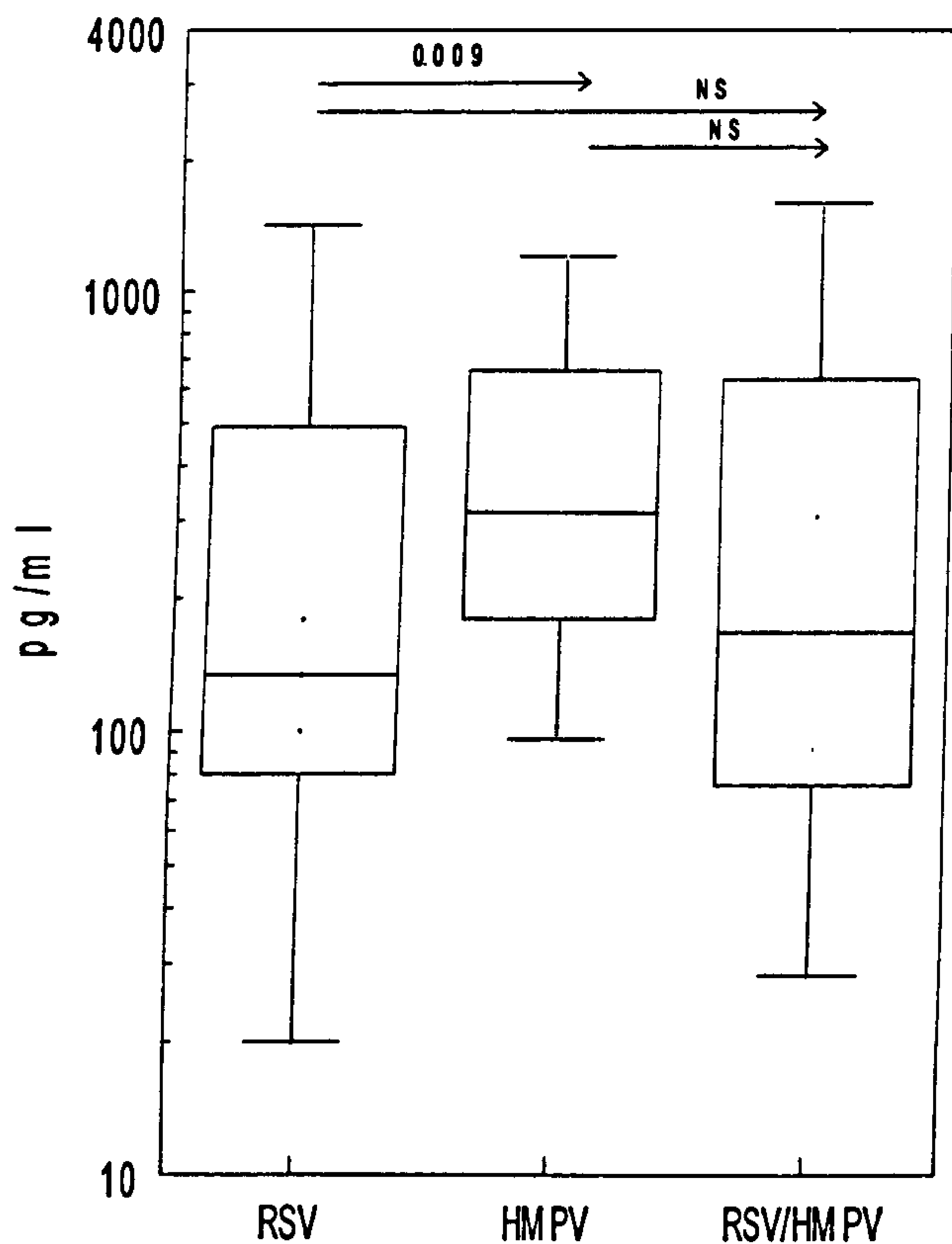
P^1= RSV vs. HMPV, P^2 = RSV vs. RSV/HMPV, P^3= HMPV vs. RSV/HMPV

Figure 5.2 IL6, TNF-α, IL7, IL10, IL12 and INF-γ concentrations of children with RSV, HMPV and RSV/HMPV (the graph shows the GM, SE (dots), 25th and 75th centiles)

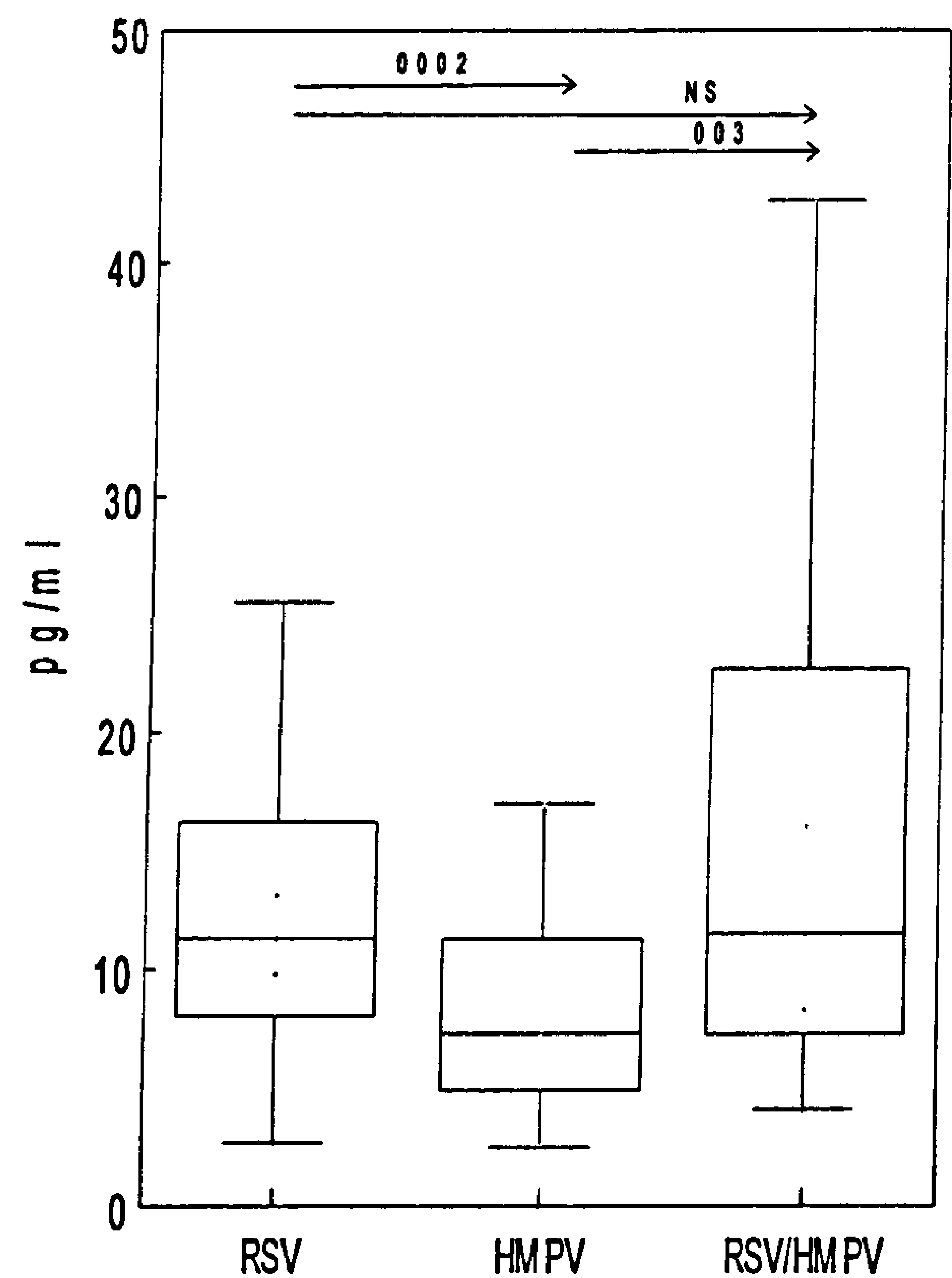
IL6



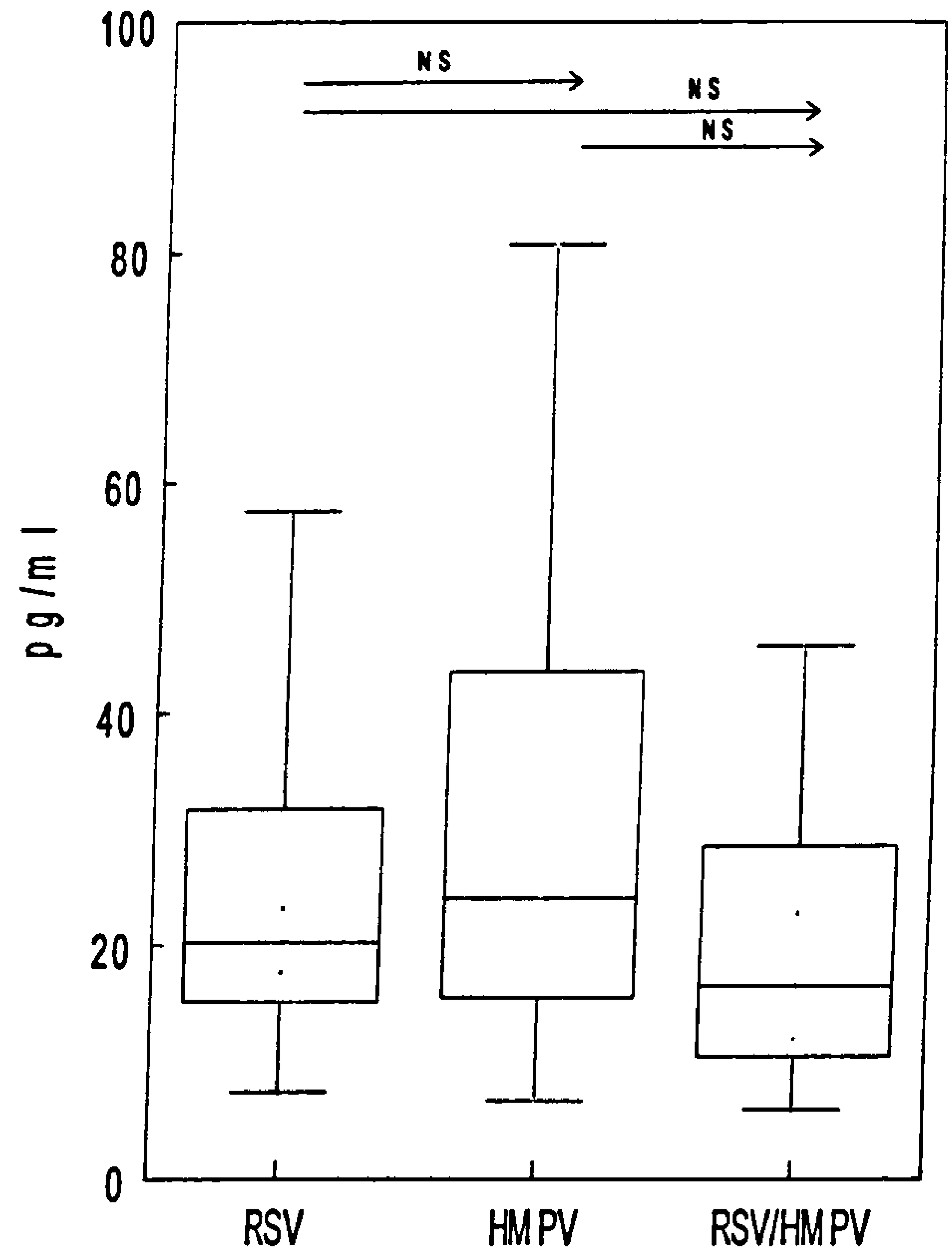
TNF-α



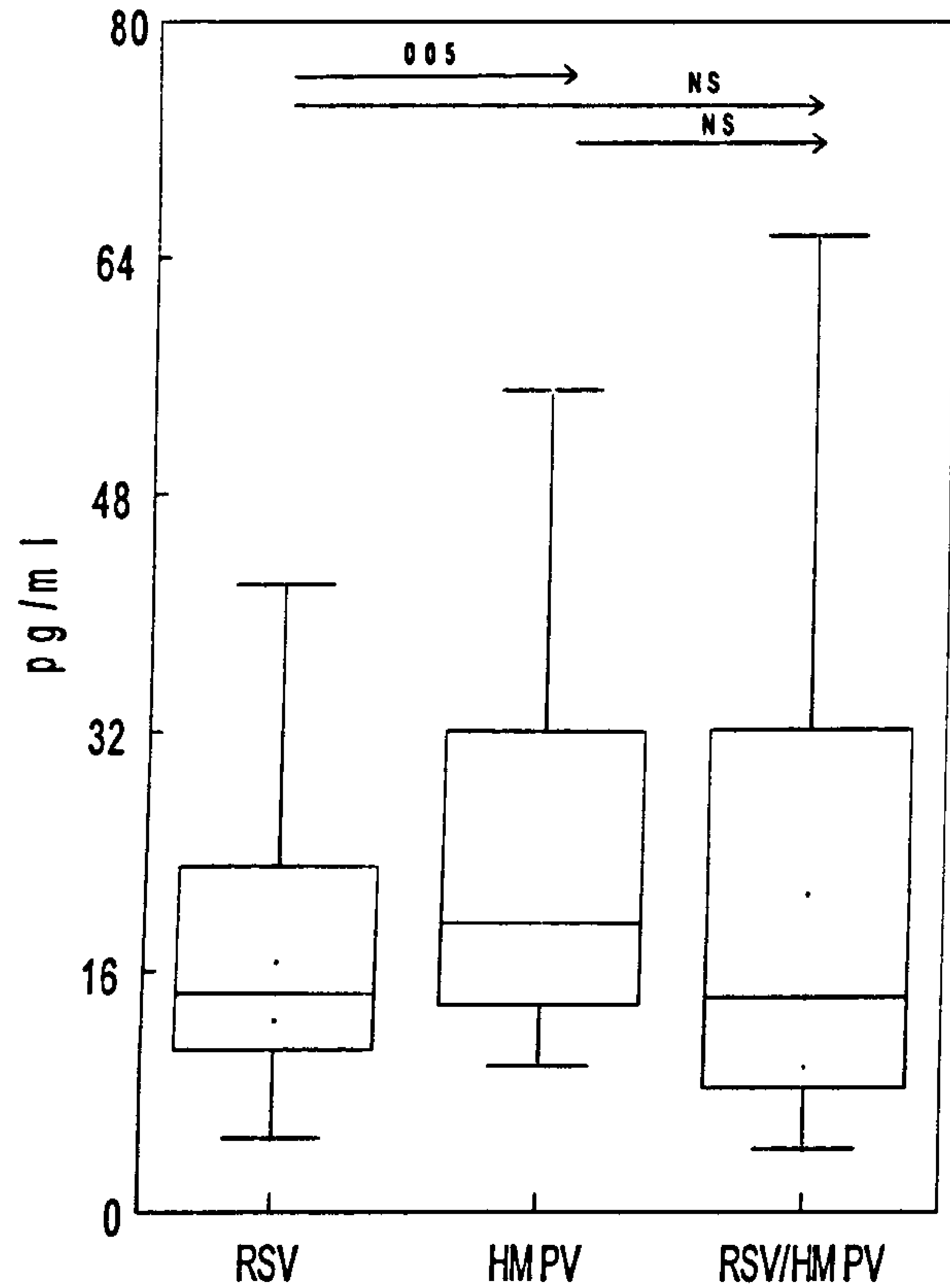
IL7



IL10



IL12



INF-γ

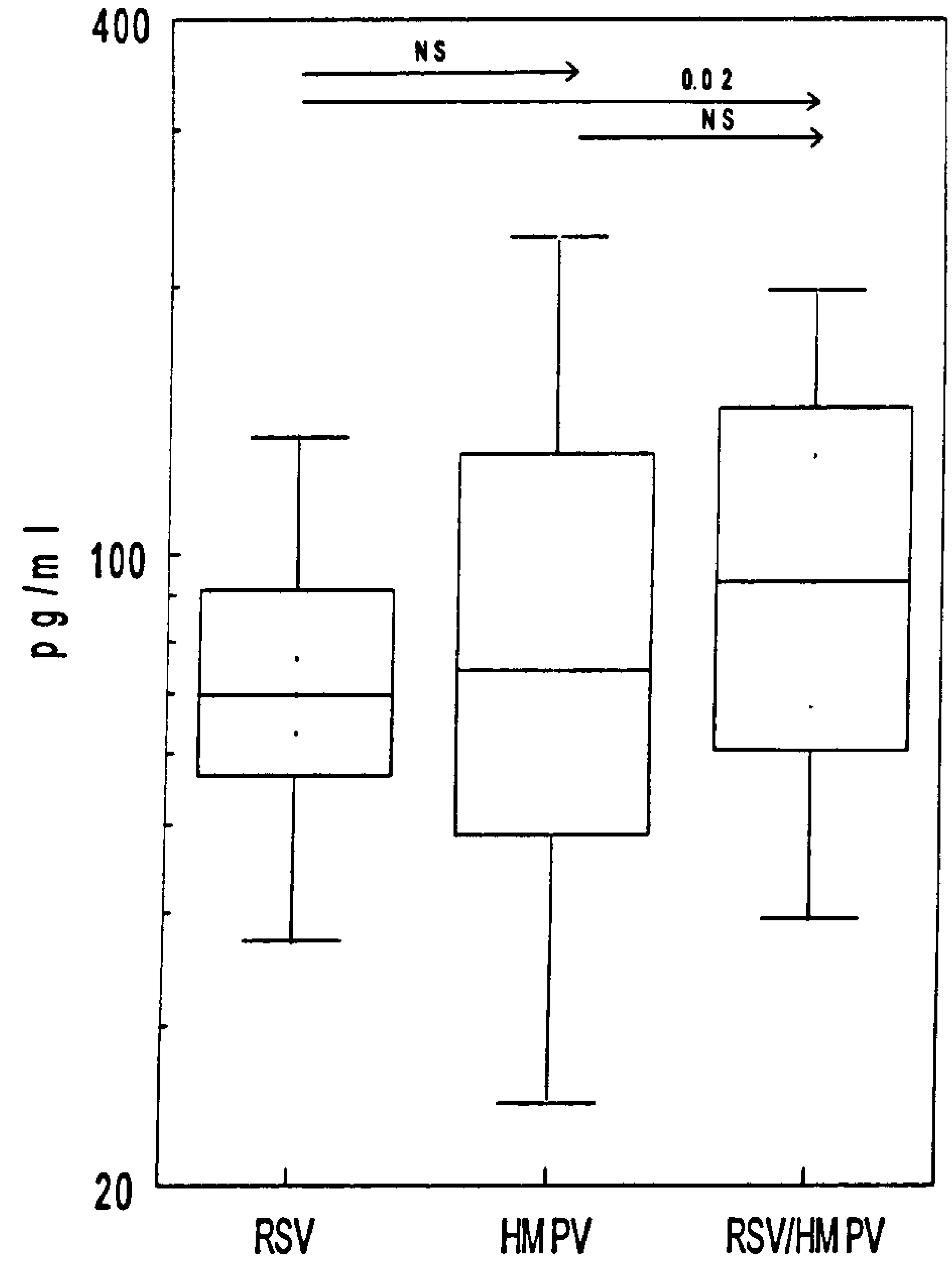
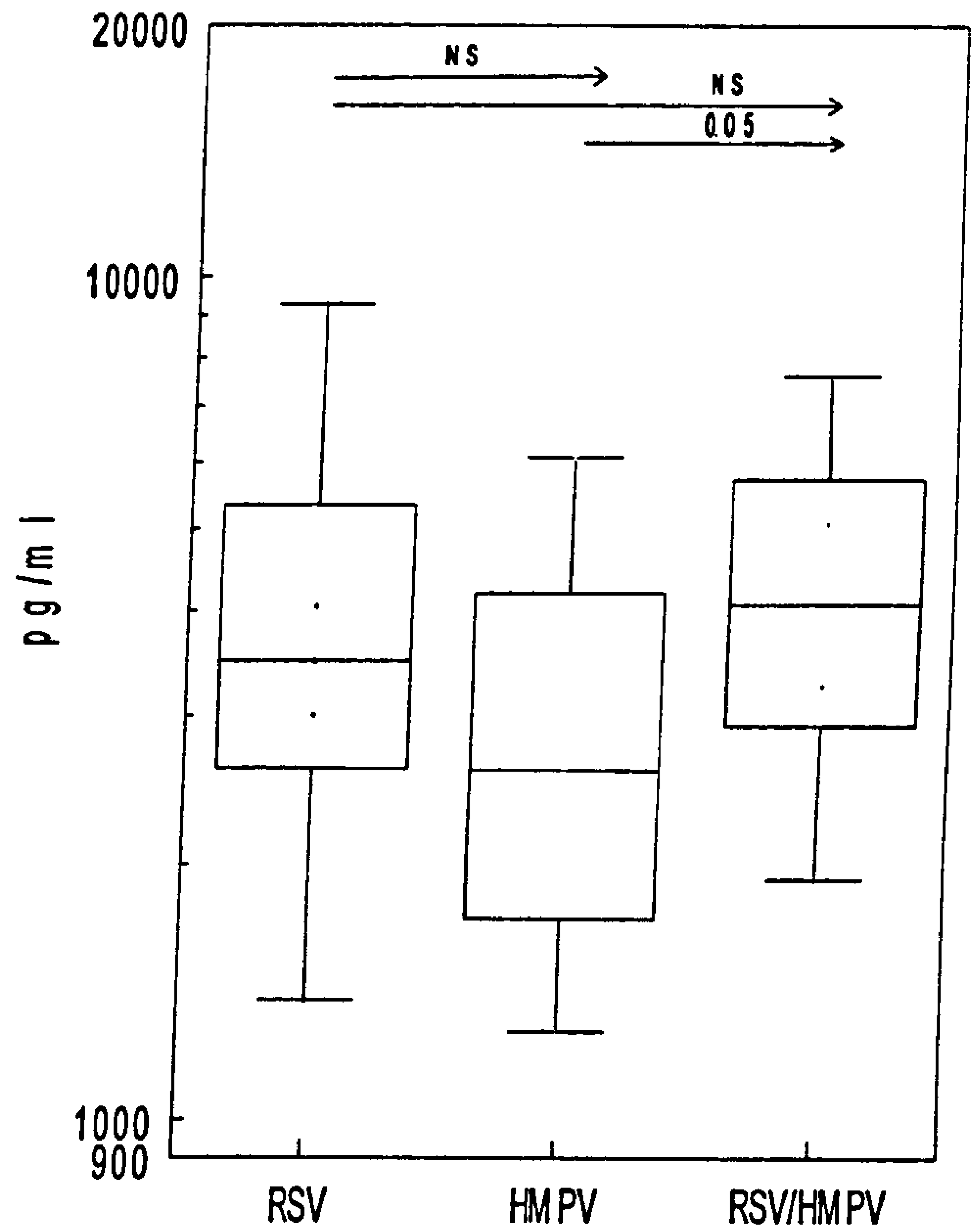
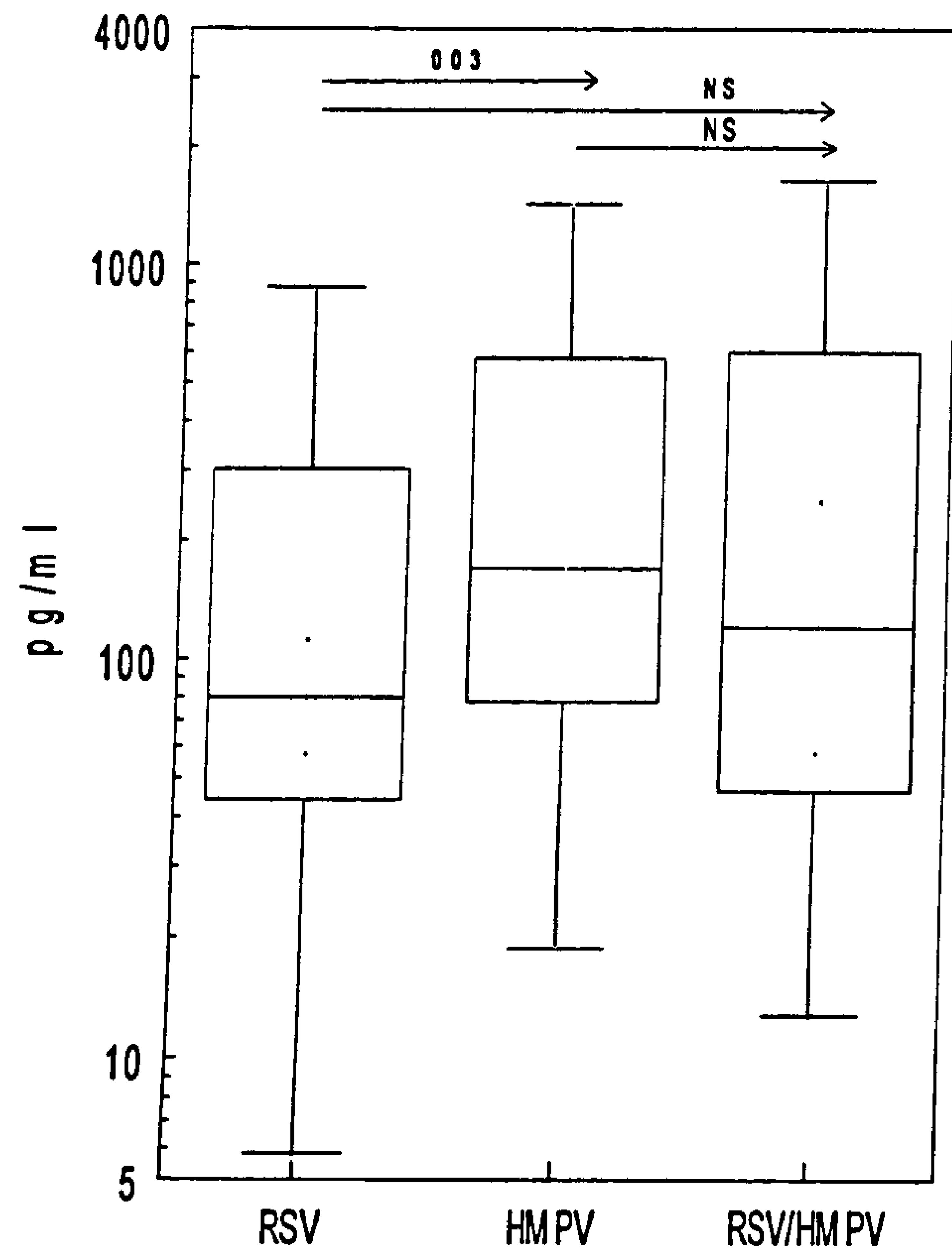


Figure 5.3 IL8 and RANTES concentrations of children with RSV, HMPV and RSV/HMPV

IL8



RANTES



Cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV infections by disease severity

The cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV coinfection were analyzed according to the clinical severity of the ARI at the time of consultation to the health services. The severity of the episode was defined on the basis of the degree of hypoxia. Children with $pO_2 > 88\%$ were considered to have mild ARI and children with $pO_2 \leq 88\%$ were considered to have severe ARI. The number of children with mild and severe ARI who had their cytokines and chemokines measured are described in table 5.4. The number of children with mild ARI due to RSV/HMPV coinfections is very small (n=3) and the statistical analysis for this group can only provide an indication of possible associations.

Table 5.4 Number of patients tested for cytokine and chemokine concentrations by disease severity

	RSV		HMPV		RSV/HMPV	
	Mild	Severe	Mild	Severe	Mild	Severe
IL6	27	66	10	23	3	20
TNF-α	27	53	6	13	3	14
IL7	25	57	9	22	2	18
IL10	26	59	10	23	2	15
IL12	28	67	9	18	3	17
INF-γ	23	59	9	21	3	15
IL8	12	52	9	20	3	15
RANTES	28	68	9	20	3	17

Children with mild and severe ARI due to RSV had significant differences in their cytokine concentrations as shown in table 3.3.4. In summary, children with severe RSV ARI had statistically higher INF-γ ($p = 0.04$) and lower IL6 and IL7 concentrations ($p = 0.01$ for both) than children with mild RSV ARI. TNF-α concentrations were higher in children with severe ARI than in children with mild ARI. In contrast, IL10 concentrations were similar in children with mild and severe ARI, with a wider range of values in children with severe ARI. Finally, IL12 were higher in children with severe RSV ARI than in children with mild presentation.

In contrast to RSV, there were no significant differences in the cytokine and chemokine concentrations in mild and severe HMPV ARI or between mild and severe RSV/HMPV coinfections ($p>0.05$ for all).

Regarding HMPV infections, children with severe disease, had higher concentrations of IL6 than children with mild presentation, although this was not statistically significant. IL7 INF-

γ, IL12 and TNF-α concentrations had wider ranges of values in children with severe ARI than children with mild presentation, but these differences were not significant.

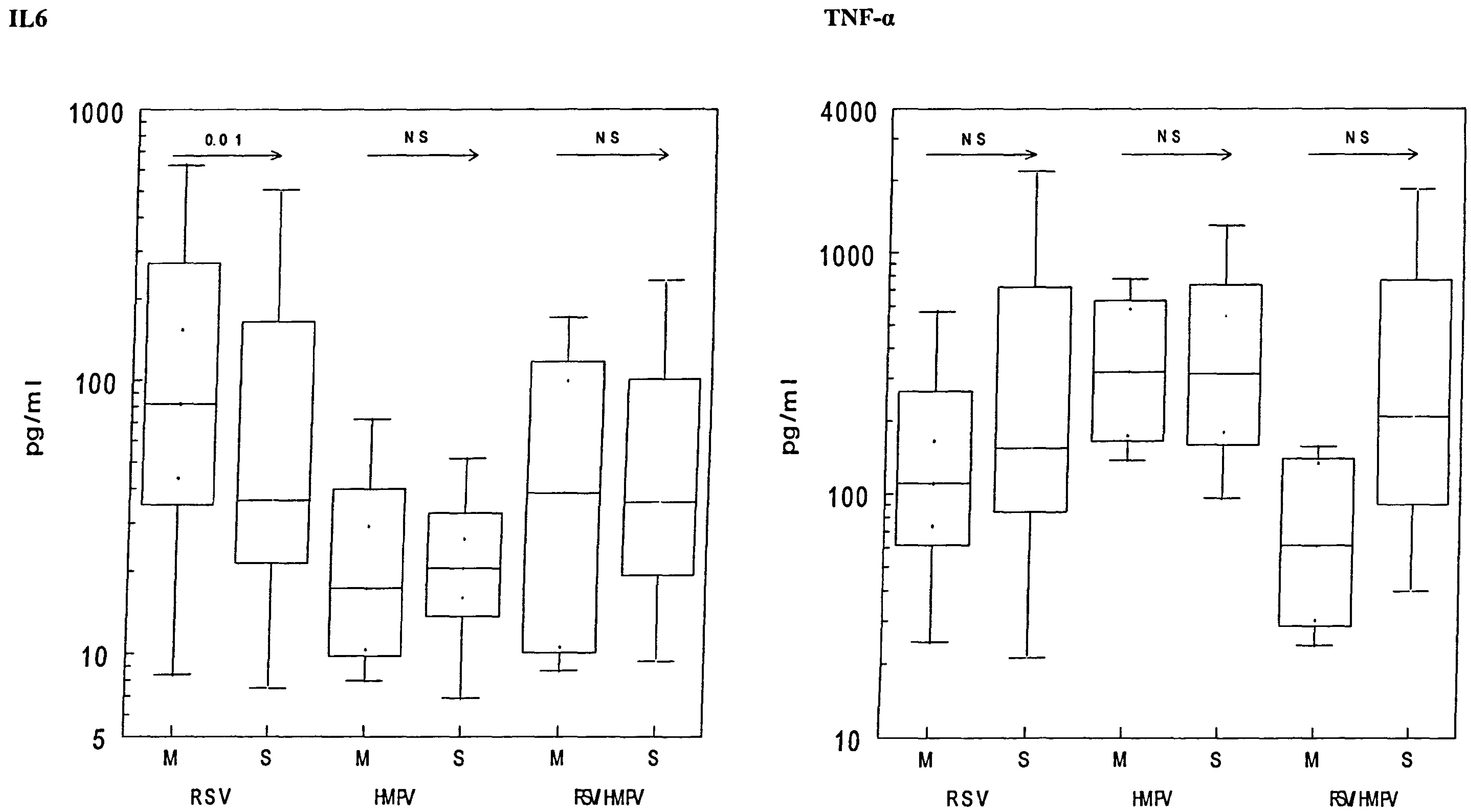
The GM and range of the cytokine/chemokine concentrations for RSV, HMPV and RSV/HMPV are shown in table 5.5. The GM, SE, 25th and 75% centiles and range for each marker by disease severity and associated viruses are illustrated in figure 5.4 for visual comparison.

Regarding, the chemokines measured, both IL8 and RANTES concentrations were lower in children with severe ARI than in children with mild ARI, independently of whether these were due to RSV, HMPV or RSV/HMPV coinfections. However, none of these differences were statistically significant. The GM and range of values for the 3 virus combinations are illustrated in figure 5.4. Severe RSV and HMPV ARI had a wider range of IL8 values, while children with mild RSV and HMPV ARI had wider ranges of values for RANTES.

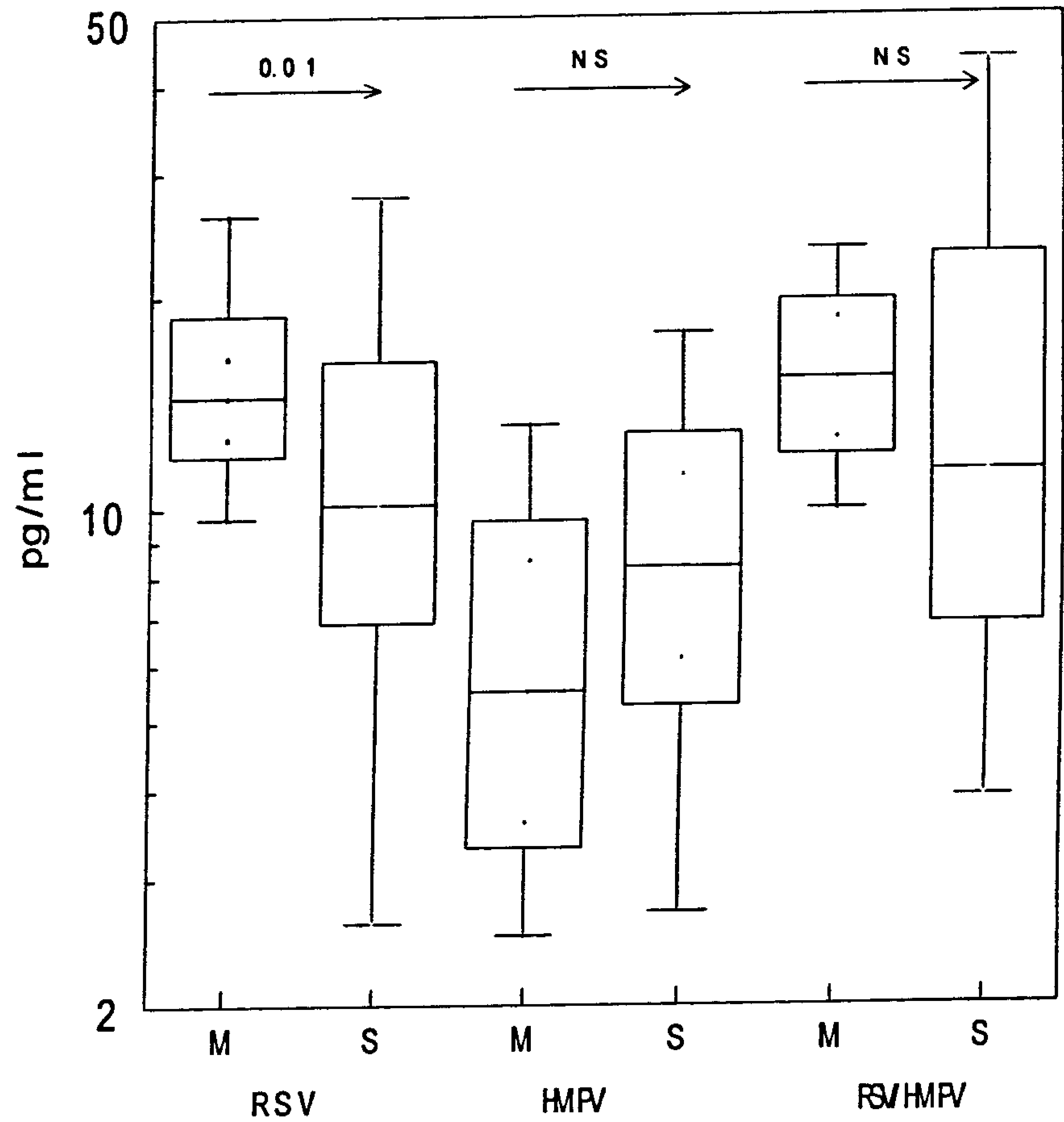
Table 5.5 Cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV by disease severity

Cytokine/chemokine GM (SE) and range	RSV		P	HMPV		P	RSV/HMPV		P
	Mild	Severe		Mild	Severe		Mild	Severe	
IL6	81.8 (1.38)	36.2 (1.19)	**0.01**	17.4 (1.3)	20.5 (1.13)	0.4	38.5 (2.1)	35.5 (1.3)	0.9
	7.3 - 2713.4	5.7 – 2488		7.6 - 92.9	6.7 - 66		8.7 - 103.9	7 - 299	
TNF-α	110.2 (1.22)	153.7 (1.2)	0.3	317.3 (1.36)	311.9 (1.32)	0.9	61 (1.6)	207.5 (1.4)	0.1
	16.5 - 1421.8	8.3 - 4196.6		121.8 - 774.5	95 - 1477.3		28.4 - 147	27 - 3269.6	
IL7	14.4 (1.07)	10.1 (1.11)	**0.01**	5.5 (1.24)	8.2 (1.16)	0.2	15.2 (1.2)	11.2 (1.2)	0.6
	8.7 - 28.4	1.9 - 61.8		2.3 - 16.3	2.3 - 39.5		12.2 - 18.9	2.4 - 49.6	
IL10	18.7 (1.14)	21 (1.09)	0.4	22 (1.2)	25 (1.19)	0.6	12.1 (1.6)	17.2 (1.19)	0.5
	5.8 - 90.4	6.1 - 90.1		8.2 - 63.8	5.0 - 97.0		7.5 - 19.5	5.7 - 47.3	
IL12	13.2 (1.12)	15.1 (1.08)	0.3	16.1 (1.2)	21 (1.17)	0.3	6.5 (1.39)	16.4 (1.24)	0.09
	3.3 - 87.6	4 - 92.4		9.9 - 46.2	9.5 - 82.3		4.2 - 12.4	3.8 - 84.4	
INF-γ	59 (1.07)	73.7 (1.06)	**0.04**	94 (1.19)	67.3 (1.2)	0.3	169.9 (2.12)	82.7 (1.14)	0.1
	37.2 - 110.2	31.2 - 173.9		36.1 - 208.1	18.4 - 450.6		54.7 - 710.8	31.1 - 158	
IL8	4197.1 (1.19)	3333.3 (1.09)	0.24	2913.5 (1.1)	2451 (1.26)	0.6	4713.2 (1.36)	3938.3 (1.13)	0.6
	1549.3 - 9483	884 - 9326.9		1988 - 5502.8	48.5 - 6431.9		2750.7 - 7925	1652.6 -7551.8	
RANTES	99.2 (1.46)	70.5 (1.2)	0.3	174.8 (1.86)	167.2 (1.37)	0.9	378.2 (2.27)	98.2 (1.5)	0.2
	3.8 - 1678.7	4.2 - 3569.1		21.4 - 3083.4	16 - 1427.6		90.8 - 1558	6 - 4916.35	

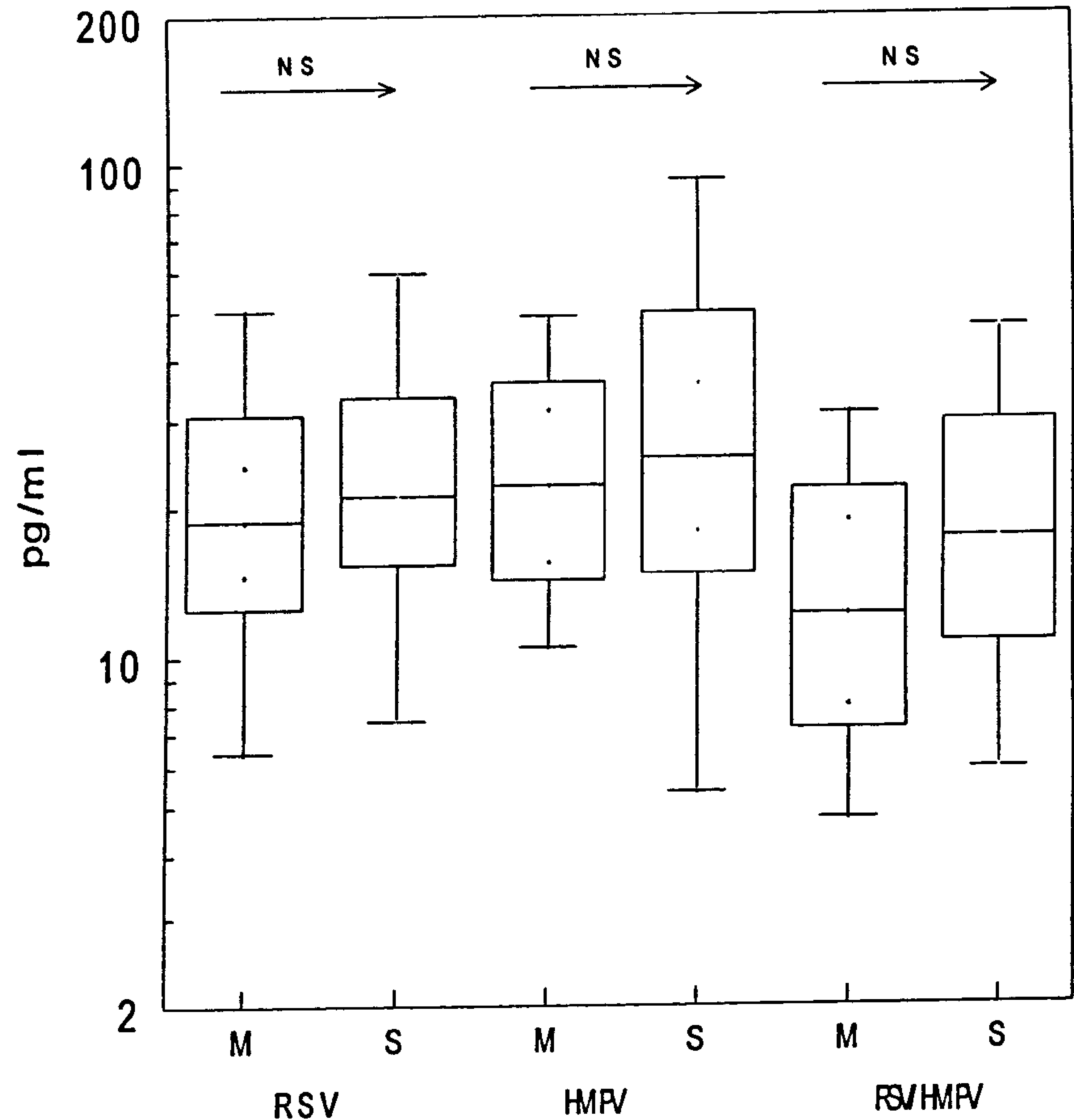
Figure 5.4 Cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV by disease severity (M= mild, S= severe ARI as defined in the text)



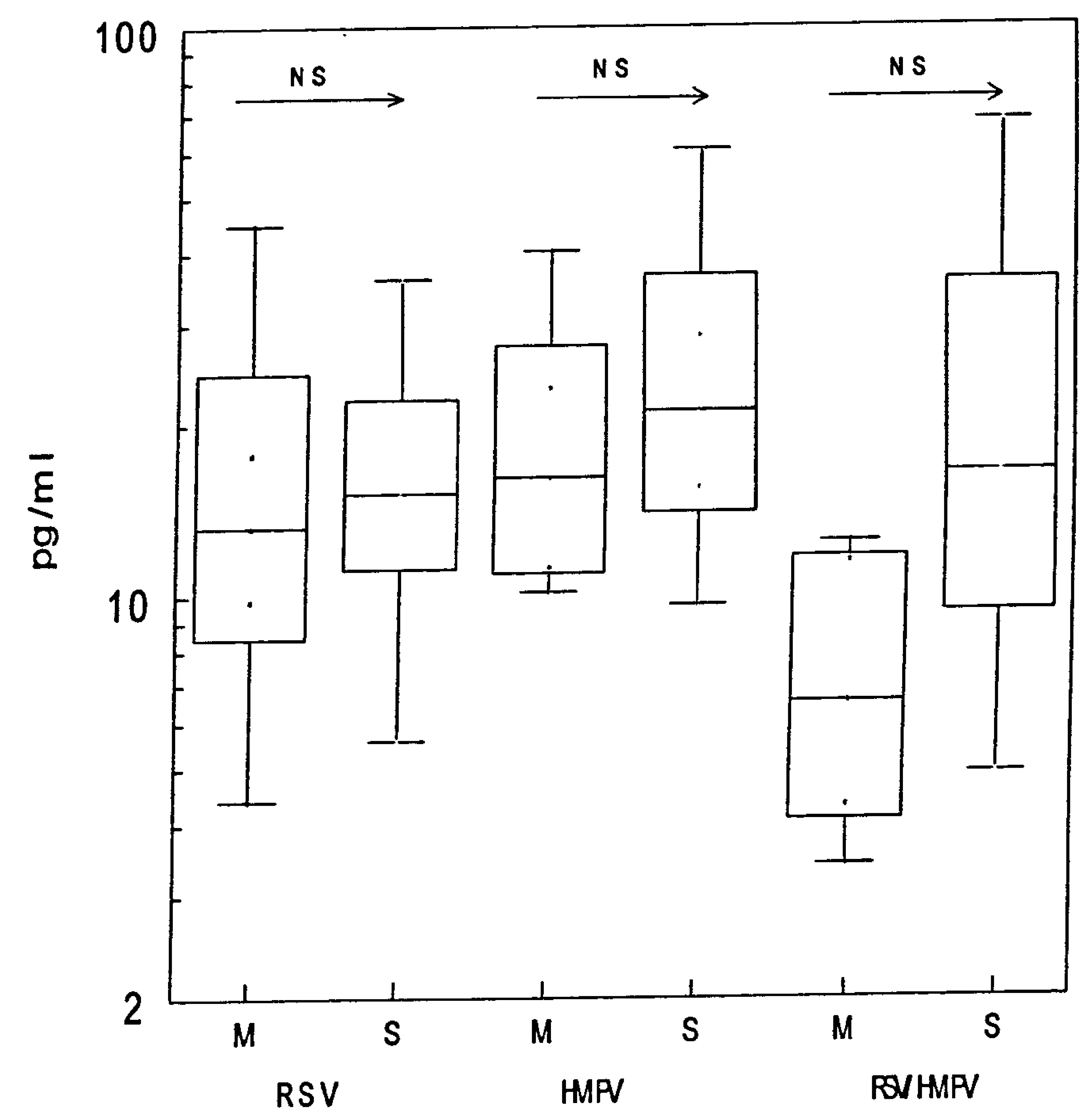
IL7



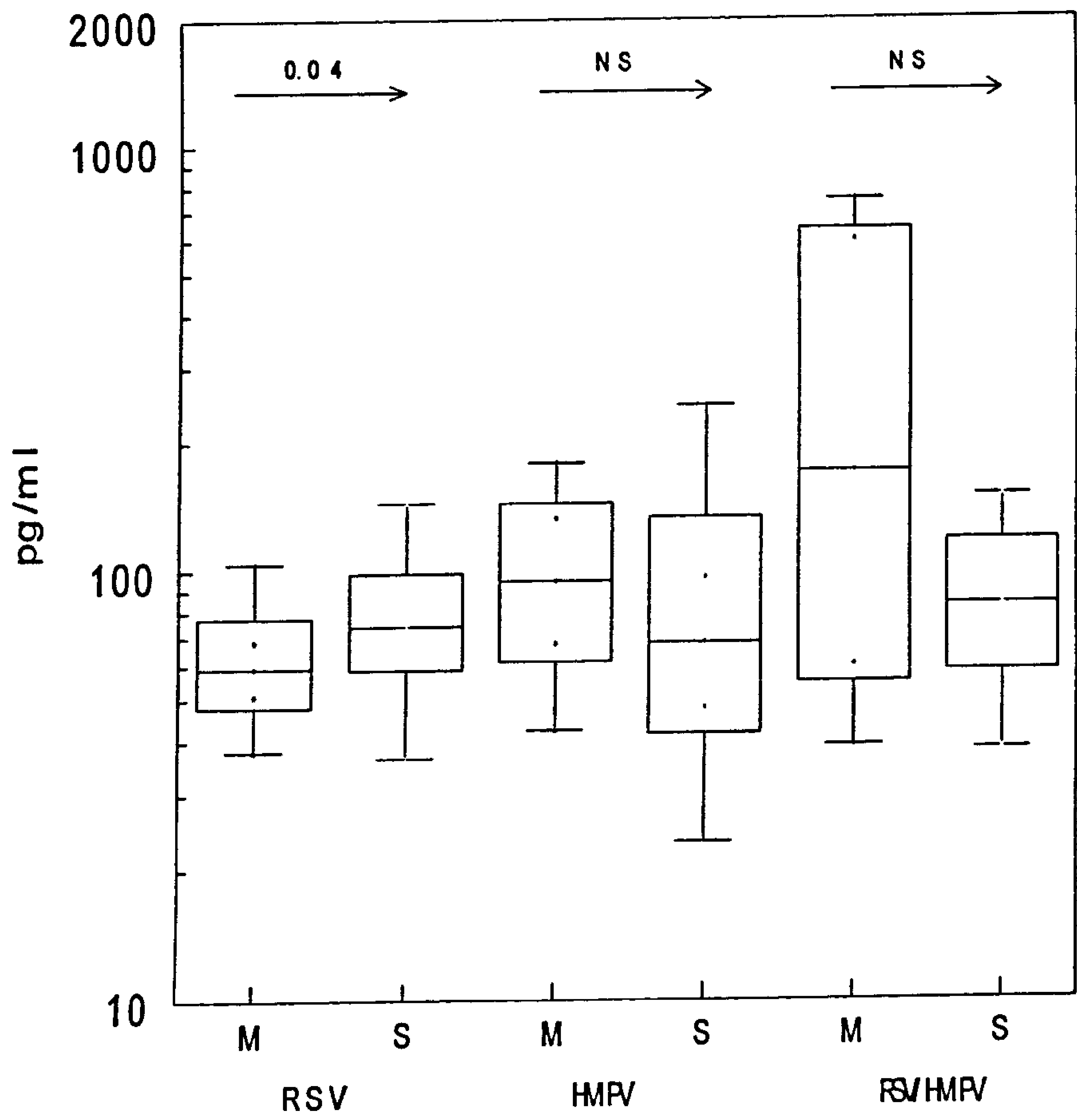
IL10



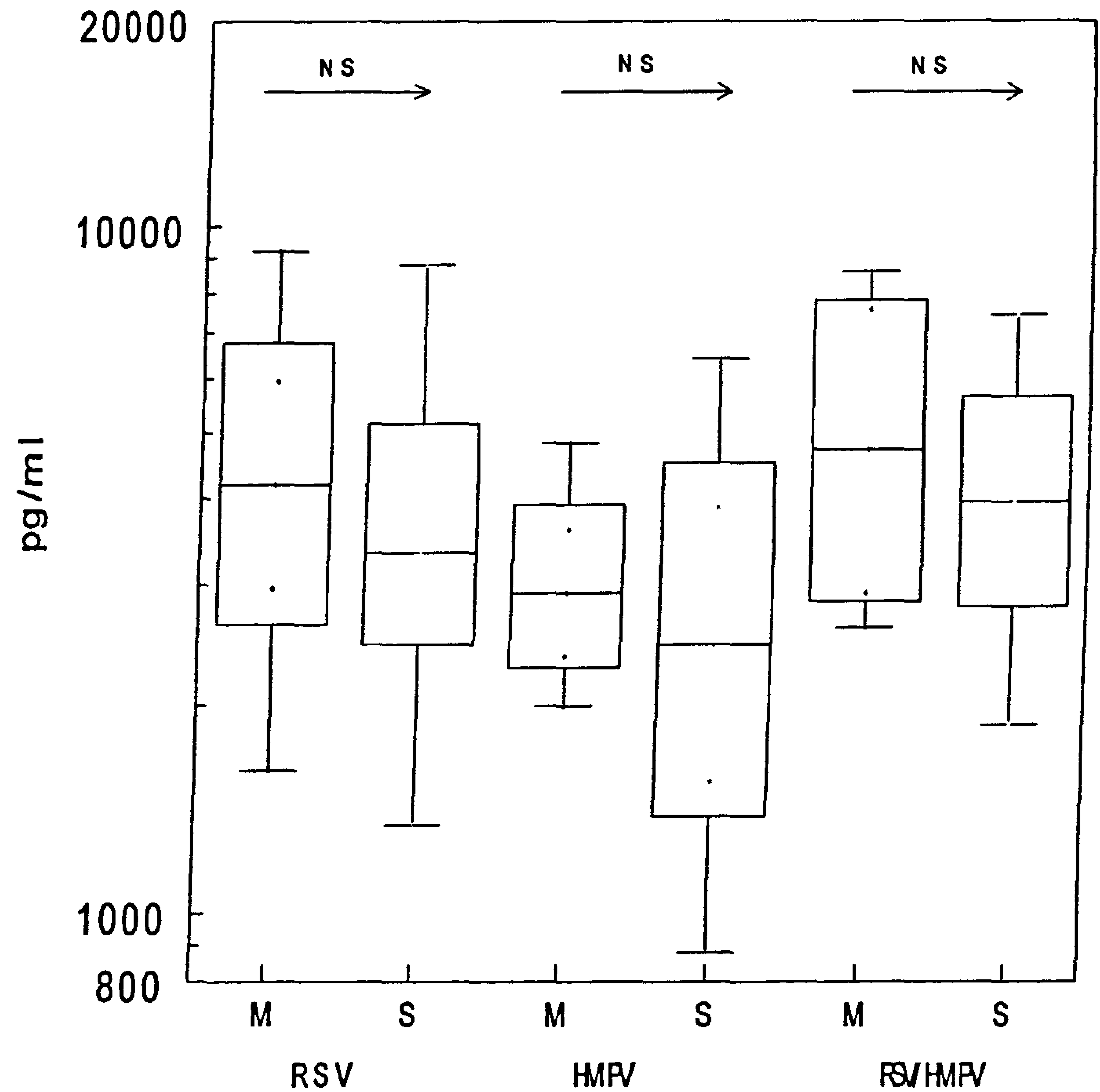
IL12



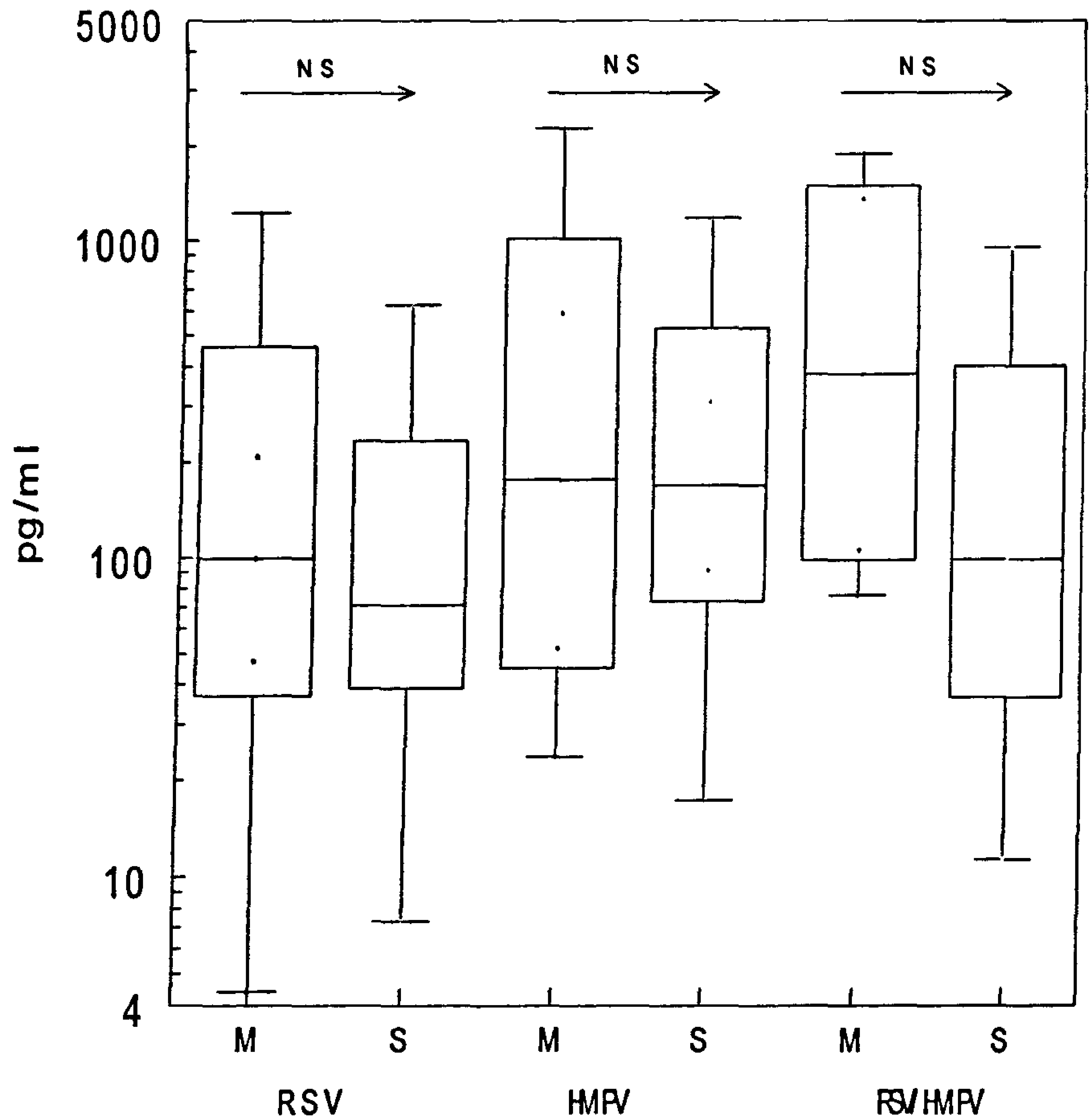
INF-γ



IL8



RANTES



M= mild ARI; S =severe ARI

Cytokine and chemokine concentrations in children with mild ARI due to RSV, HMPV and RSV/HMPV

Cytokine and chemokine concentrations were compared between children with mild ARI due to RSV, HMPV and RSV/HMPV and are described in table 5.6.

IL6 and IL7 concentrations in children with mild ARI were significantly higher in children with RSV than in children with HMPV. TNF-α and INF-γ concentrations however had an inverse pattern and were significantly lower in children with RSV than those with HMPV. There were no significant differences in the IL10, IL12, IL8 and RANTES concentrations among children with a mild presentation associated with RSV and HMPV. The cytokine and chemokine concentrations in children with mild ARI infected with RSV/HMPV had a similar pattern to RSV infections with the exception of INF-γ, which was significantly higher in children with mild RSV/HMPV coinfections.

In contrast, the cytokine and chemokine concentrations in children with mild ARI associated with HMPV were different to those observed in mild and RSV/HMPV. IL7 concentrations were higher in children with RSV/HMPV coinfections, while IL12 and TNF-α were lower. The differences in IL7 and IL12 concentrations were borderline statistically significant, while it was statistically significant for TNF-α concentration. Figure 5.5 illustrates the GM, SE, 25th and 75th centiles of the cytokines and chemokines for the children with mild ARI.

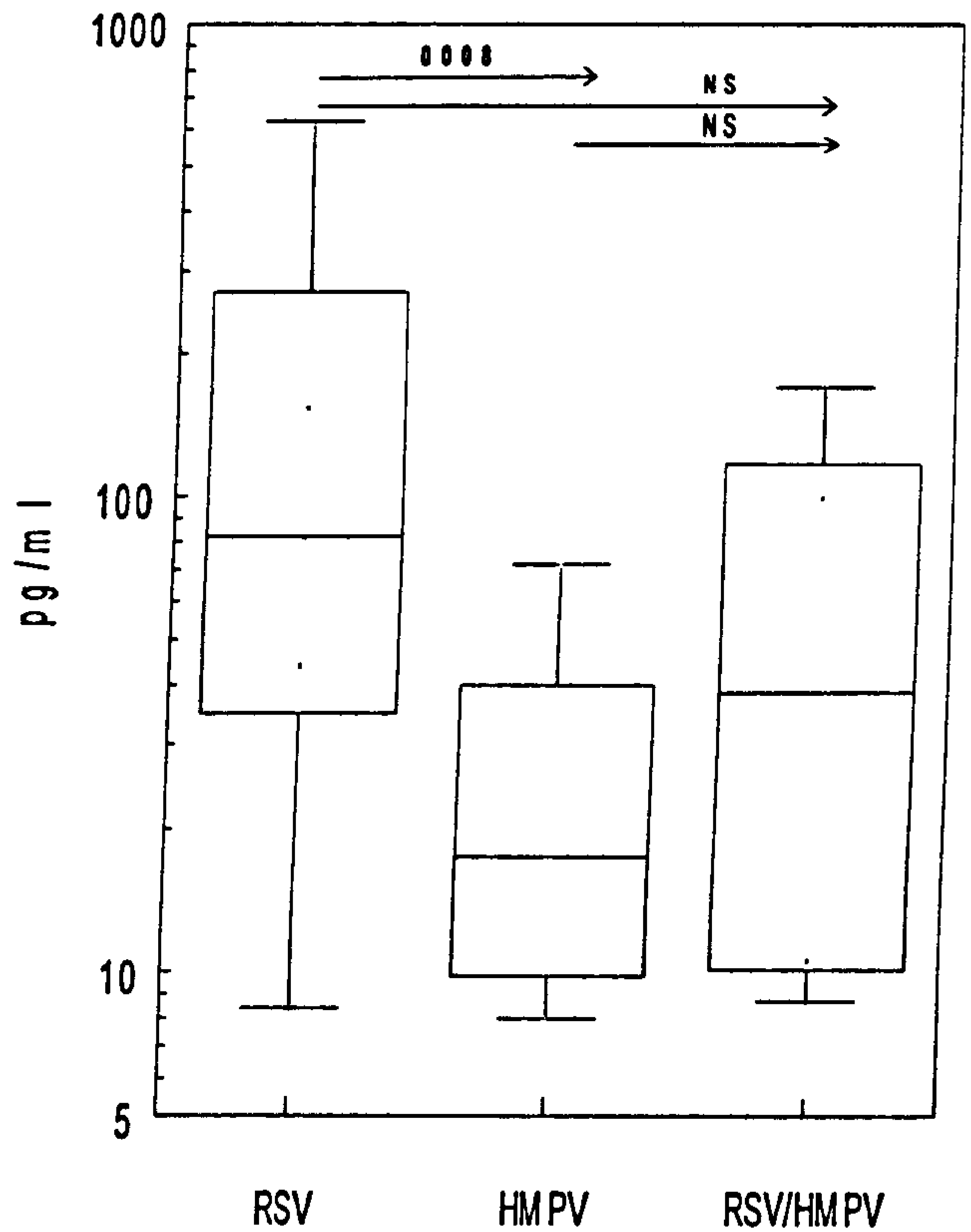
Table 5.6 Cytokine and chemokine concentrations in children with mild ARI due to RSV, HMPV and RSV/HMPV

Cytokine/chemokine	Mild ARI			P^1	P^2	P^3
GM (SE) and range	RSV	HMPV	RSV/HMPV			
IL6	81.8 (1.38)	17.4 (1.3)	38.5 (2.1)	**<0.01**	0.4	0.2
	7.3 - 2713.4	7.6 – 92.9	8.7 - 103.9			
TNF-α	110.2 (1.22)	317.3 (1.36)	61(1.6)	**0.02**	0.4	**0.02**
	16.5 - 1421.8	95 – 1477.3	28.4 - 147			
IL7	14.4 (1.07)	5.5 (1.24)	15.2 (1.2)	**< 0.001**	0.8	**0.05**
	8.7 - 28.4	2.3 – 16.3	12.2 -18.9			
IL10	18.7 (1.14)	22 (1.2)	12.1 (1.6)	0.5	0.4	0.2
	5.8 - 90.4	8.2 – 63.8	7.5 - 19.5			
IL12	13.2 (1.12)	16.1 (1.2)	6.5 (1.39)	0.5	0.1	**0.03**
	3.3 - 87.6	9.9 – 46.2	4.2 - 12.4			
INF-γ	59 (1.07)	94 (1.19)	169.9 (2.12)	**<0.001**	**0.002**	0.3
	37.2 - 110.2	36.1 – 208.1	54.7 - 710.8			
IL8	4197.1 (1.19)	2913.5 (1.1)	4713.2 (1.36)	0.1	0.7	0.08
	1549.3 - 9483	1988 – 5502.8	2750.7 - 7925			
RANTES	99.2 (1.46)	174.8 (1.86)	378.2 (2.27)	0.4	0.2	0.5
	3.8 - 1678.7	21.4 – 3083.4	90.8 - 1558			

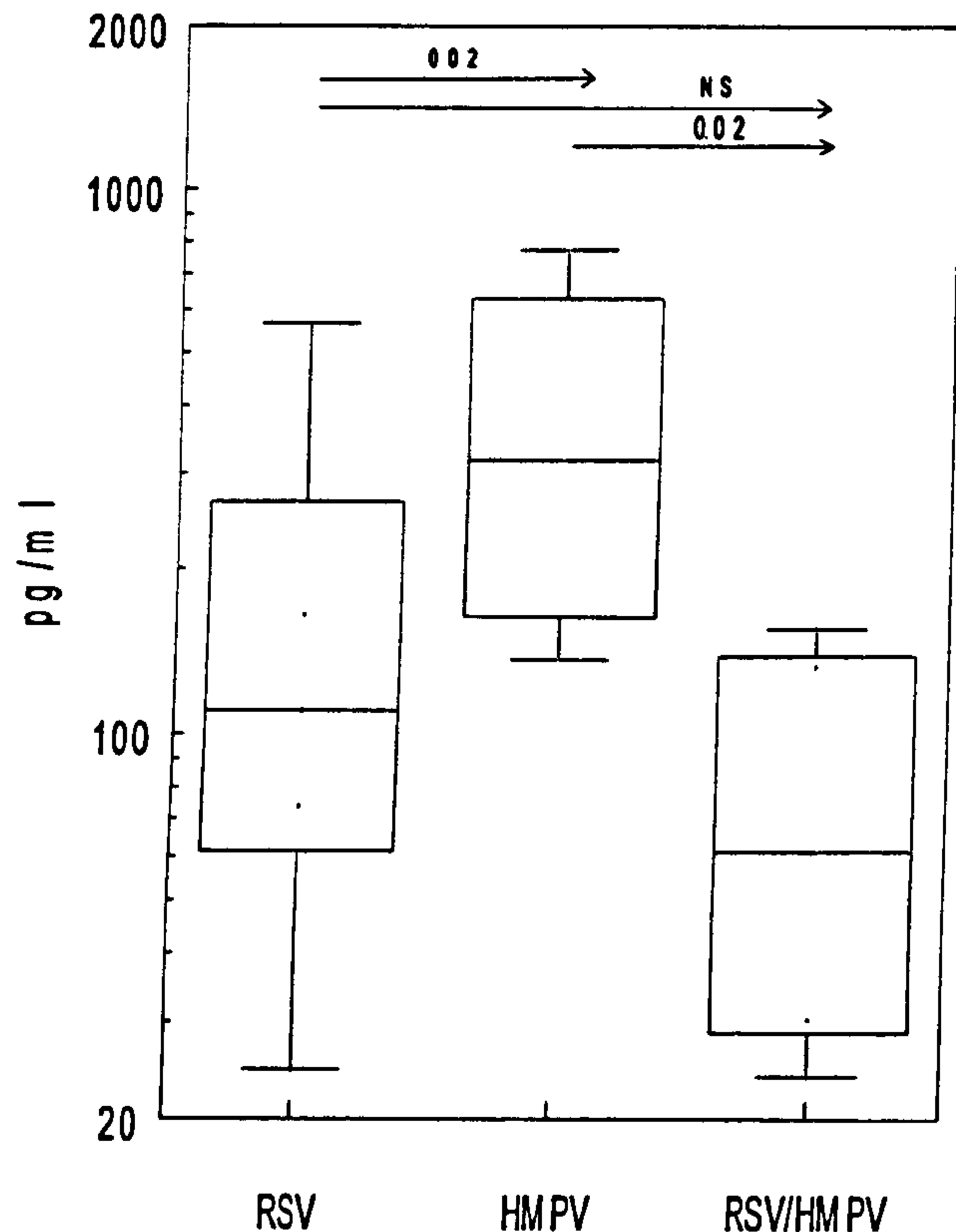
P^1= RSV vs. HMPV; P^2 = RSV vs. RSV/HMPV, P^3 = HMPV vs. RSV/HMPV

Figure 5.5 Cytokine and chemokine concentrations in children with mild ARI associated with RSV, HMPV and RSV/HMPV infections

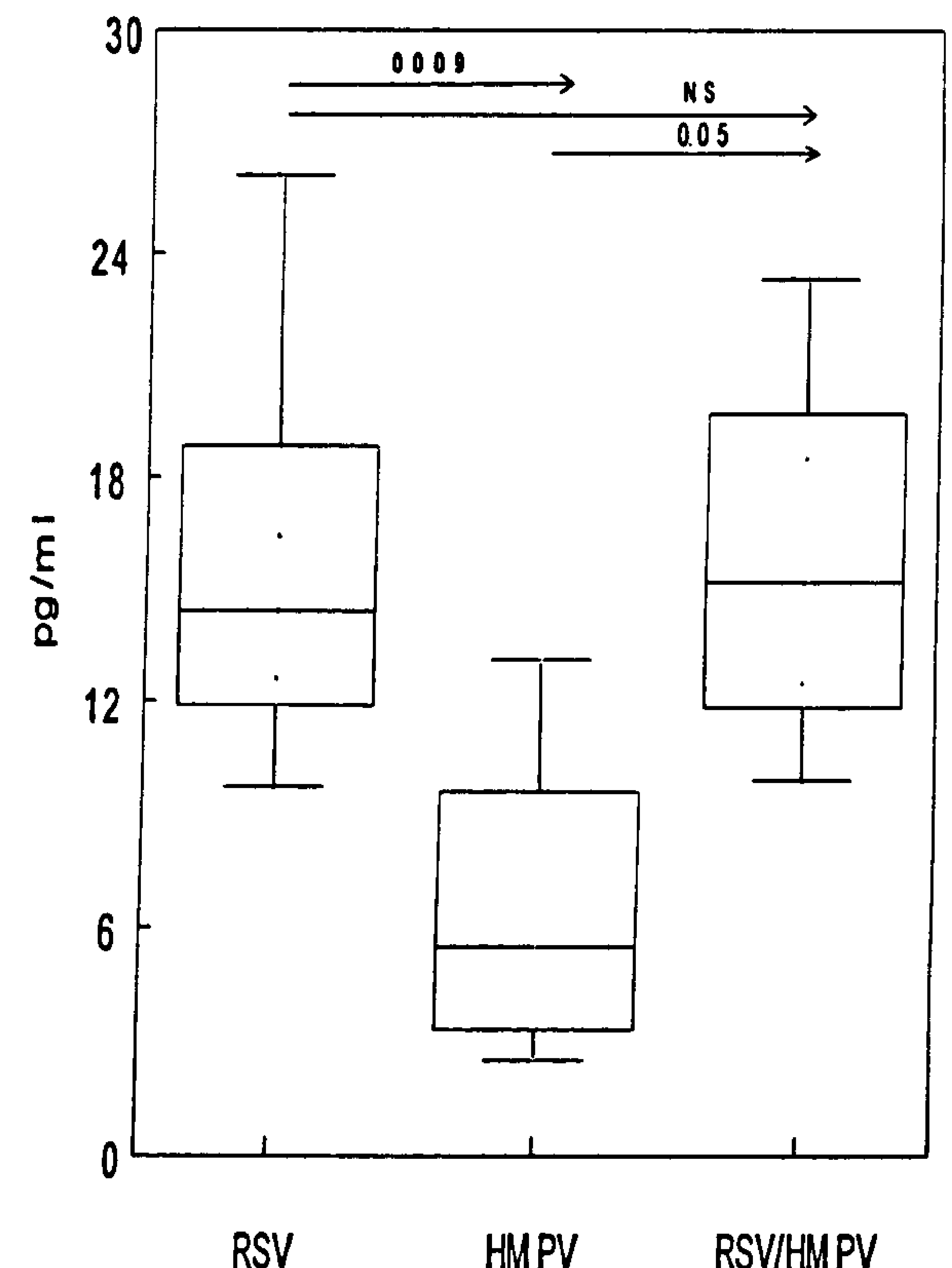
IL6



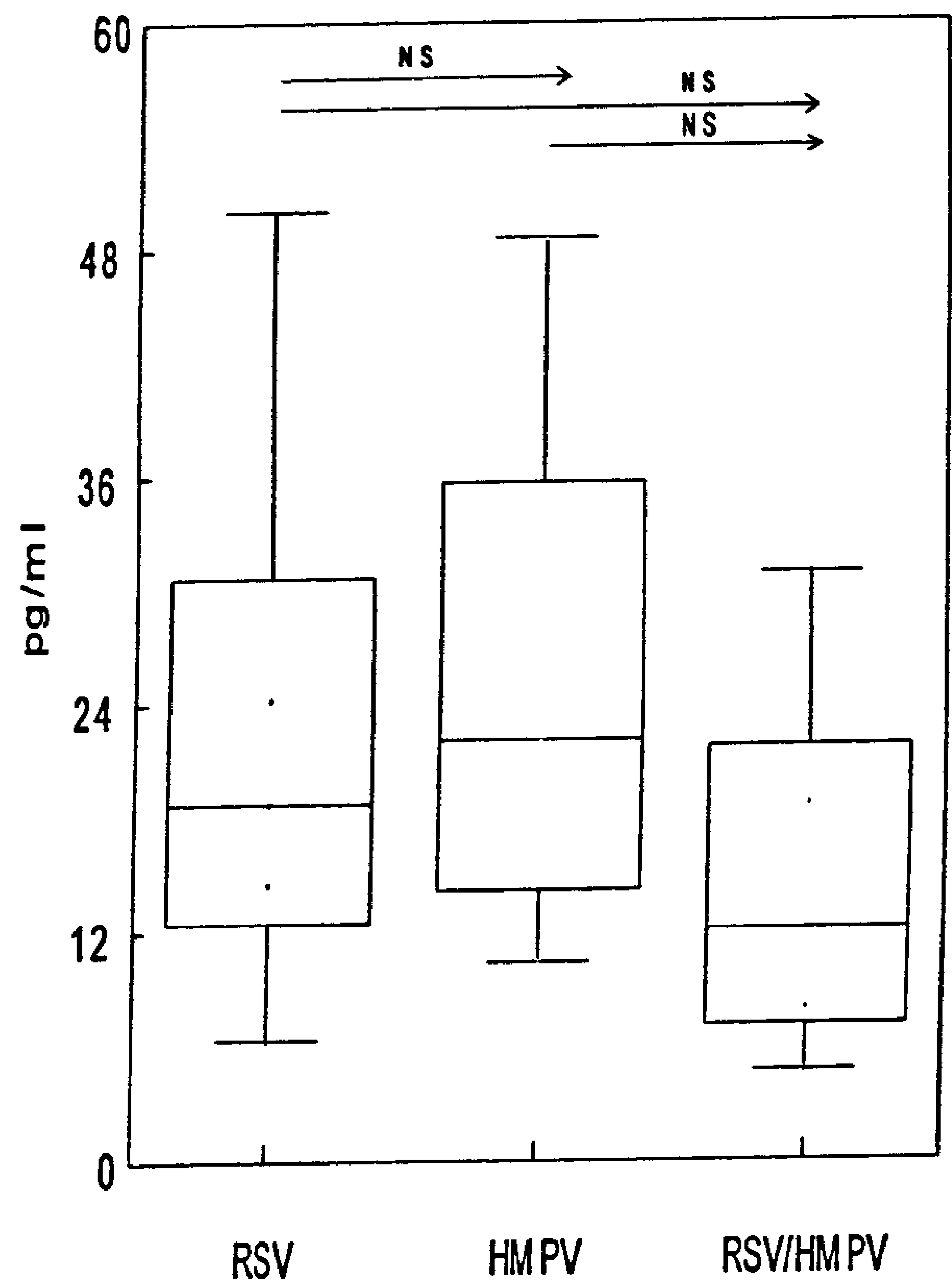
TNF-α



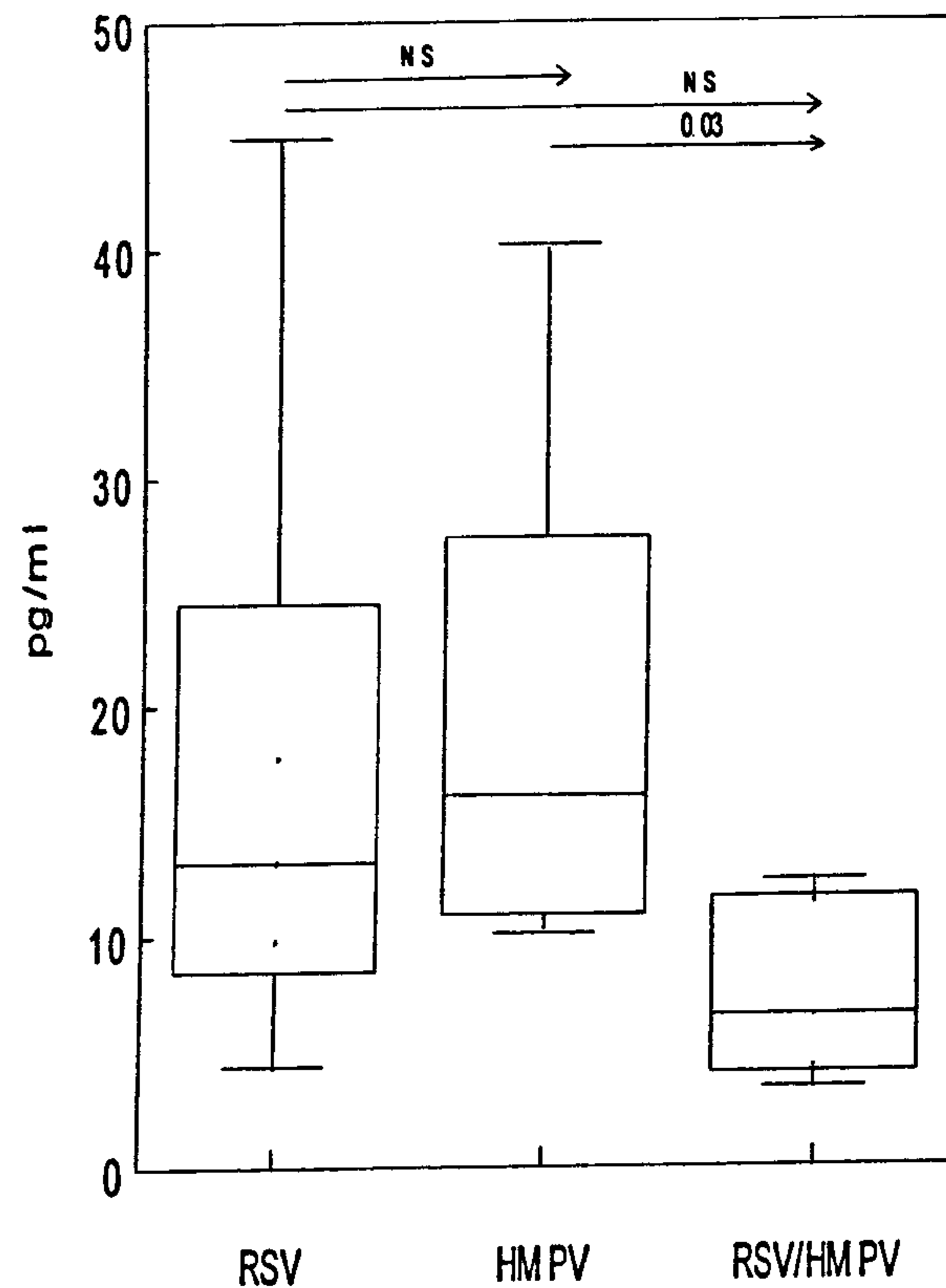
IL7



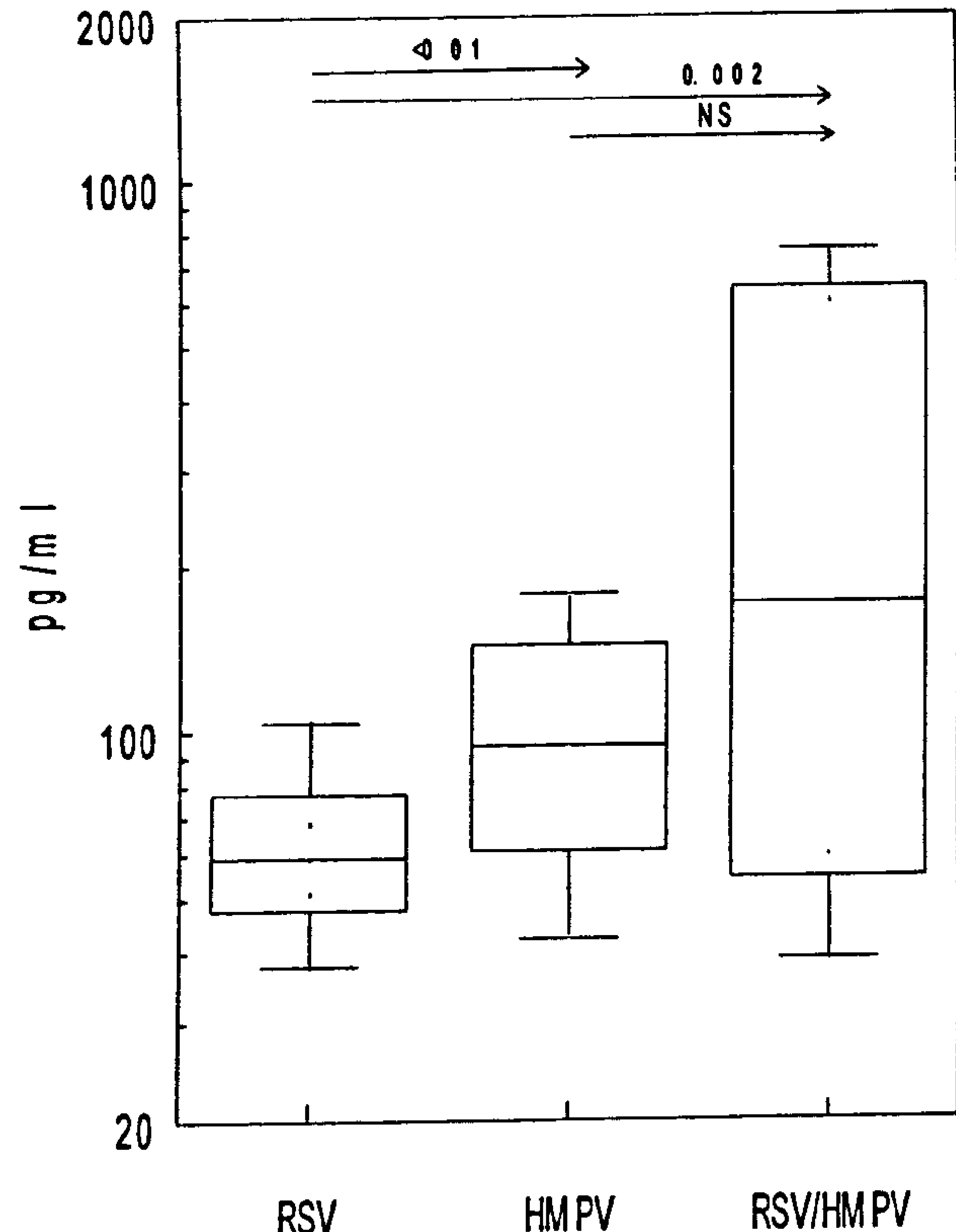
IL10



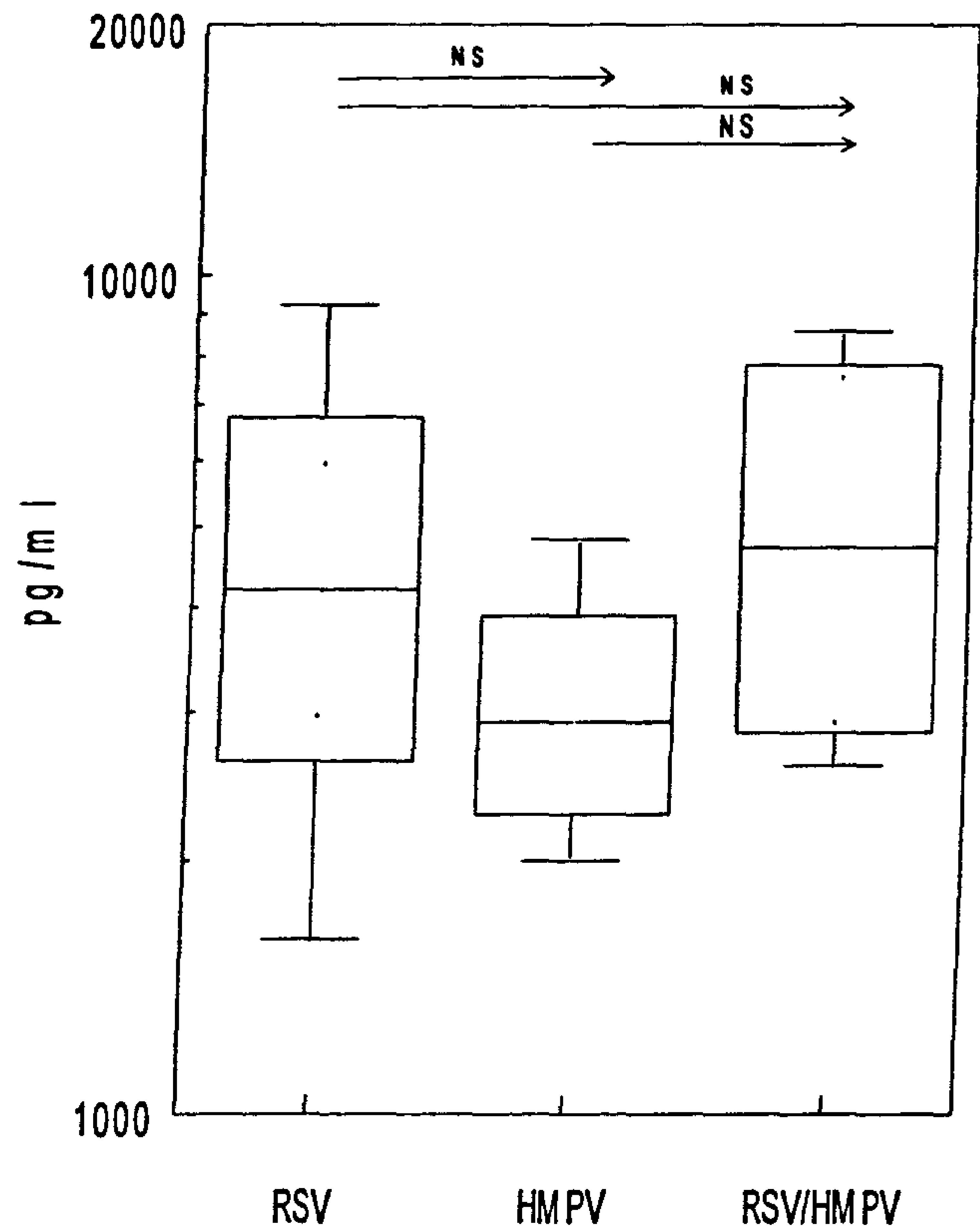
IL12



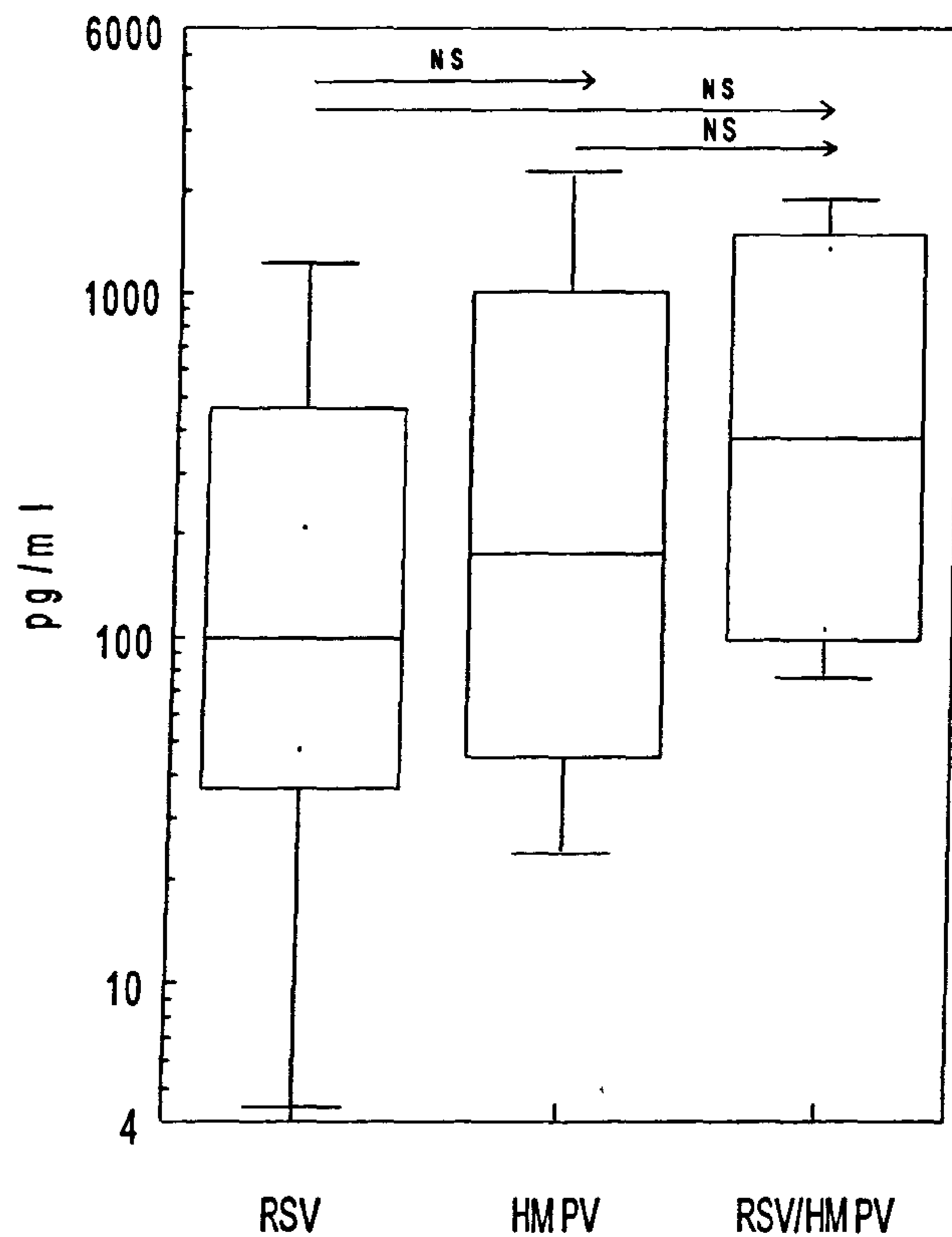
INF-γ



IL8



RANTES



Cytokine and chemokine concentrations in children with severe ARI due to RSV, HMPV and RSV/HMPV

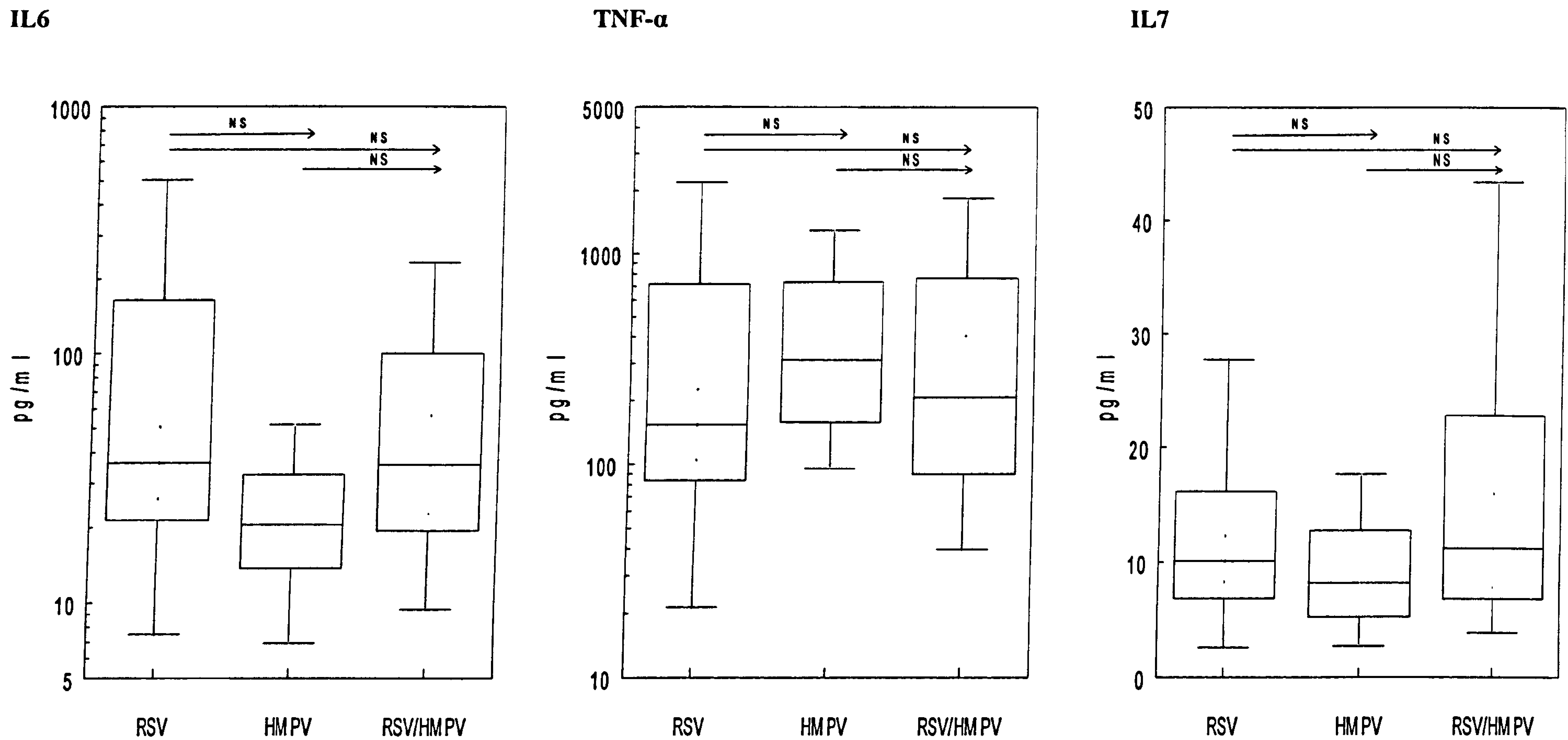
Table 5.7 describes the cytokine and chemokine concentrations of children with severe ARI associated with RSV, HMPV and RSV/HMPV. There were no significant differences in the cytokine and chemokine concentrations of children with severe ARI associated with RSV and HMPV with the exception of RANTES. This chemokine was significantly lower in children with RSV than in children with HMPV. Similarly, there were no significant differences between the cytokine and chemokine concentrations of children with severe ARI associated with RSV and RSV/HMPV or between HMPV and RSV/HMPV as shown in figure 5.6.

Table 5.7 Cytokine and chemokine concentrations in children with severe ARI associated with RSV, HMPV and RSV/HMPV

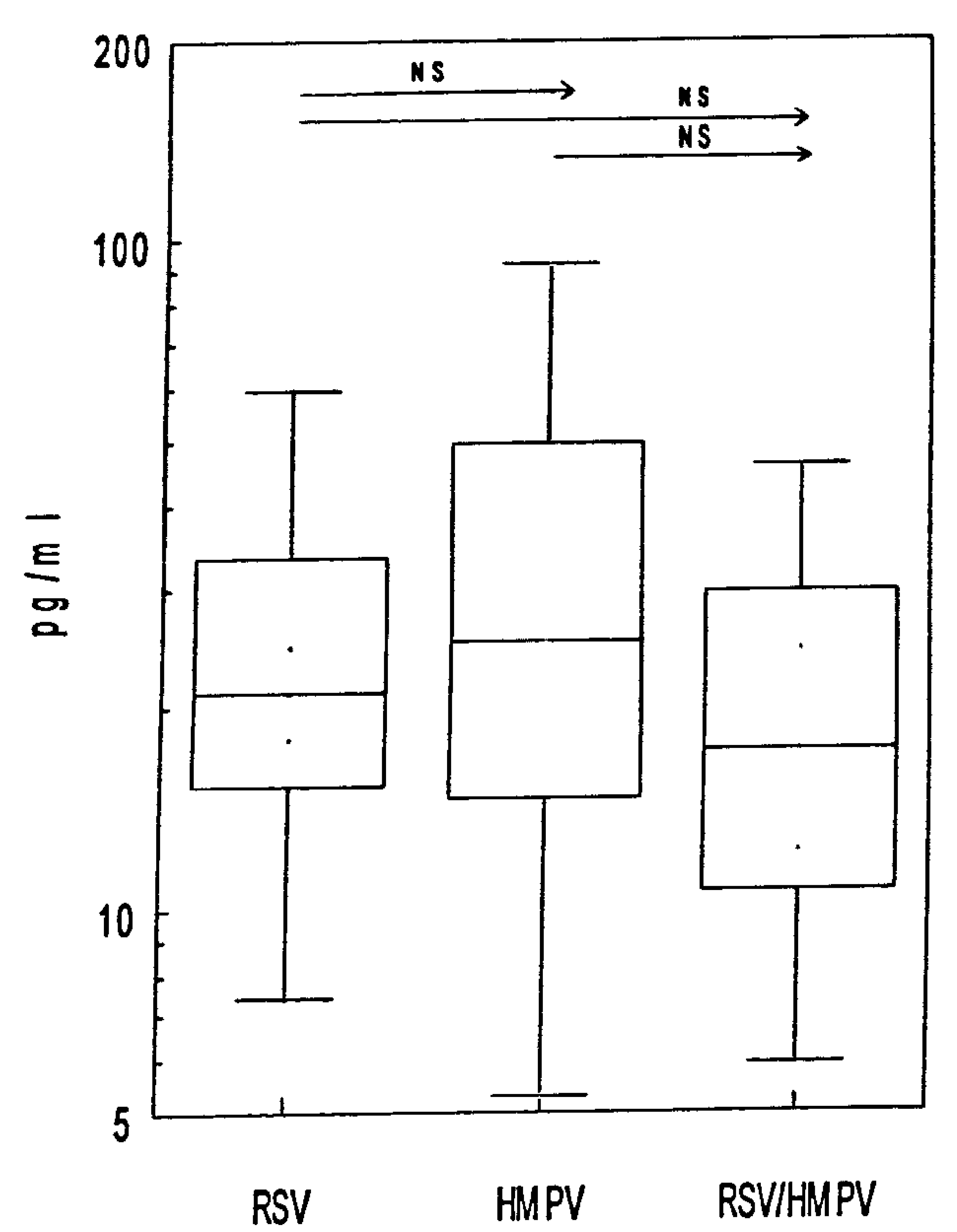
Cytokine/chemokine	Severe ARI			P[1]	P[2]	P[3]
GM (SE) and range	RSV	HMPV	RSV/HMPV			
IL6	36.2 (1.19)	20.5 (1.13)	35.5 (1.3)	0.09	0.9	0.06
	5.7 – 2488	6.7 - 66	7 - 299			
TNF-α	153.7 (1.2)	311.9 (1.32)	207.5(1.4)	0.09	0.4	0.4
	8.3 - 4196.6	95 - 1477.3	27 - 3269.6			
IL7	10.1 (1.11)	8.2 (1.16)	11.2 (1.2)	0.3	0.5	0.2
	1.9 - 61.8	2.3 - 39.5	2.4 - 49.6			
IL10	21 (1.09)	25 (1.19)	17.2 (1.19)	0.3	0.3	0.1
	6.1 - 90.1	5.0 - 97.0	5.7 - 47.3			
IL12	15.1 (1.08)	21 (1.17)	16.4 (1.24)	**0.04**	0.6	0.3
	4 - 92.4	9.5 - 82.3	3.8 - 84.4			
INF-γ	73.7 (1.06)	67.3 (1.2)	82.7 (1.14)	0.5	0.4	0.4
	31.2 - 173.9	18.4 - 450.6	31.1 - 158			
IL8	3333.3 (1.09)	2451 (1.26)	3938.3 (1.13)	0.1	0.3	0.1
	884 - 9326.9	48.5 - 6431.9	1652.6 - 7551.8			
RANTES	70.5 (1.2)	167.2 (1.37)	98.2 (1.5)	**0.02**	0.4	0.3
	4.2 – 3569.1	16 – 1427.6	6 – 4916.4			

P[1]= RSV vs. HMPV; P[2] = RSV vs. RSV/HMPV, P[3] = HMPV vs. RSV/HMPV

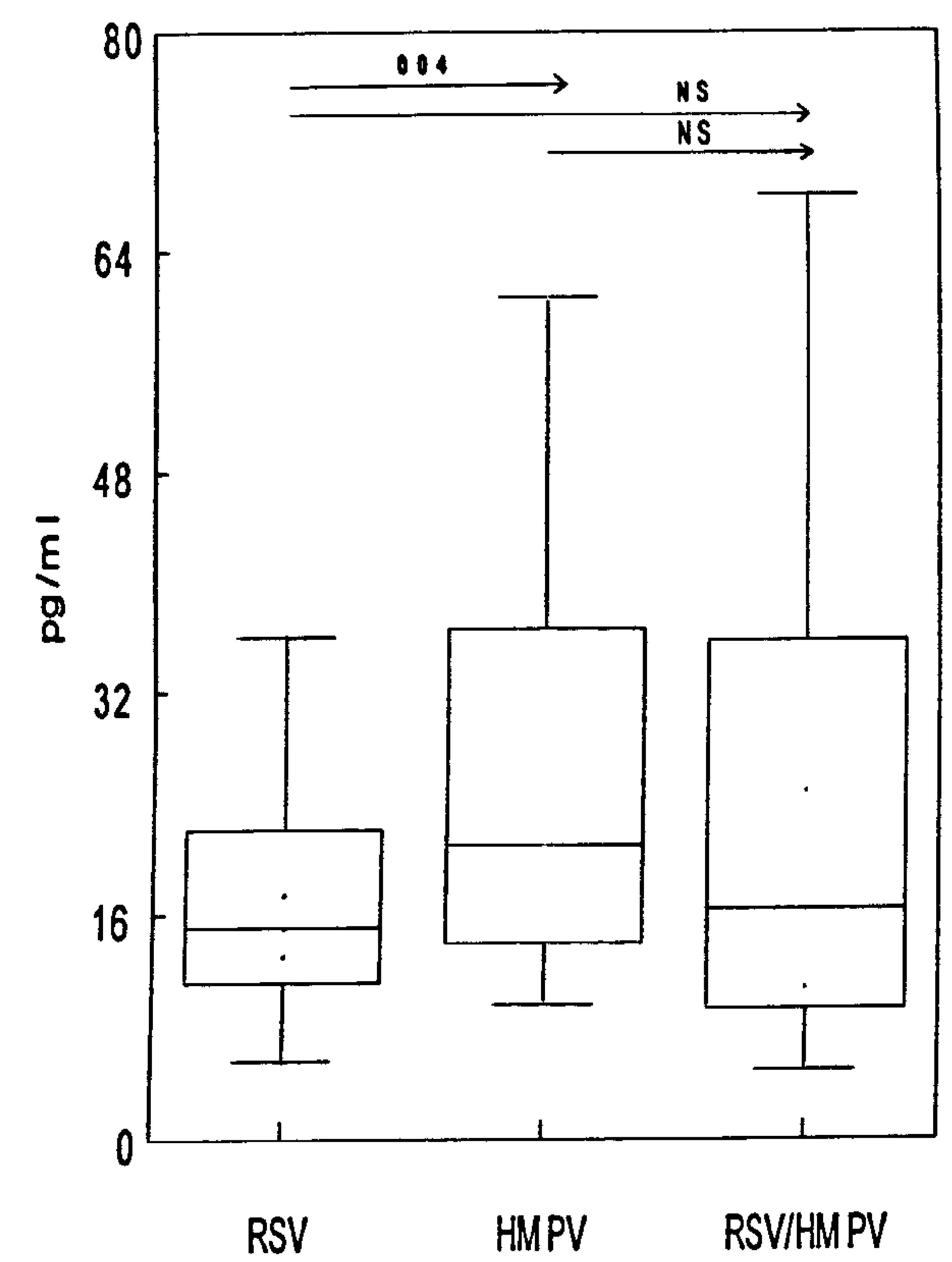
Figure 5.6 Cytokine and chemokine concentrations in children with severe ARI associated with RSV, HMPV and RSV/HMPV infections



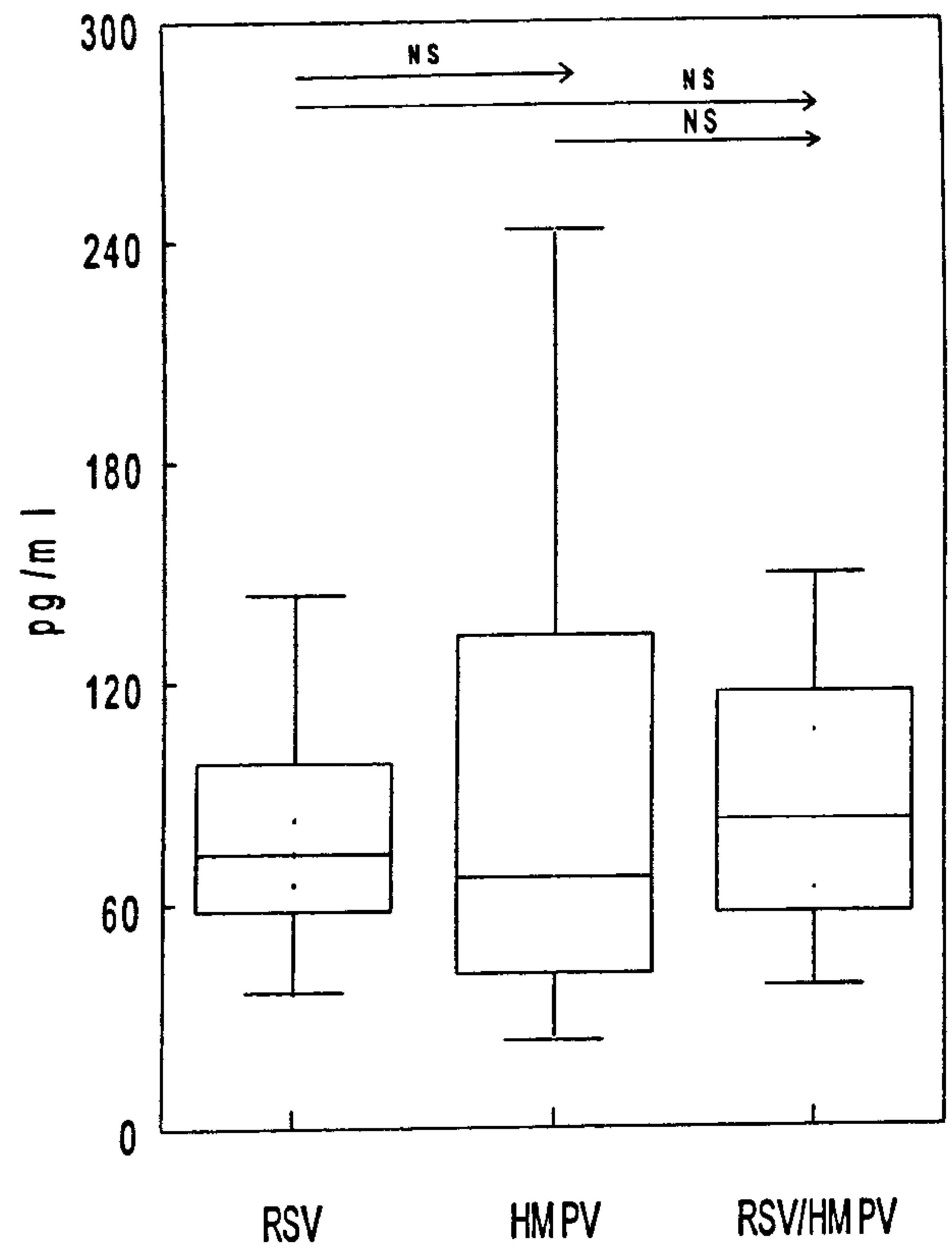
IL10



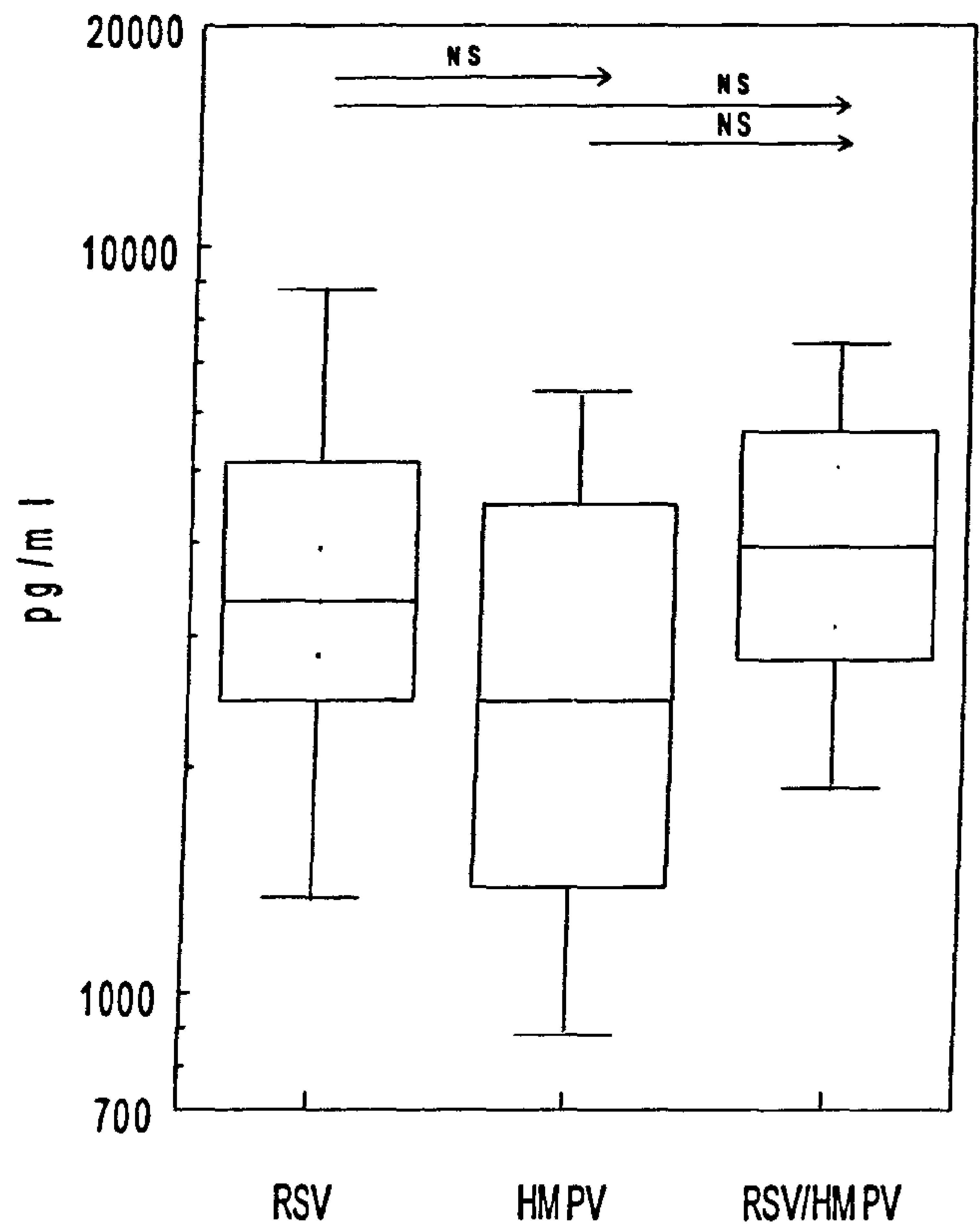
IL12



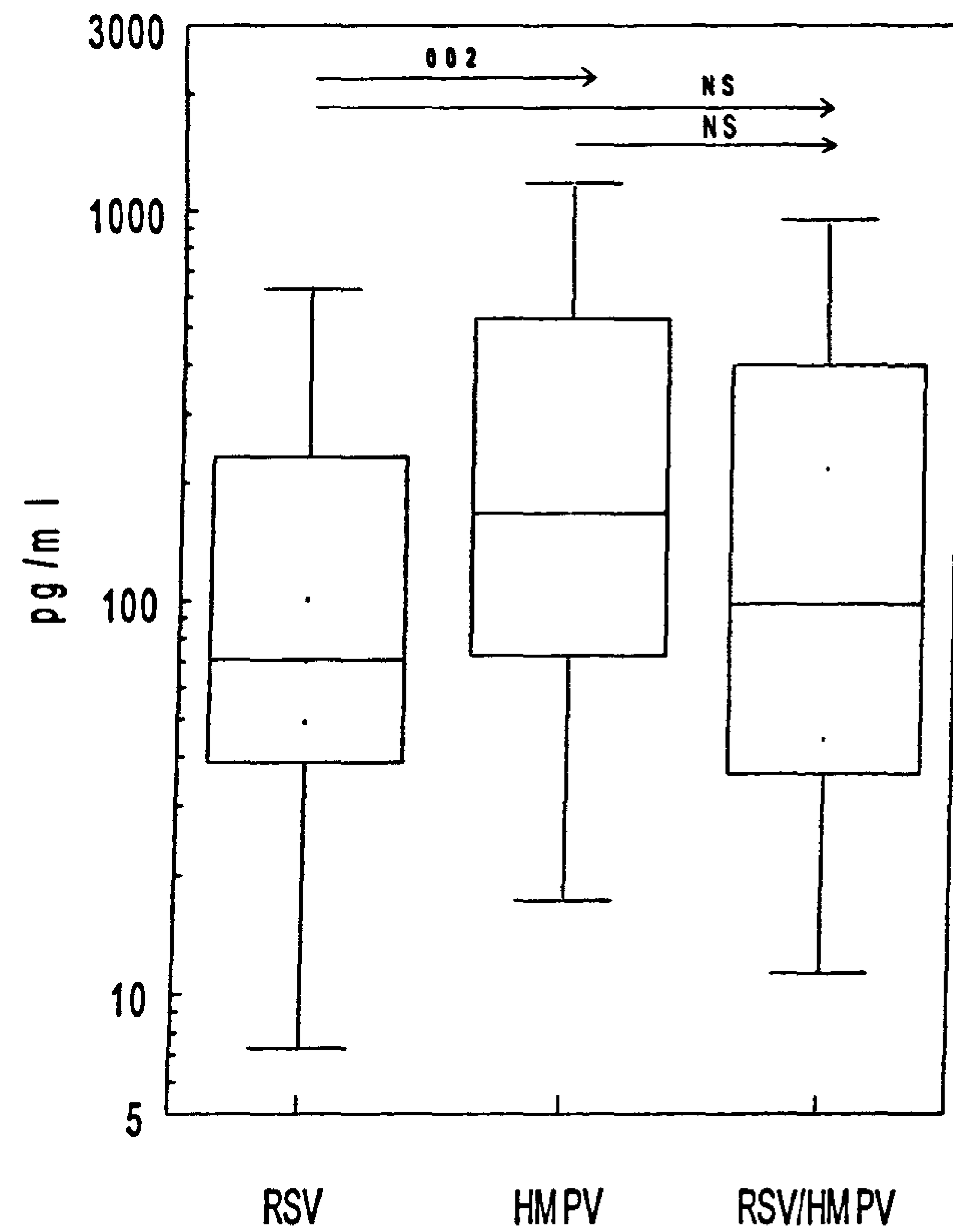
INF-γ



IL8



RANTES



Cytokine and chemokine concentrations by age

The cytokine and chemokine concentrations were also analyzed by age. A cut off point of 3 months was selected because children ≤ 3 months old were found to be at greater risk of developing severe disease than older children. As shown in chapter 3, HMPV was also more frequently isolated in children above this age, while RSV was more frequently isolated in children < 6 months old. Table 5.8 describes the cytokine and chemokine concentrations in children by age.

In children with RSV infection, IL7 concentrations were significantly higher in children > 3 months old than in children below this age. IL10 concentrations however were significantly lower in children > 3 months old than in children ≤ 3 months of age. On the other hand, age did not modify the cytokine and chemokine concentrations in children with HMPV as these were similar above or below 3 months of age.

Similarly, in children with RSV/HMPV coinfections, all cytokine and chemokine concentrations were similar above and below 3 months of age with the exception of IL12 which showed higher concentrations in children < 3 months of age.

Table 5.8 Cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV coinfections by age

Cytokine/chemokine GM (SE) and range	RSV		P	HMPV		P	RSV/HMPV		P
	≤3 m	>3 m		≤3 m	>3 m		≤3 m	>3 m	
IL6	37.4 (1.22)	63.5 (1.28)	0.1	20.7 (1.23)	20.1 (1.16)	0.9	39.6 (1.37)	32.8 (1.37)	0.6
	5.7 - 2488	7.3 - 2713.4		6.7 - 66	6.7 - 92.9		10.7 - 299	7 - 299.4	
TNF-α	144.2 (1.26)	125.5 (1.21)	0.7	250 (2.07)	327.2 (1.25)	0.6	154.9 (1.65)	176.3 (1.52)	0.8
	8.3 - 4196.6	20.2 - 2805.1		95 - 1036.3	95.9 - 1477.3		27 - 1342	28.4 - 3269.6	
IL7	9.5 (1.12)	14.1 (1.09)	**0.01**	6.8 (1.26)	7.6 (1.17)	0.6	12.2 (1.16)	10.7 (1.39)	0.7
	1.9 - 26.2	3 - 61.8		2.3 -15.4	2.7 - 39.5		4.2 - 24.8	2.4 - 49.6	
IL10	23.4 (1.1)	17.1 (1.11)	**0.02**	19.9 (1.27)	26.4 (1.17)	0.3	19.1 (1.23)	13.5 (1.3)	0.3
	6 - 90.1	5.8 - 90.4		5 - 72.7	5 - 97		6 - 47.3	5.7 - 31.6	
IL12	15.4 (1.09)	13.8 (1.12)	0.4	17.9 (1.19)	19.7 (1.17)	0.7	20.8 (1.36)	9.8 (1.23)	**0.05**
	4 - 92.4	3.3 - 87.6		12.2 - 14.8	9.5 - 82.3		5.2 - 84.4	3.8 - 25.5	
INF-γ	73.6 (1.08)	65.8 (1.07)	0.2	59.1 (1.22)	83.5 (1.19)	0.2	80.5 (1.18)	111.9 (1.36)	0.3
	31.2 - 173.9	37.7 - 123.9		23.2 - 128	18.4 - 450.6		31.1 - 158	41.1 - 710.8	
IL8	3267.6 (1.09)	3983.5 (1.14)	0.2	2002.8 (1.57)	2958.4 (1.08)	0.2	4340.1 (1.14)	3845.5 (1.12)	0.6
	1186.1 - 9840	1549.3 -9894.9		48.5 - 6431.9	1746.4 -5666.8		2495.2 -7339.7	1652.6 - 7925	
RANTES	68.8 (1.25)	94.3 (1.32)	0.4	204.6 (1.61)	155.7 (1.43)	0.6	61.3 (1.39)	208.6 (1.8)	0.1
	4.2 - 1678.7	3.8 - 3569.1		17.3 - 1004	16 - 3083.4		13.3 - 295.9	6 - 4916.4	

Cytokine and chemokine concentrations in children ≤ 3 months old with RSV, HMPV and RSV/HMPV co-infection

Table 5.9 compares the cytokine and chemokine concentrations of children ≤ 3 months old with ARI due to RSV, HMPV or RSV/HMPV. IL7 concentrations were borderline statistically significantly higher in children with RSV than in children with RSV/HMPV infections, and the latter group had higher IL7 concentrations than children with HMPV alone ($p=0.04$ and $p=0.05$ respectively). RANTES concentrations were higher in children with RSV than in children with RSV/HMPV, however children with HMPV had higher concentrations than children with RSV or RSV/HMPV coinfections ($p=0.05$ for both) as shown in figure 5.7. Children ≤ 3 months of age with RSV had a wider range of values for all the cytokines and chemokines.

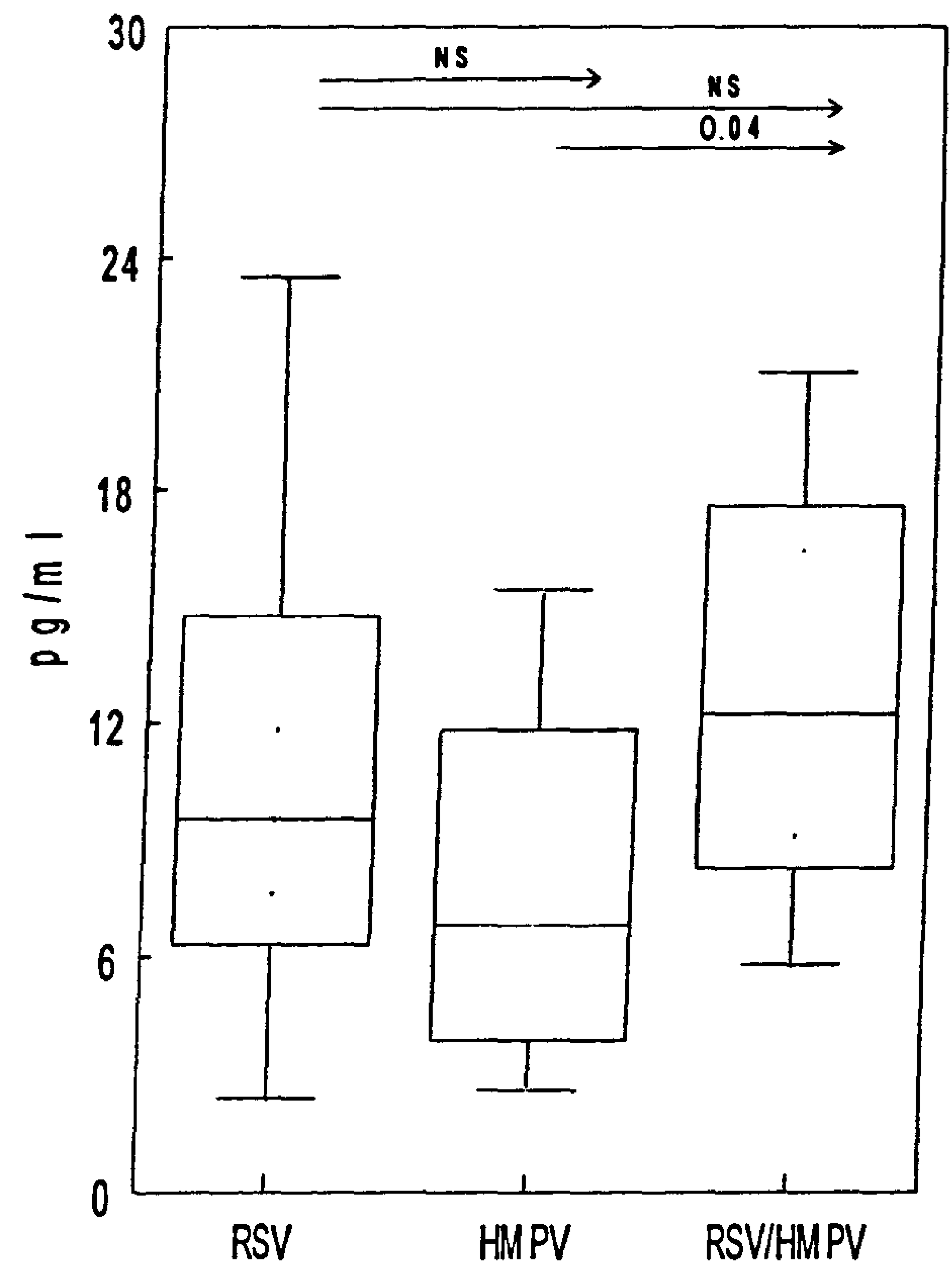
Table 5.9 Cytokine and chemokine concentrations in children ≤ 3 months old with RSV, HMPV and RSV/HMPV

Cytokine/chemokine GM (SE) and range	RSV	HMPV	RSV/HMPV	P^1	P^2	P^3
IL6	37.4 (1.22)	20.7 (1.23)	39.6 (1.37)	0.2	0.9	0.1
	5.7 – 2488	6.7 – 66	10.7 – 299			
TNF-α	144.2 (1.26)	250 (2.07)	154.9 (1.65)	0.5	0.9	0.6
	8.3 – 4196.6	95 -1036.3	27 – 1342			
IL7	9.5 (1.12)	6.8 (1.26)	12.2 (1.16)	0.2	0.3	**0.04**
	1.9 - 26.2	2.3 -15.4	4.2 - 24.8			
IL10	23.4 (1.1)	19.9 (1.27)	19.1 (1.23)	0.5	0.3	0.9
	6 - 90.1	5 - 72.7	6 - 47.3			
IL12	15.4 (1.09)	17.9 (1.19)	20.8 (1.36)	0.5	0.2	0.7
	4 - 92.4	12.2 - 14.8	5.2 - 84.4			
INF-γ	73.6 (1.08)	59.1 (1.22)	80.5 (1.18)	0.2	0.6	0.2
	31.2 - 173.9	23.2 – 128	31.1 – 158			
IL8	3267.6 (1.09)	2002.8 (1.57)	4340.1 (1.14)	0.08	0.2	0.2
	1186.1 – 9840	48.5 - 6431.9	2495.2 - 7339.7			
RANTES	68.8	204.6 (1.61)	61.3 (1.39)	**0.05**	0.8	**0.05**
	4.2 - 1678.7	17.3 – 1004	13.3 - 295.9			

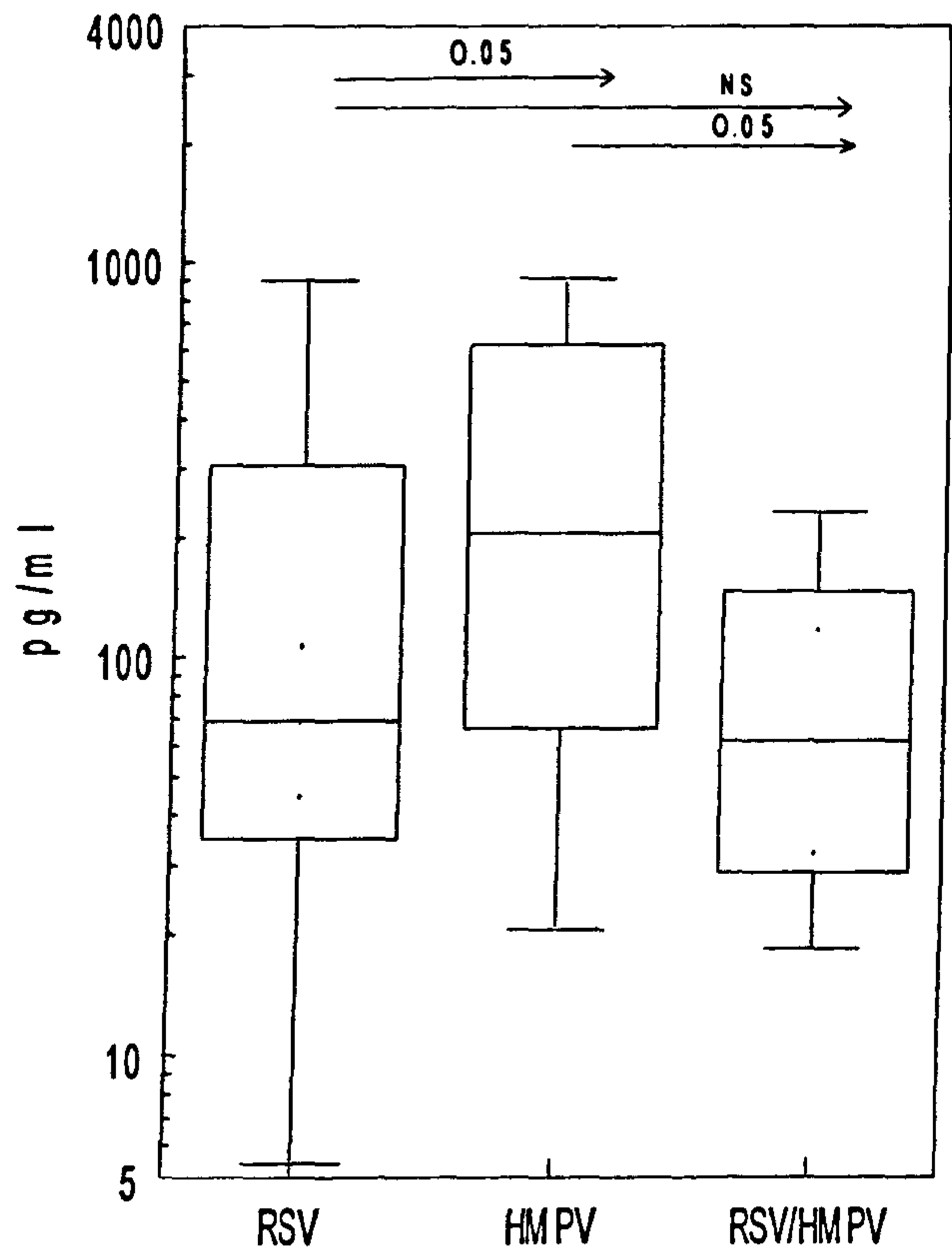
P^1= RSV vs. HMPV, P^2= RSV vs. RSV/HMPV and P^3= HMPV vs. RSV/HMPV

Figure 5.7 Cytokine and chemokine concentrations in children ≤ 3 months of age with RSV, HMPV and RSV/HMPV

IL7



RANTES



Cytokine and chemokine concentrations in children > 3 months old with RSV, HMPV and RSV/HMPV co-infection

Table 5.10 describes the cytokine and chemokine concentrations in children > 3 months old with RSV, HMPV and RSV/HMPV co-infection.

IL6, IL7 and IL8 concentrations were higher and IL10 and TNF-α concentrations were lower in children with RSV than in children with HMPV. INF-γ concentrations were lower in children with RSV than in children with RSV/HMPV coinfections. IL10 concentrations were borderline statistically significantly higher in children with HMPV than in children with RSV/HMPV ($p<0.05$ for all) as shown in figure 5.8.

Cytokine and chemokine concentrations by gender in children with RSV, HMPV and RSV/HMPV

To examine the association between gender and the immunological responses to RSV and HMPV infections, the levels of the cytokines and chemokines are described by gender in table 5.11. All the cytokine and chemokine concentrations were similar in males and females. Gender was not associated with differences in innate immune responses among the children

There are several reports of the ratios of cytokines and chemokines as markers of disease severity. These include IL6 /TNF-α ratio, among others (Hornsleth et al., 1998). However, IL6/TNF-α ratio and multiple permutations of the cytokines and chemokines did not reveal correlations that were statistically significant in this study. A few correlations were marginally significant (e.g. IL12/IL10, INF-γ/ IL12) as shown in appendix 7. The linear regression of these values however had large residuals and were not considered suitable for further analysis.

Table 5.10 Cytokine and chemokine concentrations in children > 3 months old with RSV, HMPV and RSV/HMPV

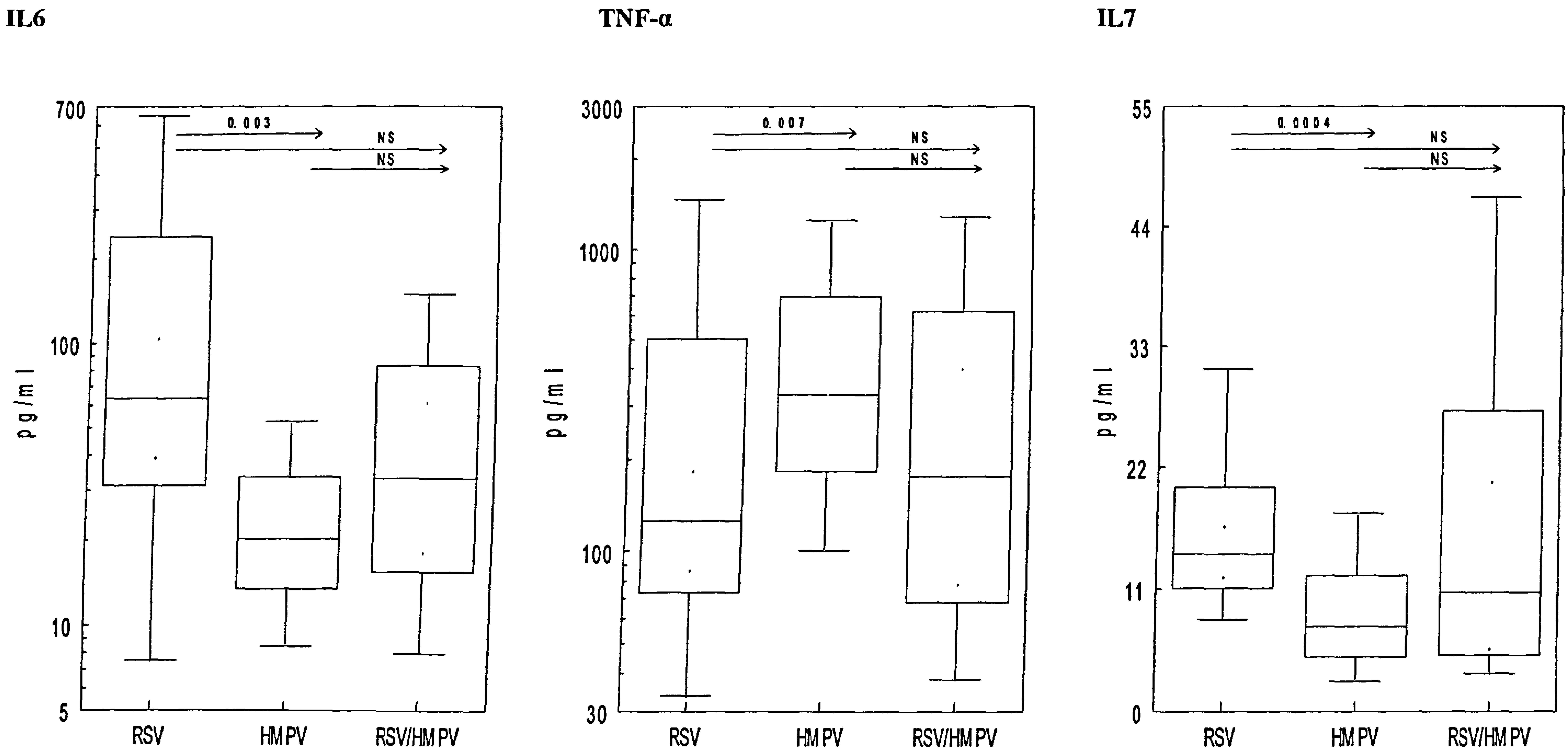
Cytokine/chemokine GM (SE) and range	RSV	HMPV	RSV/HMPV	P[1]	P[2]	P[3]
IL6	63.5 (1.28)	20.1 (1.16)	32.8 (1.37)	**0.003**	0.2	0.1
	7.3 - 2713.4	6.7 - 92.9	7 - 299.4			
TNF-α	125.5 (1.21)	327.2 (1.25)	176.3 (1.52)	**0.007**	0.5	0.2
	20.2 - 2805.1	(95.9 - 1477.3	28.4 - 3269.6			
IL7	14.1 (1.09)	7.6 (1.17)	10.7 (1.39)	**0.0004**	0.3	0.28
	3 - 61.8	2.7 - 39.5	2.4 - 49.6			
IL10	17.1 (1.11)	26.4 (1.17)	13.5 (1.3)	**0.01**	0.4	**0.04**
	(5.8 - 90.4	5 – 97	5.7 - 31.6			
IL12	13.8 (1.12)	19.7 (1.17)	9.8 (1.23)	**0.05**	0.2	**0.01**
	3.3 - 87.6	9.5 - 82.3	3.8 - 25.5			
INF-γ	65.8 (1.07)	83.5 (1.19)	111.9 (1.36)	0.1	**0.007**	0.4
	37.7 - 123.9	18.4 - 450.6	41.1 - 710.8			
IL8	3983.5 (1.14)	2958.4 (1.08)	3845.5 (1.12)	0.07	0.9	0.1
	1549.3 - 9894.9	1746.4 - 5666.8	1652.6 – 7925			
RANTES	94.3 (1.32)	155.7 (1.43)	208.6 (1.8)	0.3	0.2	0.7
	3.8 - 3569.1	16 - 3083.4	6 - 4916.4			

P[1]= RSV vs. HMPV, P[2]= RSV vs. RSV/HMPV and P[3]= HMPV vs. RSV/HMPV

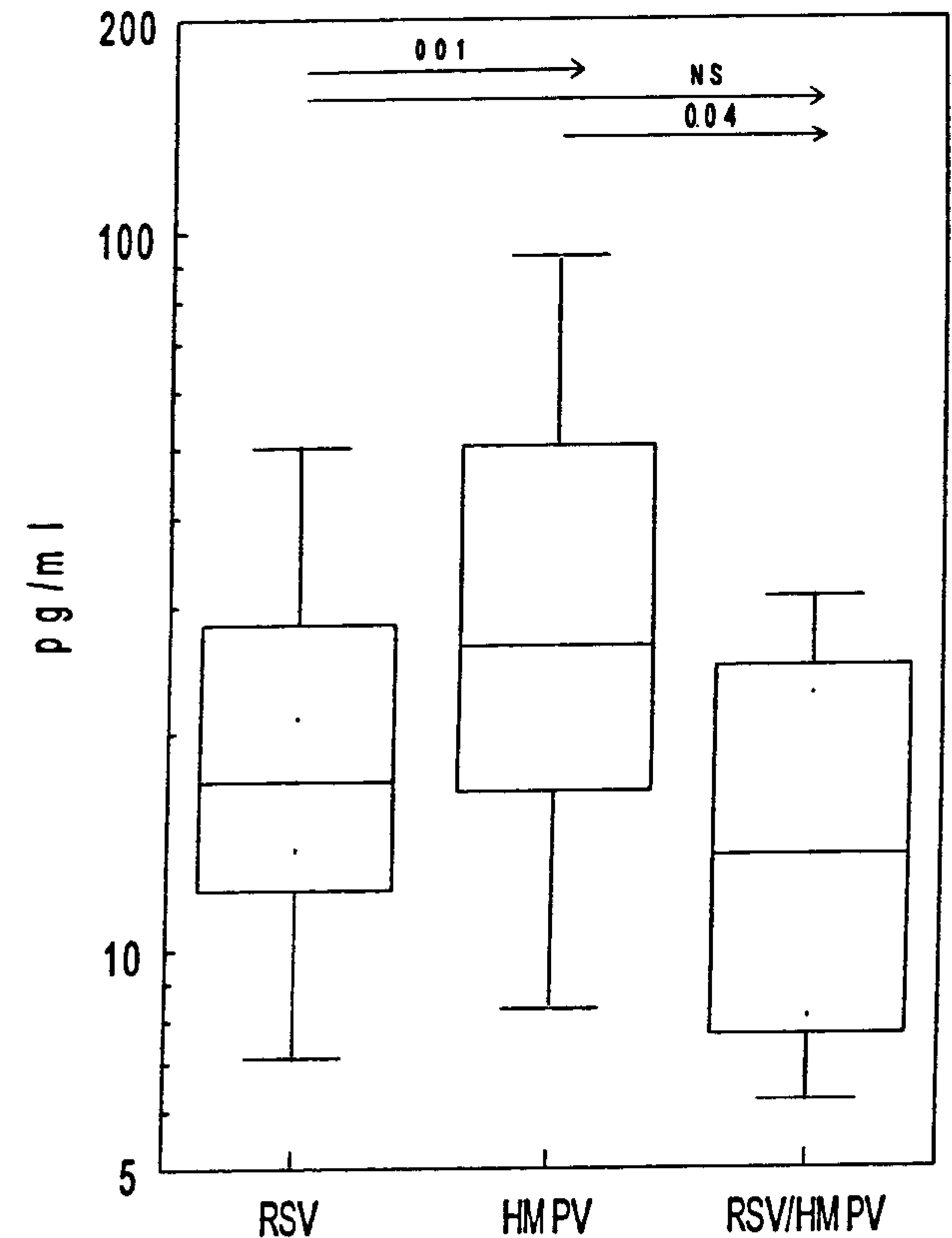
Table 5.11 Cytokine and chemokine concentrations in children with RSV, HMPV and RSV/HMPV by sex

Cytokine/chemokine	RSV		P	HMPV		P	RSV/HMPV		P
GM (SE) and range	Male	Female		Male	Female		Male	Female	
IL6	41.3 (1.2)	54.7 (1.33)	0.3	19.6 (1.16)	22 (1.3)	0.6	32.7 (1.3)	44.5 (1.49)	0.5
	7.4 - 1579.7	5.7 - 2713.4		6.7 - 92.9	6.7 - 18.2		7 - 229.4	10.7 - 299	
TNF-α	157.7 (1.21)	109.2 (1.26)	0.2	349.6 (1.3)	247.9 (1.43)	0.4	210.5 (1.43)	79.1 (1.67)	0.1
	16.7 - 4196.6	8.3 - 1421.8		95 - 1477.3	95.9 - 774.5		28.4 - 3269.6	27 - 292.4	
IL7	10.8 (1.09)	12.3 (1.14)	0.5	6.4 (1.16)	9.7 (1.24)	0.1	9.5 (1.18)	16.5 (1.4)	0.1
	1.9 - 26.2	2.4 - 61.8		2.3 - 16.3	3.2 - 39.5		2.4 - 18.9	4.2 - 49.6	
IL10	21.2 (1.09)	18.6 (1.12)	0.3	24.6 (1.17)	22.9 (1.28)	0.8	17.1 (1.23)	14.9 (1.18)	0.1
	6.1 - 90.4	5.8 - 86		5 - 94.4	5 - 97		5.7 - 47.3	10.7 -22.9	
IL12	13.6 (1.09)	16.1 (1.13)	0.2	19.9 (1.17)	17.7 (1.22)	0.6	14 (1.24)	15.3 (1.79)	0.8
	4 - 92.4	4.4 - 87.6		9.6 - 82.3	9.5 - 49.7		3.8 - 64.5	6.6 - 84.4	
INF-γ	67.7 (1.06)	72.1 (1.08)	0.5	76.1 (1.16)	70.4 (1.38)	0.7	99.1 (1.2)	75.2 (1.37)	0.5
	154.6 -173.9	37.3 - 166.5		23.2 - 243.3	18.4 - 450.6		41.1 - 710.8	31.1 - 121.6	
IL8	3422.8 (1.1)	3585.8 (1.14)	0.6	2362.5 (1.26)	3161.6 (1.13)	0.4	3853.6 (1.16)	4641.5 (1.12)	0.4
	1318.8 - 9894.9	884 - 9326.9		48.5 - 5502.8	1819.2 - 5666.8		1652.6 - 7925	3002.9 - 5802.9	
RANTES	83.1 (1.24)	69.9 (1.32)	0.6	153.1 (1.48)	212.6 (1.35)	0.6	115.2 (1.57)	142.9 (1.86)	0.8
	6.2 - 3569.1	3.8 - 1282.9		16 - 3083.4	43.4 - 779		6 - 4916.4	30.2 - 486.9	

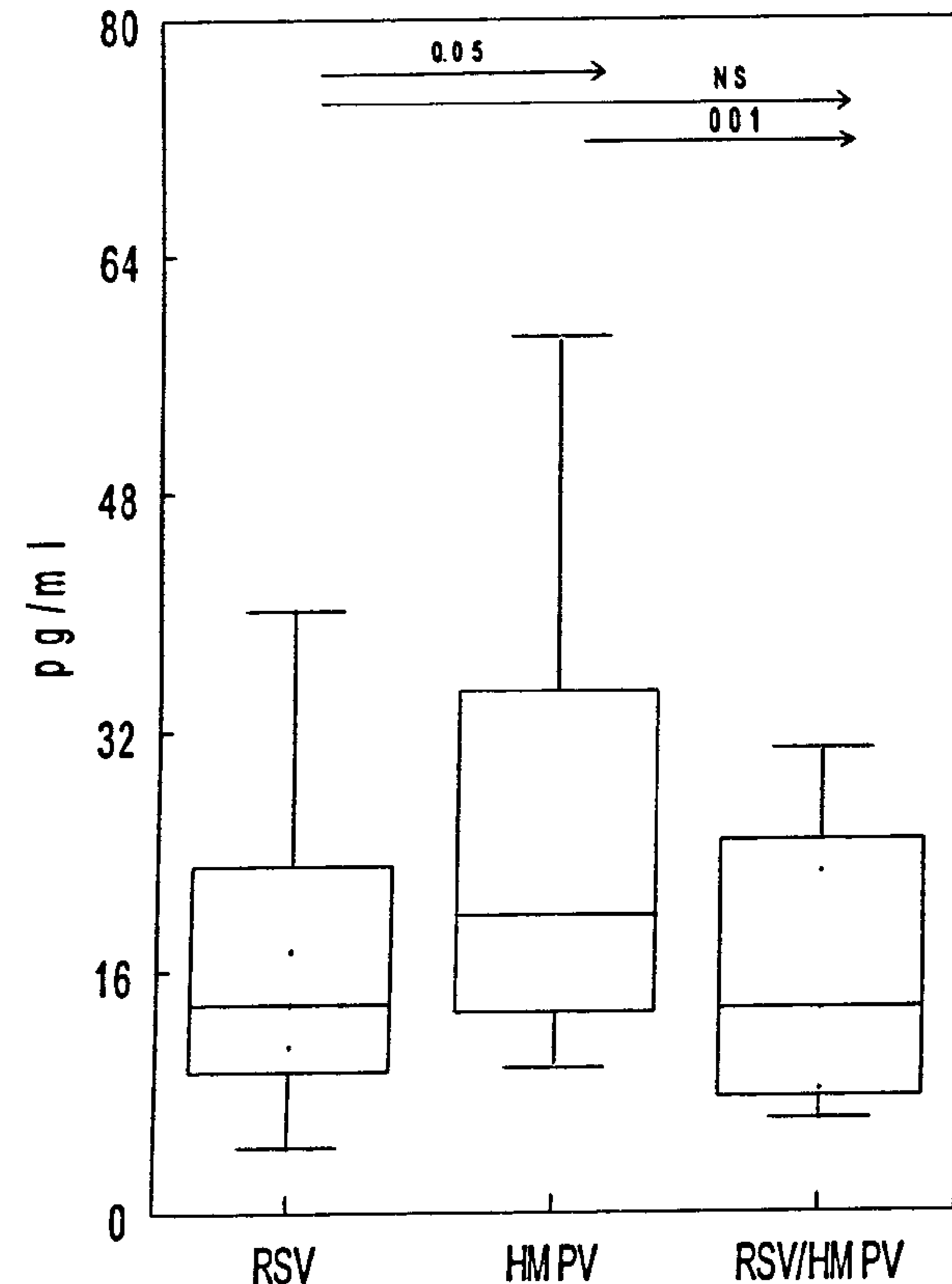
Figure 5.8 Cytokines and chemokines in children > 3 months of age with RSV, HMPV and RSV/HMPV



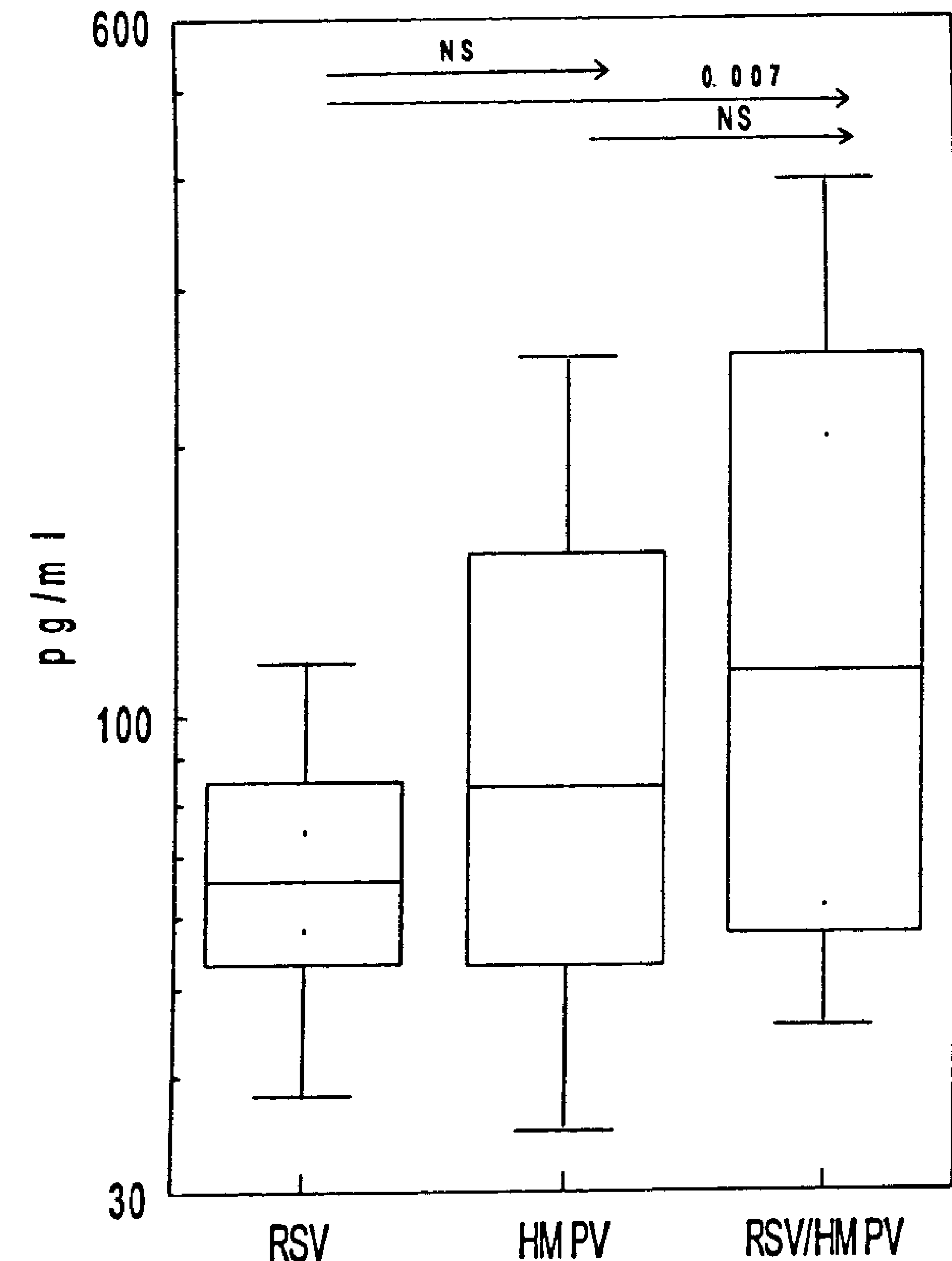
IL10



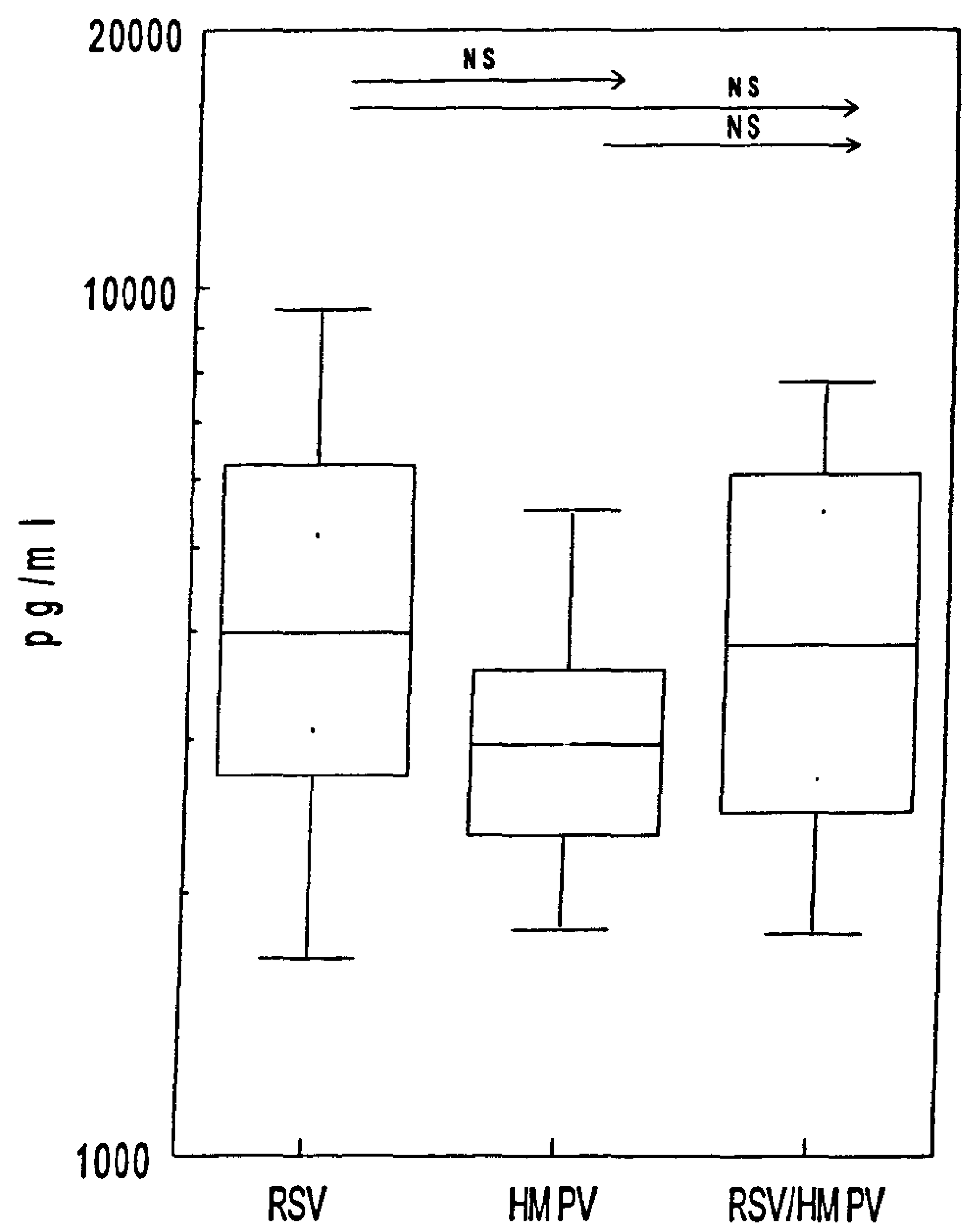
IL12



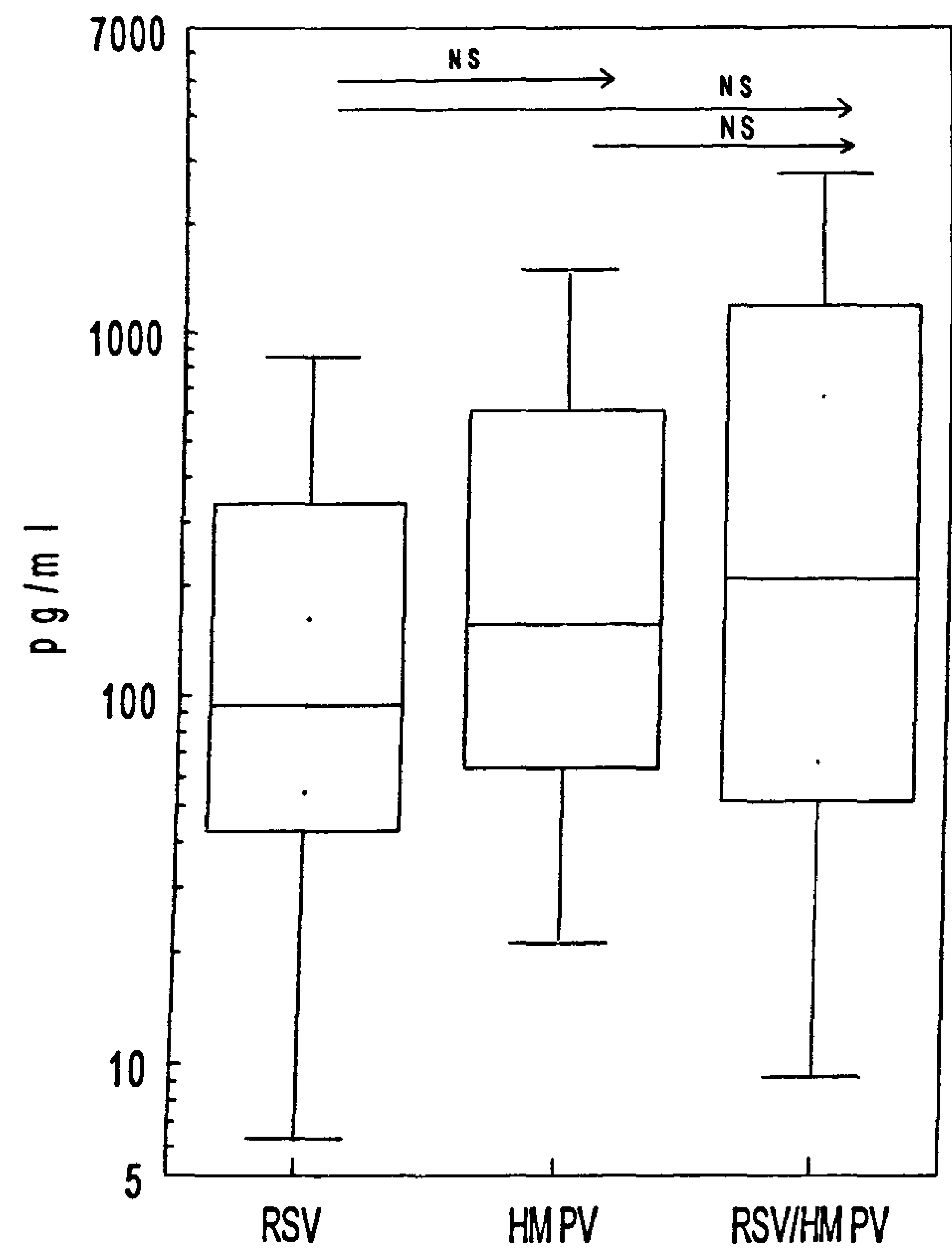
INF-γ



IL8



RANTES



Discussion

Bronchiolitis is often considered an immune-mediated disease (Openshaw, 1995). The cytokine and chemokine response of children with RSV and especially HMPV infection have not been fully elucidated. Such responses were studied in this chapter in order to understand the early events of the immune response associated with these infections.

The reliability of measurement of cytokines and chemokines in NPA was evaluated by Bont et al. who demonstrated a high correlation between cytokine and chemokine levels in NPA with those in tracheobronchial aspirates among children with lower respiratory tract infections. This method is less invasive than bronchial aspiration and seems suitable to describe even low level cytokine and chemokine concentrations in the respiratory tract (Bont et al., 2001). The analysis of NPA is a novel technique which allows the detection of *in vivo* cytokine and chemokine concentration in the respiratory tract of children with RSV or HMPV infections.

RSV and HMPV immune responses

Proinflammatory cytokines (IL6 & TNF-α)

The IL6 concentrations were significantly higher, and the TNF-α concentrations were significantly lower in children with RSV than in children with HMPV. The IL6 concentrations in our study are in agreement with Laham et al. who reported similar concentrations in a report of children with upper and lower ARI in Argentina. However, in the same study, TNF-α concentrations were higher in infants with RSV than in infants with HMPV (Laham et al., 2004). TNF-α had been shown to have a protective role against RSV infection by inhibition of its replication (Neuzil et al., 1996) and our study demonstrated that TNF-α concentration was significantly lower compared with HMPV, this may explained that fact that more severe disease was found due to RSV than HMPV.

Anti-inflammatory cytokines

Our findings illustrate for the first time that IL7 is produced in considerable amounts as a result of infection by either RSV, HMPV or their combination. However, HMPV infections resulted in a lower IL7 concentration than RSV. IL7 is a potent stimulator of CD4 antiviral T cells and CD8+ T cells in some viral infections such as CMV (Lu et al., 2005) and HSV-1 (Wiryana et al., 1997). It was therefore unexpected that HMPV infection resulted in lower concentrations than RSV. HMPV has been reported to have a milder clinical presentation than

RSV (Cuevas et al., 2003, Greensill et al., 2003) and the severity and immunological mechanisms used by RSV and HMPV in inducing IL7 may explain these differences.

Th1 & Th2 immune response

This study indicates that the early production of Th1 cytokines is weak in infants experiencing RSV ARI. In contrast, HMPV ARI is associated with a strong Th1 cytokine response and infants were able to elicit competent IL12 and INF-γ production against HMPV but not RSV infection in the first few months of life. These findings may be explained in several ways. RSV may be less immunogenic and may not efficiently prime T-cell immunity at an early age compared with HMPV. This could be due to combination of factors, including differences in antigenic motifs or different properties of the virus for induction innate immune responses. For example, RSV stimulates innate immune mechanisms through TR-4 receptors, which are primary regulators of innate immunity. TR-4 is required for RSV induced cytokine responses by macrophages *in vitro* as well as for virus elimination in a mouse model system (Kurt-Jones et al., 2000). Also, although the effect of RSV on Th1 cytokine production by monocytes and macrophages has not been investigated, it is conceivable that RSV itself effectively prevents the initiation of adequate antiviral immune responses by inhibiting IL12 and INF-γ production. Evidence for the immunosuppressive activity of the virus itself is also provided by *in vitro* studies (Bartz et al., 2003, Panuska et al., 1995). RSV may inhibit lymphocyte responses either directly or through the induction of cytokines with anti-proliferative or immunosuppressive activity such as IL10 (Panuska et al., 1995). In addition Blanco-Quiros et al. reported that infants with RSV bronchiolitis already have immunoregulatory alterations that are present at the time of delivery as they showed decreased levels of IL12 in neonates who later developed bronchiolitis (Blanco-Quiros et al., 1999). Furthermore, the RSV genome contains genes which encode non-structural proteins NS1 and NS2 which suppress the induction of α, β, and γ INF in RSV infected human epithelial cells and by macrophages (Spann et al., 2004b, Schlender et al., 2000). These genes however are absent in the HMPV genome (van den Hoogen et al., 2002).

Our findings were in agreement with a comparative study between immune responses in RSV and non-RSV ARI, where lower levels of INF-γ and INF-γ/IL10 ratio were found in RSV compared to non-RSV ARI (Joshi et al., 2003, Aberle et al., 1999). Laham et al however found that the production of IL12 increased significantly in infants with RSV compared to HMPV (Laham et al., 2004). However our *in vivo* study suggest that RSV and HMPV infections induce different Th cytokine profiles in infants with a significant increase in the production of Th1 cytokines and other related pro-inflammatory cell responses in infants with HMPV. In addition, no significant differences in the Th2 cytokines (IL10) were observed

between the two groups. This is consistent with the results of Laham et al. who also reported that IL10 concentrations in infants with RSV and HMPV were similar (Laham et al., 2004).

Chemokines

Our results have demonstrated that IL8 concentrations were higher, and RANTES concentrations were lower in children with RSV compared to children with HMPV infection (p=0.06 and 0.05 respectively). This is in contrast to a Finnish study by Jarti et al, who found that IL8 concentrations were significantly lower and RANTES concentrations significantly higher in children with RSV than in children with HMPV (Jartti et al., 2002). The comparison control however consisted of historical cohort of children infected with RSV and only included children who clinically had wheezing which may be associated with different immunological responses in children with ARI with no wheeze. However, our findings were in agreement with Laham et al., who reported that RSV infections induce higher IL8 concentrations than HMPV (Laham et al., 2004). Furthermore, RSV has also been reported to increase the production of both plasma IL8 (Bont et al., 1999a) and in the airways of infants with bronchiolitis (Sheeran et al., 1999).

It has been reported that RSV induces a more severe clinical presentation than HMPV (Cuevas et al., 2003). This might be reflected by deficient type 1 immune responses with RSV infection whereas HMPV elicits a more competent type 1 cytokine response. This is in agreement with previous studies that suggested that severe RSV ARI is associated with deficient type 1 and or excess type 2 cytokine responses (Aberle et al., 1999, Bendelja et al., 2000, Legg et al., 2003). Moreover, IL8 was induced in higher concentrations in children with RSV than in children with HMPV infection. High IL8 concentrations have been considered to be a marker for disease severity (Bont et al., 1999a) and this is discussed in a subsequent section of the thesis.

Innate immune responses of children with RSV/HMPV coinfections

Innate immune responses in children with RSV/HMPV coinfection did not differ from those of children with single RSV infections with the exception of INF-γ concentrations. This cytokine was present at significantly higher levels in children with dual infections. This increased response could be as the result of the modulation of the innate immune stimulation by the two viruses. HMPV has been reported to be a more potent stimulant for the induction of INF-γ than RSV and this might affect the net amount of INF-γ concentrations in children with coinfections.

Similarly, children with single HMPV infections only differed from children with dual RSV/HMPV infection in the concentration of IL6 and IL8. Similar to children with RSV, children with RSV/HMPV had higher concentrations of IL6 than children with HMPV. It seems that the coinfection modified the production of the cytokines according to two different stimuli and mechanisms by the two viruses. For example, RSV G glycoprotein contains a CX3C chemokine motif capable of interacting with the CX3CR1 receptors, inhibiting fractalkine-mediated responses and altering trafficking of CX3CR1+ cells, which include T lymphocytes, NK cells, monocytes and macrophages and thus may modulate the innate immune response (Tripp et al., 2001). The HMPV G protein does not contain a CX3C chemokine motif (van den Hoogen et al., 2002) , and it is unlikely to similarly antagonize fractalkine-mediated responses and modulate the immune response in a similar manner to RSV.

RSV/HMPV coinfection induced the highest concentrations of IL8 which is in agreement with the clinical diagnosis as most of the children with dual infections had a diagnosis of severe ARI. Dual infection has been reported to increase disease severity and further studies should be conducted to confirm this finding.

In summary, our results suggest that RSV and HMPV infections in infants differ significantly in regard to the type of induced immune response elicited.

Immune response of children with RSV and HMPV by disease severity

It is still not known why some infants develop bronchiolitis, whereas other children have only mild symptoms when exposed to RSV or HMPV.

Proinflammatory and anti-inflammatory cytokines

In our study, IL6 concentrations were significantly higher in children with mild than in children with severe RSV ARI. This in agreement with previous reports that have demonstrated that high IL6 concentrations are present in children with mild URTI (Gentile et al., 2001, Noah et al., 1995). TNF-α was found to be higher in children with severe RSV ARI than in children with mild clinical presentation. This is consistent with Fujita et al, who reported that children with severe RSV pathology produced high levels of TNF-α (Fujita et al., 2001).

IL7 concentrations were significantly higher in children with mild RSV ARI than in children with severe RSV ARI. This finding supports the hypothesis that IL7 may play a protective

role from severe clinical illness as suggested for other viruses, CMV (Lu et al., 2005) and HSV-1 (Wiryana et al., 1997).

Th1 and Th2 immune responses

The inverse relationship between high IL12 responses and the duration of mechanical ventilation in previous studies suggests that IL12 may play a protective role among infants with RSV infections and respiratory failure (Bont et al., 1999a). However, our study did not identify significant differences in IL12 concentrations between children with mild and severe RSV ARI. Our results however are consistent with those of Garofalo et al. who also reported that there were no significant differences in IL12 concentration between subjects with mild, non-hypoxic or hypoxic RSV bronchiolitis (Garofalo et al., 2004).

We speculated that the cell's tendency to produce higher concentration of INF-γ in infants with severe RSV bronchiolitis and pneumonia contributes to the pattern of illness through an as yet unknown mechanism. An exuberant CD8+ CTL response producing high concentration of INF-γ has been shown to result in severe haemorrhagic pulmonary RSV disease (Cannon et al., 1988). Our study has demonstrated that Yemeni infants with clinically severe RSV infection had significantly higher INF-γ concentrations than those with mild disease. This is consistent with a predominantly Th1 immune response, with more inflammation in the lungs, but they do not exhibit a more pronounced RSV-specific type 2 like T- cells response than infants with mild RSV infection. This is in agreement with previous studies that have reported that INF-γ concentrations were higher in children with severe RSV disease (Bendelja et al., 2000, Tripp et al., 2002). However, the main finding in the study of (Bont et al., 2001) which was that mechanically ventilated infants with RSV LRTI had greatly decreased INF-γ levels, when compared with hospitalised infants with RSV LRTI who did not required mechanical ventilation was not found in our study.

IL10 concentrations were higher in children with severe RSV associated diseases than in children with milder diseases. These differences however were not statistically significant and our data do not support the hypothesis that severe infection is associated with type -2 like T cellular was responses in naturally acquired RSV bronchiolitis.

Chemokines

Children with severe RSV disease requiring ventilation have been reported to produce higher concentrations of IL8 than children who are not ventilated (Bont et al., 1999a, Smyth et al., 2002). In our study, however IL8 and RANTES concentrations did not correlate with severity.

Our findings are in agreement with reports from others, who did not find such differences in their patients groups (Laham et al., 2004, Noah et al., 2002, Oh et al., 2002). Moreover, in some studies, levels of IL8 and RANTES were inversely related to the clinical severity of RSV disease (Sheeran et al., 1999) and further studies are needed to clarify their relationship to disease severity.

Immune responses of children with mild and severe ARI due to HMPV and RSV/HMPV coinfection

Children with mild and severe HMPV did not show significant differences in concentrations of any of the cytokines and chemokines. These findings however are consistent with the results of Laham et al. who did not observe differences in series of patients with upper and ALRI (Laham et al., 2004). The numbers of children with mild and severe RSV/HMPV were however small and no significant differences in the concentrations of any cytokines or chemokines were detected.

Immune response of infants with RSV, HMPV and RSV/HMPV by age

Most infants ≤ 3 months of age in our study developed clinically severe disease. This is consistent with previous reports from other geographical areas (Kaneko et al., 2001, Henderson et al., 1979). Thus, it is of interest to compare the cytokine and chemokine profiles of infants ≤ 3 months of age with those who were above this age to determine whether there are immunological bases for severity of disease associated with young age. With the exception of IL7 and IL10 we could not detect any significant differences in the cytokine and chemokine concentrations in infants ≤ 3 and > 3 months of age with RSV ARI.

The significant increment in the Th2 cytokine IL10 in infants ≤ 3 compared to infants > 3 months of age may explain why young infants exhibit severe disease more frequently than older children. An excess of type 2 cytokines could result in an inability to control the disease severity and to more severe pulmonary pathology (Martinez et al., 1997). Age and host genetics certainly affect the balance of the immune responses during primary infection, and the first RSV infection sometimes resembles secondary disease. In the neonatal period, Th1 responses are generally poor or short lived and IL12 production is weak. One possibility is that both Th1 and Th2 responses are developed during the initial infections; however, there is specific IL4 dependant apoptosis of Th1 cells in the neonatal period (Li et al., 2004). This may explain why at this age, there is a natural tendency towards a strong Th2 response.

IL7, which is known to have a stimulant effect on the cellular immune response against viral diseases (Lu et al., 2005) was also significantly lower in infants ≤ 3 months of age and this may explain why young infants develop more severe forms of the disease than older children. However, the low IL7 concentrations may characteristic of the immature immune system of young infants.

Interestingly, there were no significant differences in the cytokine and chemokine concentrations between infants ≤ 3 and > 3 months of age who had HMPV ARI. There are, to date, no comparable studies published in this regard.

Infants with RSV/HMPV had no significant differences in the cytokine and chemokine concentrations before and after 3 months of age with the exception of IL12, which was increased in infants ≤ 3 months of age. This is consistent with a predominant Th1 immunological response. This may be due to the different effect of the stimulation of innate immune responses by HMPV that might lead to a shift in the immune response from Th2 cytokine responses (IL10) to Th1 cytokine responses (IL12) in young infants with RSV/HMPV co-infection. Further studies to evaluate this response however are needed.

In summary, our study demonstrated that an excess in the type 2 immune response (IL10) is associated with the pathology of disease in young infants with RSV whereas an excess in type 1 immune responses (IL12) was associated with severe disease presentation in children with RSV/HMPV.

It was also noticed that gender did not play a significant role in the immunopathogenesis of disease severity due to RSV, HMPV or RSV/HMPV since no significant differences in cytokine and chemokine concentrations were observed between male and female children with RSV, HMPV or RSV/HMPV coinfections.

Chapter 6

Antioxidant status of children with RSV, HMPV and RSV/HMPV co-infection

Literature review

Malnutrition still remains a major public health problem and is a significant contributor to childern's deaths despite numerous advances and improvements in child health worldwide (Black, 2003). A number of factors may influence micronutrient deficiencies in developing countries. These include poor body stores at birth as a consequence of maternal factor leading to intrauterine malnutrition, high intake of inhibitors of absorption or lack of food. Micronutrient deficiencies have significant adverse consequences on key functional aspects of the immune system and resistance to infection (Coutsoudis et al., 1991, Field et al., 2002). In developing countries, malnutrition and micronutrient deficiencies are associated with an increased incidence and severity of ARI (Victora et al., 1999). We have, however, a poor understanding of the role of specific micronutrient such as zinc, selenium, copper, vitamin A and E in relation to disease severity caused by RSV and other viral infections and there are no reports on micronutrient changes in children with HMPV. This chapter describes the micronutrient status of children with RSV, HMPV and RSV/HMPV co-infections and their association with severity of disease.

Micronutrient status and inflammation

The term "acute phase response" is used to describe a short term metabolic change evidenced by increased plasma concentrations of certain proteins - positive acute phase proteins (APPs) - such as C-reactive protein (CRP), haptoglobin, fibrinogen and α-1 antitypsin and decreased concentrations of certain proteins – negative APPs- such as albumin, retinol binding protein (RBP), transthyretin (TTR) and high density lipoprotein-apolipoprotein A1, which tend to fall during infection (Tomkins, 2003). An acute phase protein has been defined as one whose plasma concentration increases (positive acute phase protein) or decreases (negative acute phase proteins) by at least 25% during inflammatory disorders (Morley and Kushner, 1982). The magnitude of these increases varies from 50% in the case of ceruloplasmin and several complement components to as much as 1000 fold in the case of CRP (Gabay and Kushner, 1999).

The changes in plasma levels of APP may be due to alterations in dietary intake, absorption, synthesis or metabolism of the individual inflammatory protein together with change in

urinary losses, alteration in plasma volume or extrusion from intravascular into tissue spaces are all possibilities. Changes of all these have been documented during inflammation but there are remarkable powers of adaptation, and deficiency in micronutrient level does not necessarily mean that the micronutrient status and physiology are disturbed (Tomkins, 2003).

C-reactive protein

CRP activates the classical pathway of complement, one of the main mechanisms in providing host defence. It also interacts with cells of the immune system by binding to Fc gamma receptors. It may thus bridge the gap between the innate and adaptive immunity and provide an early effective bacterial response. CRP appears to protect against the damaging inflammatory response induced by lipopolysaccharide and cytokines (Andersson et al., 1983, Wessels and Moldawer, 2000). Measurements of inflammatory responses have been used to detect systemic infection in several clinical conditions. For example children with elevated CRP who had acute or persistent diarrhoea were more likely to have a severe complicating clinical illnesses such as meningitis, septicaemia or pneumonia (Tomkins, 2003).

Zinc

Zinc is one of the essential trace elements and a member of one of the major subgroups of micronutrients that have attained high predominance in human nutrition and health. Zinc deficiency is often seen in the context of malnutrition and zinc supplementation in children in developing countries has been demonstrated to cause a significant reduction in the incidence of diarrhoea and pneumonia (Bhutta et al., 1999b, Sazawal et al., 1998).

Zinc and the immune system

Dietary zinc deficiency impairs the overall immune function and resistance to infection suppressing thymic function, T-lymphocyte development, lymphoproliferation, and T – cell-dependent B- cells functions (Nielsen et al., 2002). Although the understanding of the role of zinc in immune function has increased, the specific mechanisms by which zinc acts in the prevention and treatment of infectious diseases in humans are still unclear. Zinc deficiency might play a yet unexplored role in the immune alterations that modify the predominantly cellular Th1 responses, required for their control and humoral Th2 responses (Maitreyi et al., 2000, Sprietsma, 1997). An imbalance between Th1 and Th2 functions has been reported during zinc deficiency. In human studies, zinc deficiency resulted in decreased production of IL 2 and INF-γ (Th1 cytokines), whereas the production of IL 4, 6, and 10 (Th2 cytokines)

was not affected (Becker et al., 1997, Maitreyi et al., 2000). These changes, however, are readily reversed with zinc supplementation (Becker et al., 1997).

Zinc status during inflammation

Zinc status is often assessed by measurement of zinc in plasma, white blood cells or hair (Krebs et al., 1995). However many dietary and physiological factors such as exercise, eating, pregnancy and rapid growth in childhood may alter plasma zinc levels (Armour et al., 1994). Whether this really represents zinc deficiency is arguable. A redistribution of zinc appears to be one result of the inflammatory process that occurs during the acute phase response to serious illnesses (Shenkin, 1995). Mean plasma zinc levels in a population can be used to indicate deficiency however, the level of systemic inflammation need to be taken into account (Wieringa et al., 2002). Plasma zinc is reduced during acute phase response, in addition there are considerable urinary losses of zinc in systemic infection, particularly in those with a pronounced metabolic stress leading to breakdown of muscle (Kreuzer et al., 1996, Dinarello, 1984) and children with raised CRP often have relatively low plasma concentrations of zinc (Wieringa et al., 2002).

Zinc and ARI

Initial clinical trials to evaluate the effect of zinc supplementation on the prevention of ALRI showed in a double blind randomised controlled trial in India, that zinc supplemented children had 0.19 ALRI episodes/ child/year compared with 0.35 episodes/child/year in the control children. There was a reduction of 45% (95% CI, 10% - 67%) in the incidence of ALRI in the zinc supplemented children (Sazawal et al., 1998). It also showed that zinc reduced the incidence of serious ARI (OR 0.59, 95% CI, 0.41 – 0.83) (Bhutta et al., 1999b).

The effect of weekly zinc supplementation on the incidence of pneumonia in children was assessed in a randomised controlled trial of 1665 Bangladeshi children aged 2-12 months. There were significantly fewer cases of pneumonia in the zinc group than in the control group (RR 0.83, 95% CI, 0.73 to 0.95) (Brooks et al., 2005). In addition, in a double blind, randomised placebo controlled trial of 2482 Indian children aged 6 to 30 months, supplemented with a single dose of vitamin A plus daily zinc supplementation or placebo for four months, the proportion of children with ALRI during the follow up was not different between the groups (absolute risk reduction -0.2%, 95% CI, -3.9% to 3.6%). After correction for multiple episodes in the same child, the odds ratio was 0.74 (95% CI, 0.56 to 0.99) (Bhandari et al., 2002). Table 6.1 describes the main finding of selected studies assessing the effect of zinc on ARI in children.

Table 6.1 Studies assessing the effect of zinc on ARI

Country	Type of study	Characteristics on enrolment	No	Outcome	Age (months)	Main findings	Refernce
India	RCT	Severe LRTI	38 zinc + vit. A, 38 vit. A 39 zinc, 38 placebo	Recovery from LRTI	2-24	Earlier resolution of fever and very ill status in boys but not in girls.	(Mahalanabis et al., 2004)
India	RCT	After measles	42/43	Measles-related pneumonia	9 m–15 y	No beneficial effect	(Mahalanabis et al., 2002)
India	RCT	Normal	1241/1241	LRTI, pneumonia	6 – 30	Lower incidence of pneumonia	(Bhandari et al., 2002)
India	Cohort	Recovering from diarrhoea	116	ALRI	12-59	Higher prevalence of LRTI in those with low zinc at enrolment	(Eugene-Ruellan et al., 1998)
Bangladesh	RCT	Normal	152/149	ALRTI, growth and morbidity	1-6	Lower risk of LRTI in infants with low baseline zinc	(Osendarp et al., 2002)
Bangladesh	Cluster RCT	With diarrhoea	3974/4096	LRTI	3-59	Lower incidence of subsequent LRTI	(Baqui et al., 2002)
Bangladesh	RCT	Malnourished, acute diarrhoea	32/35	URTI LRTI	3 -24	Lower respiratory morbidity 2 months after supplementation	(Roy et al., 1999)
Guatemala	RCT	Normal	45/44	Diarrhoea and ARI morbidity	6-9	Higher ARI incidence and prevalence in zinc-supplemented group (not statistically significant)	(Ruel et al., 1997)
Ecuador	RCT	Malnourished	25/25	URTI and LRTI	12-59	Less fever, cough and upper respiratory tract secretions	(Sempertegui et al., 1996)

RCT = randomised control trial, vit. = vitamin. (Cuevas and Koyanagi, 2005)

Zinc and viral infections

Zinc has been shown to mediate antiviral effects against certain viruses that infect the human respiratory tract, including the enveloped virus Herpes Simplex virus (HSV) and the nonenveloped virus rhinovirus (Geist et al., 1987, Korant and Butterworth, 1976). The mechanism of inhibition of rhinovirus is likely to involves the direct binding of zinc to the virus particles (Korant and Butterworth, 1976). Clinical studies has shown that zinc supplementation significantly shortens the duration of symptoms during rhinovirus infection (Mossad et al., 1996, Maitreyi et al., 2000) and the topical application of zinc sulfate was reported to be effective in the treatment of HSV infection (Wahba, 1980).

Although zinc supplementation reduces the incidence of severe clinical pneumonia in the developing world, the effect of zinc on the replication of the primary agents of viral pneumonia in infants such as RSV is unknown. The *in vitro* inhibitory effect of three zinc salts (acetate, lactate, and sulfate) on RSV in virus yield or virus plaque reduction assays were determined by treatment of wild-type RSV strain A2 at various concentrations. The experiments suggest that zinc inhibits RSV by altering the ability of the cells to support RSV replication rather than by a direct effect on the virus. Also they reported that zinc salt significantly prevented RSV plaque formation by preventing cell to cell spread on addition of zinc salts to the semisold overlay media. This inhibitory effect was concentration dependent (Suara and Crowe, 2004).

Copper

Copper is an essential micronutrient involved in numerous biochemical interactions in the body, including iron mobilisation, the maintenance of the electron transport system, and the formation of collagen (Fisher, 1975). Copper deficiency can occur in humans and may result in depressed growth, abnormal bone development and alopecia (Fisher, 1975). Copper deficiency can also have adverse effects on the immune response resulting in decreases of both numbers and function of lymphocytes derived from the thymus (T cells) in rats (Rabalais et al., 1992) and mice (Lukasewycz et al., 1985).

Copper status during inflammation

Copper is measured within the copper-binding protein complex of ceruloplasmin. This is a positive APP that is elevated during the acute and chronic inflammatory response (Periquet et al., 1995). For example, patients with pulmonary TB have significantly higher copper concentration than individuals without TB (Koyanagi et al., 2004).

Copper and zinc interactions

Although the population – level copper deficiency is unknown (Rosado, 2003), it is biologically plausible that increases in zinc intake through supplementation or fortification could limit the absorption of copper, thus resulting in deficiency (Abdel-Mageed and Oehme, 1990). A clinical trial from Pakistan and India assessing the effect of daily zinc supplementation on copper levels in adults reported that copper level decreased after six weeks with high zinc supplements and returned to normal six weeks after supplementation ended (Abdulla and Suck, 1998). However, no significant differences were noted in 115 infants randomised to receive zinc or placebo for 120 days (Sazawal et al., 2004). Copper status and the effect of copper deficiency on RSV or HMPV infection have not been described to affect disease severity.

Selenium

The essential trace element selenium has an important function in maintaining immune status and antioxidant defence (Field et al., 2002).

Selenium and immune functions

Selenium is essential for the function of several selenoproteins, because of the selenocysteine residues present in their active sites. Glutathione peroxidase, is a selenoprotein that acts as a major an oxidant scavenger that protects against oxidative stress (Xing et al., 1992). In addition to its antioxidant functions, selenium has other immunological properties involving membrane receptor expression. The stimulation of T cell proliferation, CTL and macrophage cytotoxicity and NK activity by selenium may be a result of the ability of selenium to enhance the expression of the α and/or β subunits of the interleukin2 receptor (IL2R) on activated immune cells. This results in a greater number of functional IL2R/cells and an enhanced proliferation and clonal expansion of cytotoxic precursor cells (Kiremidjian-Schumacher et al., 1996).

Selenium status during inflammation

Selenium status is often measured directly in serum, plasma, whole blood and hair. It has also been measured indirectly by assays of plasma glutathione peroxidase levels (Diplock, 1993). Both serum and whole blood levels are decreased during the acute phase response. Low levels occur in several infections (Beck, 1999, Sammalkorpi et al., 1988). Several viral infections appear to stimulate the production of selenoproteins, leading to low serum levels of selenium.

Patients with the lowest levels of selenium have the highest levels of morbidity and mortality but a causal effect has not yet been demonstrated (Baum and Shor-Posner, 1997). Morbidity and mortality appear to be less vulnerable to changes in inflammatory response, but short- and long-term studies are not available. Overall, selenium levels are low in infection. It has been postulated that this is due to the consumption of selenium as an antioxidant as part of the process of quenching free radicals (Bates et al., 1994). Whether plasma selenium is also reduced as part of the inflammatory process has not been studied intensively and there are no published attempts to control for changes in selenium due to changes in inflammatory proteins.

The role of selenium deficiency on RSV and HMPV infections is not known and this study describes selenium status in relationship to the development of severe RSV, HMPV and RSV/HMPV coinfections

Vitamin A

Attention has recently focused on the role of vitamin A in viral infections because it is an essential micronutrient with important roles in immunity and maintenance of normal epithelial cell differentiation (Coutsoudis et al., 1991). Vitamin A deficiency is associated with increased frequency of clinical respiratory and diarrhorreal illnesses and may be a key determinant in overall childhood mortality rates (Sommer, 1984). Vitamin A supplementation improves the linear growth of children who have a low intake vitamin A but this impact is muted with increasing levels of respiratory infections (Cappel et al., 1972).

Vitamin A and immune functions

Vitamin A modulates the immune response by altering the pattern of Th1 and Th2 cytokines (Cui et al., 2000). High dose vitamin A supplements may enhance Th2 mediated immune responses (IL10) and decrease the production of Th1 cytokines (INF-γ). This may explain the lack of effectiveness of vitamin A in treating acute pneumonia (Cui et al., 2000).

Vitamin A status during infalmmation

The effect of inflammation on plasma retinol appears to depend on the underlying nutritional status (Tomkins, 2003). Inflammatory stress reduced plasma retinol in children eating a consistently deficient diet (Filteau et al., 1993) but a reduction in retinol was not marked in South African children with severe metabolic stress after accidental kerosene poisoning (Willumsen et al., 1997). The relative dose response, a method for assessing vitamin A status

that involves measurement of two forms of vitamin A, the naturally occurring form and a nonmetabolizable analog, might indicate vitamin A status, was unaffected by the presence of inflammation. However, measurements of the relative dose response taken shortly after a metabolic stress resulting from the accidental kerosene poisoning study in South Africa showed that the response changed along with the expected changes in inflammatory proteins and plasma retinol (Willumsen et al., 1997).

Plasma retinol changes quickly as inflammation starts and CRP rises. In Indonesia, children with raised CRP had relatively low plasma concentrations of vitamin A (Wieringa et al., 2002). At a cellular level there was some evidence of reduction of liver RBP synthesis in animals injected with endotoxin (Langley et al., 1994). Several studies have also demonstrated urinary losses of retinol and RBP (Kreuzer et al., 1996).

Vitamin A and respiratory infections

Vitamin A is inexpensive, has been safely administered to children worldwide and can be given orally. Vitamin A is being used increasingly in developing countries for a variety of indications. Recent trials of vitamin A for treatment of tuberculosis (Range et al., 2005) and for all acute lower respiratory infections (Kjolhede et al., 1995) have shown no benefit. Some studies have not found any benefit but probably had an inadequate sample size to detect a clinically significant changes (Quinlan and Hayani, 1996).

Vitamin A and RSV

Very few studies have evaluated the status and role of vitamin A in RSV infections. Serum retinol concentrations are low in children with RSV infection and severely ill children have the lowest concentrations (Dowell et al., 1996, Neuzil et al., 1994, Quinlan and Hayani, 1996).

The concept that vitamin A may be useful for treating RSV infection originated by analogy to studies of children with measles. Early studies demonstrated that measles infection was associated with acutely lowered serum concentrations of vitamin A in developing (Reddy et al., 1986, Markowitz et al., 1989) and developed countries (Arrieta et al., 1992, Butler et al., 1993). Furthermore the lowest concentrations of vitamin A were associated with the most severe morbidity (Butler et al., 1993).

Like measles, RSV is a paramyxovirus that infects the respiratory epithelium and can cause severe complications, including pneumonia, respiratory failure and death. High doses of

vitamin A can been administered safely to children with acute RSV (Neuzil et al., 1994, Neuzil et al., 1995, Quinlan and Hayani, 1996). In a USA multicentre, randomised placebo-controlled trial of high dose vitamin A of 239 children, vitamin A supplementation did not significantly modify, the number of days during which supplemental oxygen was required, the need for steroids, ribavirin, ICU care or mechanical ventilation (Bresee et al., 1996). Additionally there were no differences between treatment groups in the number of days required for participants to achieve normal O_2 saturation or normal respiratory rates. Children who received vitamin A had significantly longer hospital stays than children who received placebo, those found to be older than 1 year or at low risk for severe RSV (Bresee et al., 1996). Dowell et al however, hypothesised that if vitamin A has a beneficial effect on the course of RSV disease, it may be seen only in more severely ill children (Dowell et al., 1996). In their study, there was no significant benefit from vitamin A treatment for the overall study group (89 infants received vitamin A and 91 placebo) in duration of hospitalisation, need for supplemental oxygen or time to resolve hypoxemia. For the subgroup of children with significant hypoxemia on admission (room air oxygen saturation level $\leq 90\%$), those given vitamin A had more rapid resolution of tachypnoea ($P = 0.01$) and a shorter duration of hospitalisation (5.5 vs. 9.3 days, $P = 0.09$). No toxicities were seen, including excess vomiting or bulging fontanelle. The results of this study highlight the need for independent and critical evaluation of each potential new indication for vitamin A therapy.

Vitamin A and zinc interactions

Christian and West reviewed the interaction of vitamin A and zinc in a cross sectional, observational and supplementation clinical trial and concluded that zinc deficiency could impose a secondary vitamin A deficiency in protein energy deficient population (Paton et al., 1998). To evaluate the effect of simultaneous zinc and vitamin A supplementation on acute lower respiratory infections in Bangladeshi children a randomised double blind placebo controlled trial, 800 children, aged 12-35 months were randomly assigned to one of four intervention groups: 20 mg zinc once daily for 14 days; 200 000 IU vitamin A, single dose on day 14; both zinc and vitamin A; placebo. The children were followed up once a week for six months and morbidity information was collected. Incidence (1.62; 95% CI, 1.16 to 2.25) and prevalence (2.07; 95% CI, 1.76 to 2.44) of ALRTI were significantly higher in the zinc group than in the placebo group. The interaction term had rate ratios of 0.75 (95% CI, 0.46 to 1.20) for incidence and 0.58 (95% CI, 0.46 to 0.73) for prevalence of ALRT. Zinc was associated with a significant increase in ALRTI, but this adverse effect was reduced by the interaction between zinc and vitamin A (Rahman et al., 2001).

Vitamin E

Vitamin E is a liposoluble antioxidant, which may have an important role in scavengering free radicals and in stabilising the cell membranes, thus maintaining its permeability (Navarro et al., 1999, Bjorneboe et al., 1990). Vitamin E is a powerful antioxidant in the lung, where it protects against oxidative damage (Hybertson et al., 1995, Hybertson et al., 2005). Few studies investigating blood levels of vitamin E in relation to lung function; have found inconsistent results (Cook et al., 1997, Grievink et al., 1999). A study found that antioxidant vitamins may play a role in respiratory health and that vitamin E appear to have stronger correlation of lung function than other antioxidant vitamins (Schunemann et al., 2001).

Vitamin E and immune function

Vitamin E is an oxidant scavenger that acts to protect cell membrane from damage by reactive oxygen species (Field et al., 2002). Supplementation with vitamin E enhance cell-mediated and humoral immune responses in both animal and human models (Meydani et al., 1986, Meydani, 1990b). Meydani et al have shown that supplementation with vitamin E significantly increases antibody titres to hepatitis B and tetanus vaccines, and the delayed-type hypersensitivity skin response, IL-2 production and the mitogenic responses to an optimal dose of concanavalin A, and decreases prostaglandin (PG) E2 synthesis by peripheral-blood mononuclear cells in elderly human subjects (Meydani, 1990a, Meydani et al., 1997).

The immunostimulatory effect of vitamin E seems to be mediated by either reducing PGE_2 synthesis and/or decreasing free radical formation by preventing lipid peroxiadtion (Prasad, 1980). Vitamin E supplementation has been shown to affects some of the immunological factors involved in the control of influenza infection, including NK cells, Th1 cytokine (IL 2 and IFN-γ) production, and production of suppressive factors (e.g. PGE_2) which regulate CTL activity. Wang et al showed that in retrovirus-infected C57BL/6 mice, vitamin E supplementation restored the virus-induced decrease production of IL 2 and IFN-γ by splenocytes, and prevented virus-induced suppression of NK activity (Wang et al., 1994). Vitamin E supplementation also prevents the sheep erythrocyte-induced suppression of NK activity (Meydani et al., 1988). Decreased PGE_2 synthesis observed with vitamin E supplementation may be one of the mechanisms through which vitamin E exerts its effect on NK activity, since PGE_2 has been shown to inhibit NK and CTL activity (Parhar and Lala, 1988). Furthermore, vitamin E influences lymphocyte maturation, possibly by stabilising

membranes and allowing enhanced binding of antigen-presenting cells to immature T cells via increased expression of intracellular adhesion molecule-1 (ICAM-1) (Moriguchi, 1998).

The immunostimulatory effect of vitamin E has been shown to be associated with increased resistance to infections. Animal studies reported a protective effect (Hayek et al., 1997, Hybertson et al., 2005).

α-tocopherol status during inflammation

Plasma levels of tocopherol are known to be reduced during inflammation (Strayer et al., 1998) but there is no information of the association between tocopherol and APPs or chronic phase proteins.

This chapter aims to describe the micronutrients status of children with RSV and HMPV and its relation to disease severity.

Result

A total of 246 (79%) out of the 310 children with RSV and/or HMPV infections were randomly selected to measure the serum concentrations of zinc, selenium, copper, retinol, α-tocopherol and CRP. One hundred and eighty three (74%) of the children were infected with RSV, 39 (16%) with HMPV and 24 (10%) with RSV/HMPV. Table 6.2 shows the number of patients tested for serum micronutrients and CRP. Three samples had technical reading errors and were excluded from the analysis.

Table 6.2 Number of patients tested for serum micronutrients and CRP

Micronutrient/ CRP	All n = 246	RSV n = 183 (74%)	HMPV n = 39 (16%)	RSV/HMPV n = 24 (10%)
Zinc	240	181 (75)	38 (16)	21 (9)
Selenium	242	183 (76)	38 (16)	21 (8)
Copper	239	180 (75)	38 (16)	21 (9)
Retinol	213	150 (70)	39 (18)	24 (11)
α-tocopherol	165	124 (75)	21 (13)	20 (12)
CRP	188	133 (71)	38 (20)	17 (9)

Table 6.3 shows the geometrical mean serum concentrations and the range of each micronutrients and CRP, and the number of children with micronutrient deficiencies.

The GM zinc concentrations in patients with RSV and RSV/HMPV coinfections were similar and higher than in children with HMPV. A higher proportion of children with HMPV (81%) and dual RSV/HMPV infection (55%) had hypozincaemia (< 0.98 μmol zinc/l) than children with RSV (38%).

The GM selenium concentrations of children with RSV, HMPV and RSV/HMPV were similar and similar proportions of children with RSV, HMPV and RSV/HMPV had selenium concentration < 1.21 μmol/l (cut-off point selected for deficiency) (Lockitch et al., 1988) (Figure 6.1a).

In agreement with the zinc concentrations, the GM copper concentrations of children with RSV were lower than in children with HMPV (17.5 versus 24.3 μmol/l, $p < 0.001$) and RSV/HMPV (18.3 μmol/l, $p = 0.4$). Thirty one (17%), 1 (3%) and 3 (14%) children with RSV, HMPV and RSV/HMPV respectively were copper deficient with copper concentrations < 12.6 μmol/l (Lockitch et al., 1988) (Figure 6.1a).

GM zinc/copper ratio of children with RSV was significantly higher than in children with HMPV (0.6 vs. 0.4, $p<0.001$). However, children with HMPV also had significantly lower zinc/copper ratios than children with coinfections (0.4 vs. 0.6, $p<0.001$) (Figure 6.1b).

The GM retinol concentration of children with RSV was significantly lower than in children with HMPV (0.5 vs. 1.6 μmol/l, respectively $p<0.01$). Ninety (60%) children with RSV, 3 (8%) children with HMPV and 10 (44%) children with RSV/HMPV had serum retinol concentrations ≤ 0.7 μmol/l , the usual cut off point for deficiency (Figure 6.1b).

The GM α-tocopherol concentration of children with RSV were significantly lower than in children with HMPV (17.6 vs. 27.3 μmol/l, $p<0.001$). Children with RSV/HMPV coinfections also had lower concentrations (21.3 μmol/l, $p=0.3$) but the difference between these latter groups was not statistically significant. Forty three (35%) children with RSV, 4 (19%) children with HMPV and 6 (30%) children with RSV/HMPV coinfection had α-tocopherol deficiency (i.e. serum concentrations ≤16.3 μmol/l) (figure 6.1b).

The GM serum CRP concentrations in children with RSV, HMPV and RSV/HMPV were 13.5, 10.6 and 13.7 mg/l respectively and these values were not statistically different ($p > 0.05$) (Figure 6.1c).

Table 6.3 Micronutrients and CRP concentrations in children with RSV, HMPV and RSV/HMPV

GM (SE), range (µmol/l) and number (%) with deficiency	Normal ranges (µmol/L)	RSV	HMPV	RSV/HMPV	P¹	P²	P³
Zinc	9.8 - 18.1	11.11 (1.03)	9.2 (1.04)	11.1 (1.12)	**0.01**	0.9	0.06
		2.6 - 37.1	5.69 -19.91	6.8 - 36.1			
		67 (38 %)	26 (81%)	11 (55%)	**<0.001**	0.07	0.1
Selenium	1.22 - 2.05	0.9 (1.03)	0.9 (1.05)	0.9 (1.07)	0.6	0.8	0.8
		0.1 - 2.1	0.44 -1.8	0.5 - 1.6			
		153 (84%)	33 (89%)	17 (81%)	0.3	0.3	0.3
Copper	12.6 - 23.6	17.5 (1.02)	24.3 (1.05)	18.3 (1.08)	**<0.001**	0.7	**0.002**
		6.3 - 45	9.86 - 42.43	5.9 - 26.5			
		31 (17%)	1 (3%)	3 (14%)	**<0.01**	0.3	0.1
Zinc/Copper ratio		0.6 (1.03)	0.4 (1.06)	0.6 (1.12)	**<0.001**	0.8	**<0.001**
		0.18 - 2.8	0.16 - 1.3	0.3 - 1.6			
Retinol	0.3 - 1.2	0.5 (1.07)	1.6 (1.07)	0.8 (1.14)	**<0.001**	**0.05**	**<0.001**
		0.1 - 2.78	0.5 - 2.78	0.2 - 2.78			
		90 (60%)	3 (8%)	10 (44%)	**<0.001**	0.06	**0.001**
α-tocopherol	5.5 - 18	17.6 (1.06)	27.3 (1.12)	21.3 (1.12)	**<0.001**	0.3	**0.05**
		0.62 - 37.12	6.17 - 37.12	6.18 - 37.12			
		43 (35%)	4 (19%)	6 (30%)	0.12	0.3	0.3
CRP	0-0.8*	13.5 (1.12)	10.6 (1.25)	13.7 (1.27)	0.3	0.9	0.4
		3 - 320	3 - 446	3 - 58			

P¹= p value for RSV vs. HMPV, P²= p value for RSV vs. RSV/HMPV and P³= p value for HMPV vs. RSV/HMPV

Normal range were quoted from (Lockitch et al., 1988), * mg/l

Figure 6.1a Zinc, selenium and copper concentrations of children with RSV, HMPV and RSV/HMPV (the graph shows the GM, SE (dots), 25th and 75th centiles)

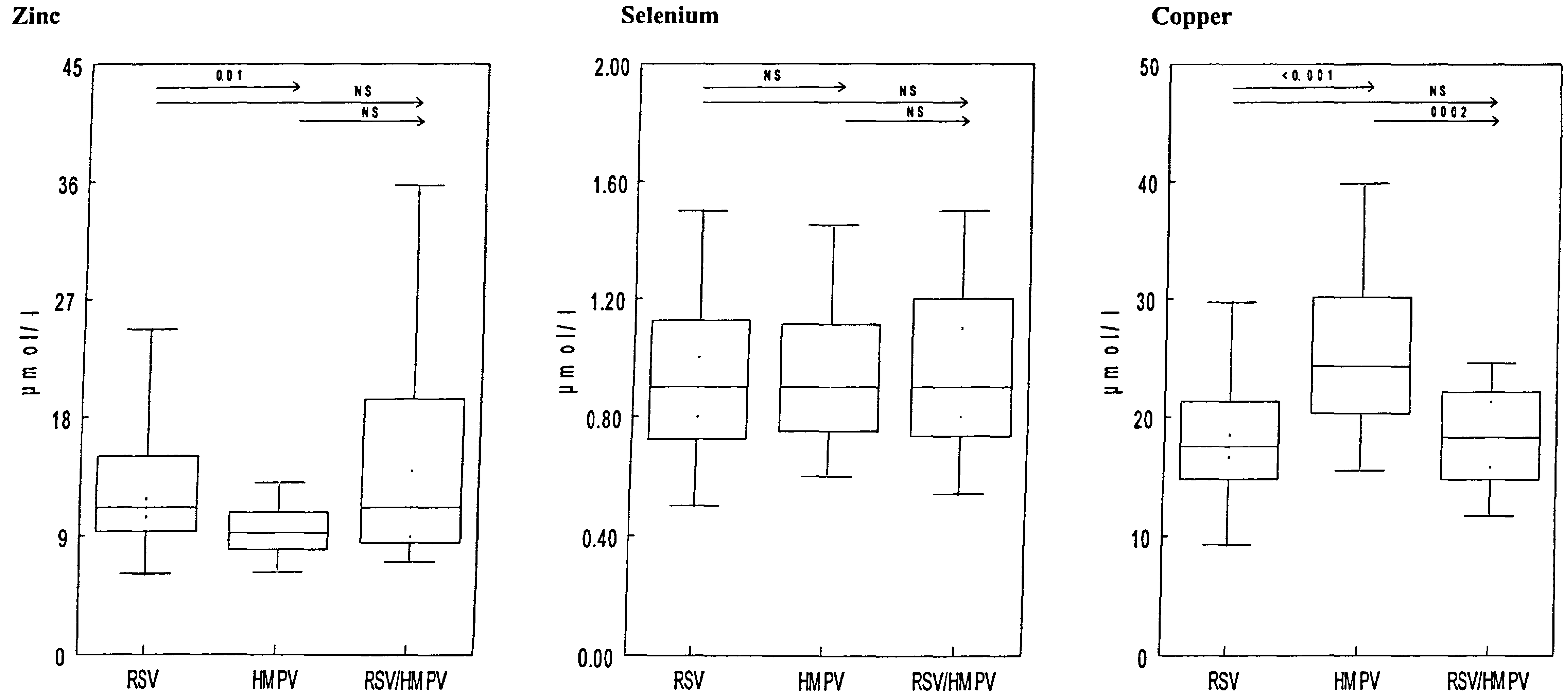


Figure 6.1b Zinc/copper ratios, retinol and α-tocopherol concentrations of children with RSV, HMPV and RSV/HMPV (the graph shows the GM, SE (dots), 25th and 75th centiles)

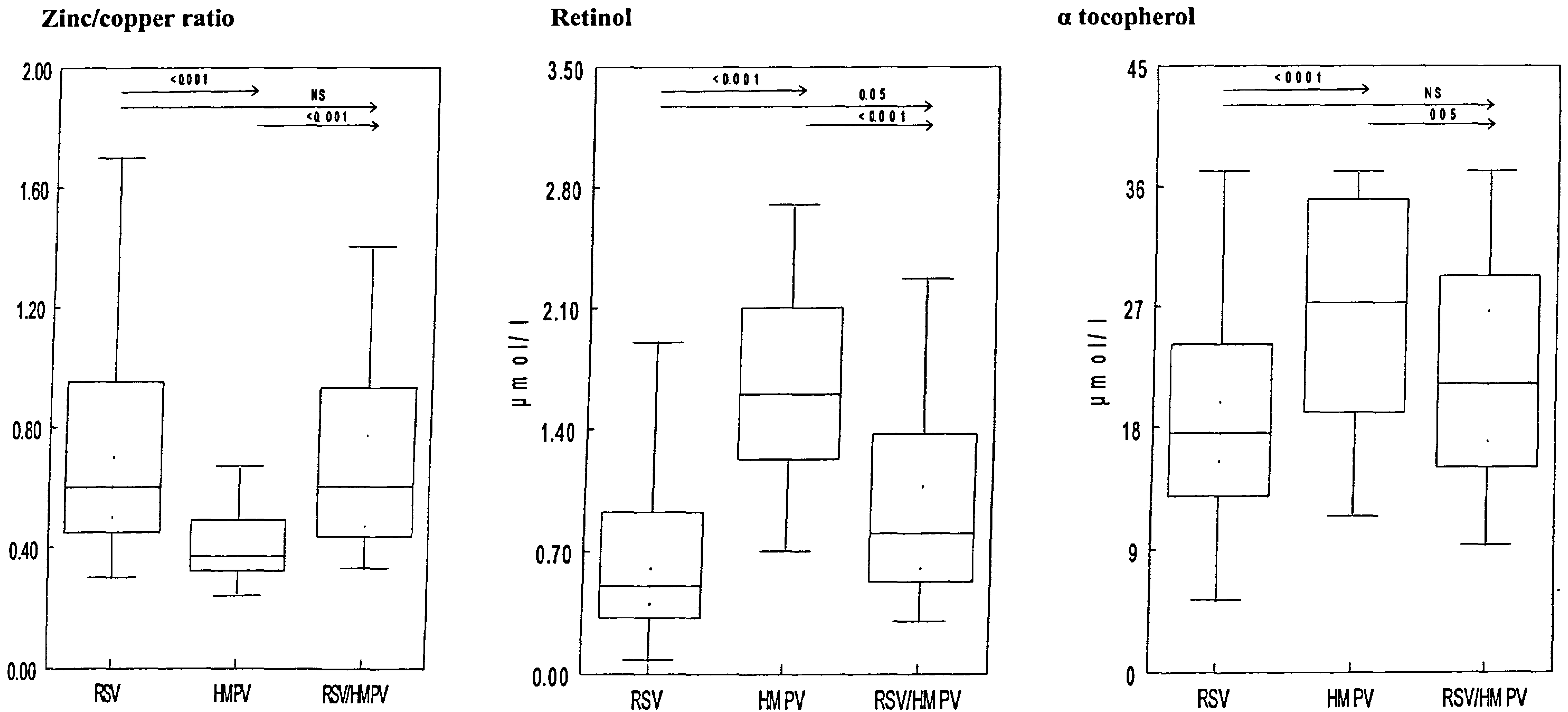
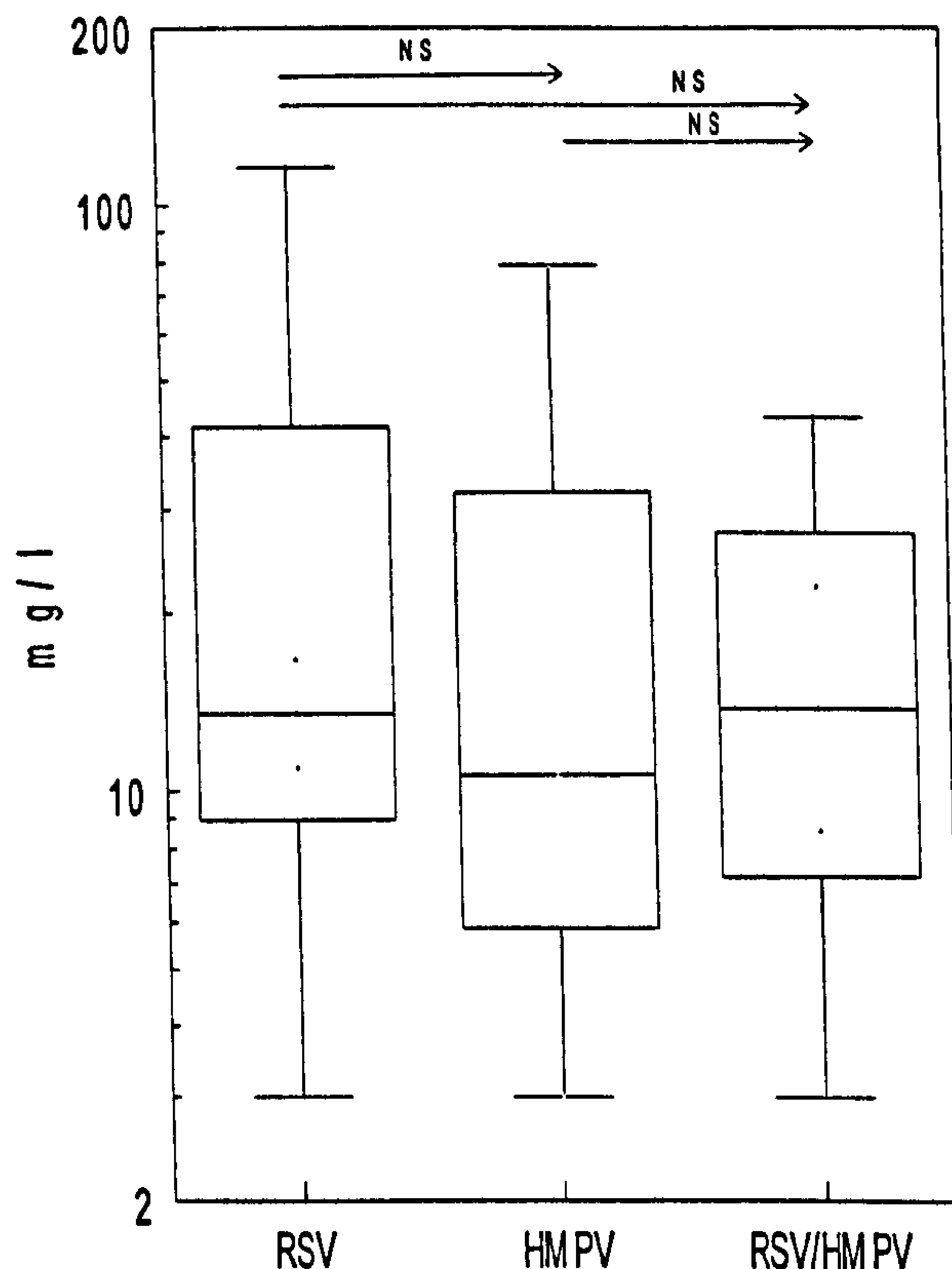


Figure 6.1c CRP concentrations of children with RSV, HMPV and RSV/HMPV (the graph shows the GM, SE (dots), 25th and 75th centiles)



Patients were further analysed accorded to whether they had CRP/l > or ≤ 5 mg/l . concentrations (Gabay and Kushner, 1999). Table 6.4 shows the numbers of samples with zinc, selenium, copper, retinol and α-tocopherol results according to these CRP, cut-offs.

Table 6.4 Number of samples tested for micronutrients by CRP cut-offs

	RSV		HMPV		RSV/HMPV	
	*CRP ≤ 5	CRP >5	CRP ≤ 5	CRP >5	CRP ≤ 5	CRP >5
	n = 40	n = 92	n = 16	n = 22	n = 4	n = 11
Zinc	40 (100%)	90 (98%)	16 (100%)	22 (100%)	4 (100%)	11 (100%)
Selenium	40 (100%)	92 (100%)	16 (100%)	22 (100%)	4 (100%)	11 (100%)
Copper	40 (100%)	92 (100%)	16 (100%)	22 (100%)	4 (100%)	11 (100%)
Retinol	27 (68%)	62 (67%)	16 (100%)	20 (91%)	4 (100%)	11 (100%)
α-tocopherol	23 (58%)	52 (57%)	10 (63%)	10 (45%)	3 (75%)	10 (91%)

CRP ≤ 5 or > 5 mg/l

Table 6.5 and Figure 6.2 show the GM (SE) serum and range of the micronutrients by viruses and CRP ≤ 5 or > 5 mg/l. The GM copper concentrations were significantly higher in children with RSV and CRP > 5 mg/l than in children with CRP ≤ 5 mg/l (p=0.01) and the zinc/copper

ratios were lower in children with raised CRP ($p=0.002$). The GM zinc, selenium, retinol and α-tocopherol concentrations were not significantly different between the children with RSV and raised or normal CRP (Table 6.5).

The GM zinc concentrations were significantly lower and the copper concentrations significantly higher in children with HMPV and CRP concentrations > 5 mg/l than in children with normal CRP. The zinc/copper ratios of children with raised CRP were also significantly lower (0.5 vs. 0.3, $p=0.001$). The GM selenium, retinol and α-tocopherol concentrations were similar in children with raised and normal CRP.

Children with RSV/HMPV coinfections and raised CRP had higher, copper and α-tocopherol concentrations than children with normal CRP, while zinc, selenium and retinol concentrations were not significantly different between the children with raised and normal CRP.

Table 6.5 Serum micronutrient and CRP concentrations of children with RSV, HMPV and RSV/HMPV by CRP cut-offs

Micronutrient GM (SE) and range	RSV		P	HMPV		P	RSV/HMPV		P
	CRP ≤ 5	CRP > 5		CRP ≤ 5	CRP > 5		CRP ≤ 5	CRP > 5	
Zinc	12.2 (1.06)	10.7 (1.05)	0.09	10.2 (1.06)	8.5 (1.05)	**0.03**	13.3 (1.18)	12.3 (1.2)	0.8
	7.5 - 31.5	4.5 - 37.1		7.3 – 19.9	10.9 - 5.93		8.1 - 16.7	6.8 - 36.1	
Selenium	0.9 (1.03)	0.9 (1.03)	0.5	0.9 (1.08)	0.9 (1.06)	0.6	1.1 (1.19)	0.9 (1.09)	0.2
	0.6 - 1.5	0.3 - 2.1		0.44 - 1.47	0.53 - 1.8		0.7 - 1.6	0.54 - 1.5	
Copper	16.6 (1.06)	19.6 (1.03)	**0.01**	20.4 (1.08)	27.5 (1.06)	**0.003**	16.5 (1.16)	21.8 (1.04)	**0.02**
	8.2 - 36.1	7.5 - 45		9.86 - 32.82	17.4 - 42.43		11.7 - 22.5	17.3 - 26.5	
Zinc/copper ratio	0.7 (1.08)	0.5 (1.05)	**0.002**	0.5 (1.09)	0.3 (1.05)	**0.001**	0.8 (1.3)	0.6 (1.2)	0.3
	0.3 - 2.8	0.18 - 2.6		0.2 - 1.3	0.16 - 0.5		0.3 - 1.43	0.3 - 1.6	
Retinol	0.6 (1.21)	0.5 (1.11)	0.07	1.6 (1.12)	1.7 (1.1)	0.5	0.9 (1.47)	0.9 (1.23)	0.9
	0.1 - 2.78	0.1 - 1.9		0.5 - 2.78	0.6 - 2.78		0.3 - 1.67	0.2 - 2.78	
α-tocopherol	17 (1.16)	15.6 (1.11)	0.4	25.6 (1.22)	31.7 (1.11)	0.4	10.3 (1.29)	23 (1.16)	**0.04**
	3.6 - 37.12	0.6 - 37.12		6.1 - 37.12	12.7 - 37.12		6.1 - 14.4	9.6 - 37.12	

Figure 6.2a Zinc, selenium and copper concentrations of children with RSV, HMPV and RSV/HMPV by CRP cut-offs (A= CRP ≤ 5 µmol/l ; B = CRP > 5µmol/l) (the graph shows the GM, SE (dots), 25th and 75th centiles)

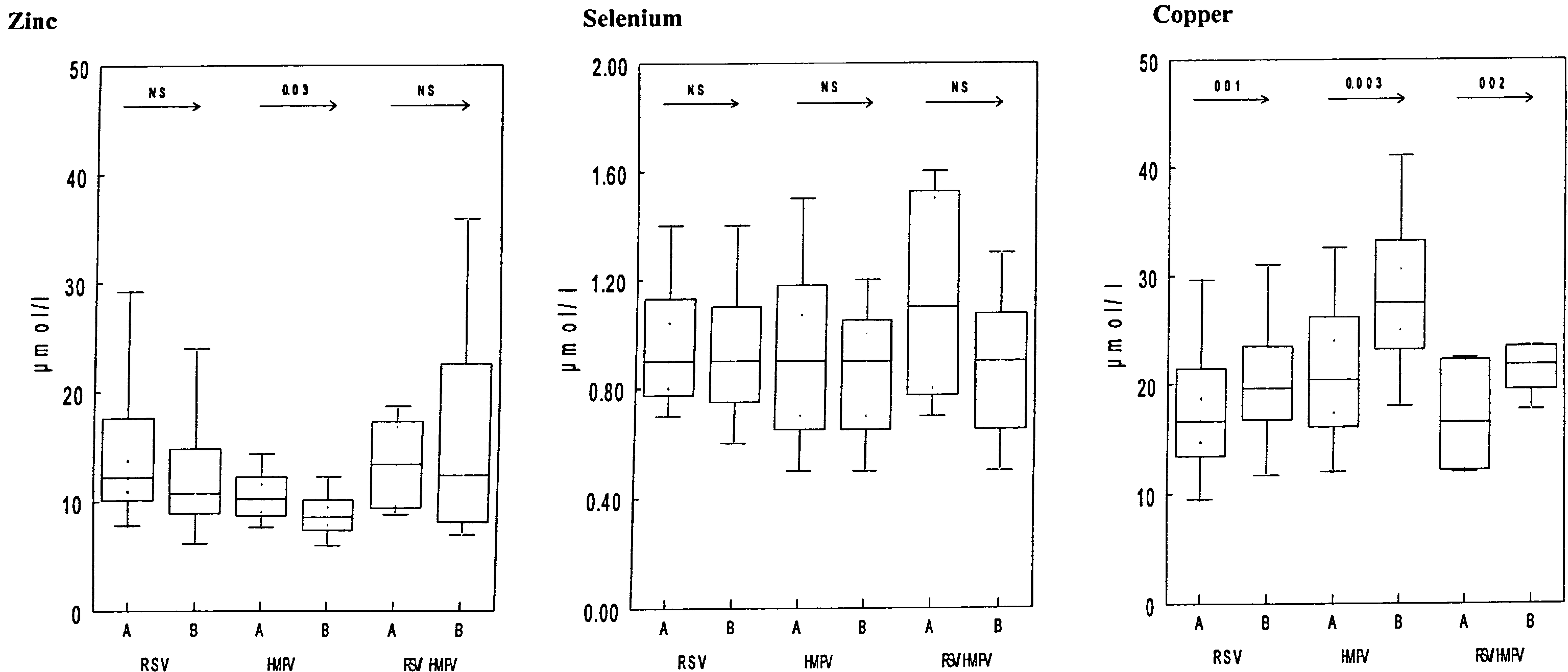
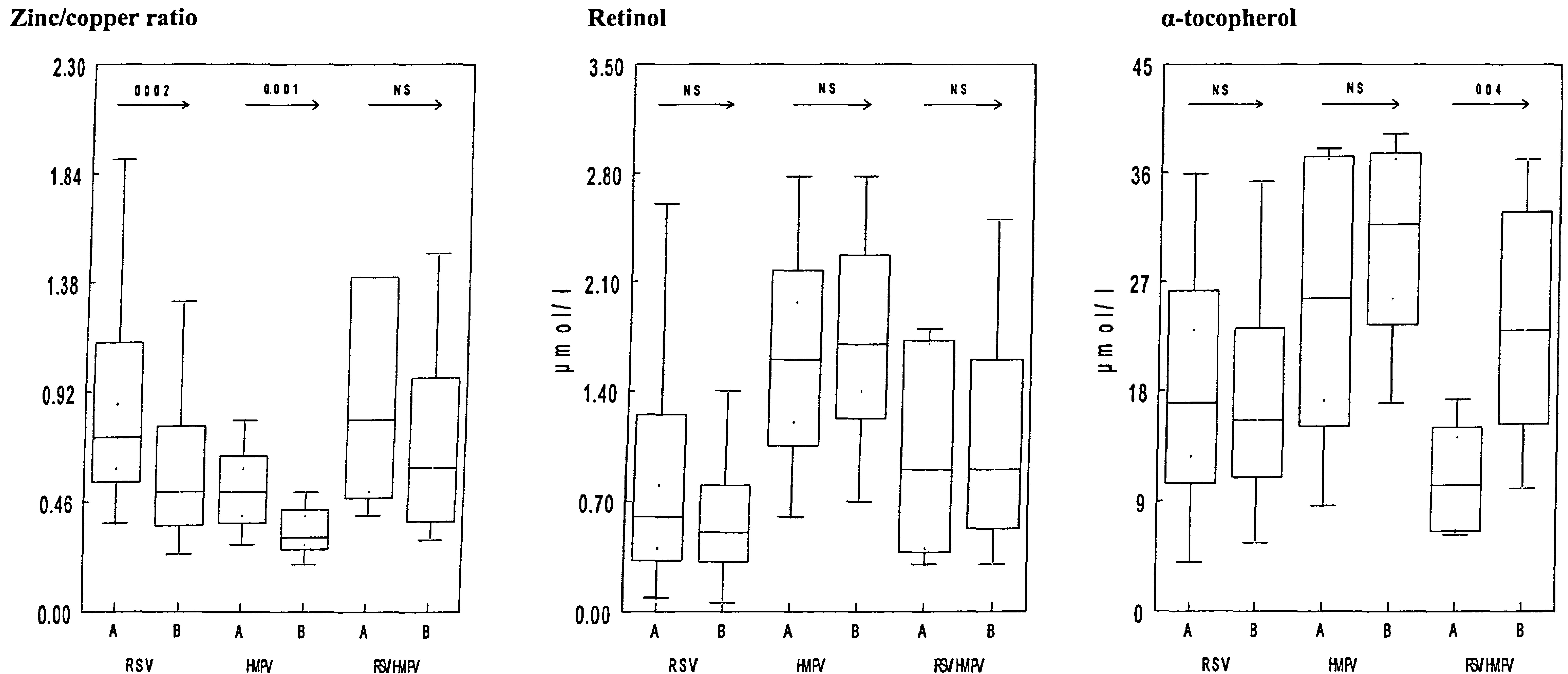


Figure 6.2b Zinc/copper ratio, retinol and α-tocopherol concentrations and zinc/copper ratios of children with RSV, HMPV and RSV/HMPV by CRP cut-offs (A= CRP ≤ 5 µmol/l ; B = CRP > 5µmol/l) (the graph shows the GM, SE (dots), 25th and 75th centiles)



Micronutrient and CRP concentrations and disease severity

Table 6.6 shows the numbers of samples tested for the micronutrients according to the disease severity of the children.

Table 6.6 Number of samples tested for micronutrients and CRP by disease severity

	RSV		HMPV		RSV/HMPV	
	Mild	Severe	Mild	Severe	Mild	Severe
	n = 31	n = 210	n = 17	n = 24	n =3	n = 22
Zinc	26 (83%)	152 (72%)	17 (100%)	21 (88%)	3 (100%)	18 (82%)
Selenium	26 (83%)	155 (74%)	17 (100%)	21 (88%)	3 (100%)	18 (82%)
Copper	26 (83%	152 (73%)	17 (100%)	21 (88%)	3 (100%)	18 (82%)
Retinol	26 (84%)	123 (56%)	16 (94%)	22 (92%)	3 (100%)	21 (95%)
α-tocopherol	16 (52%)	107 (91%)	14 (82%)	7 (29%)	2 (67%)	18 (82%)
CRP	17 (55%)	114 (54%)	17 (100%)	21 (88%)	2 (67%)	15 (68%)

Children with RSV, the GM zinc concentrations and zinc/copper ratios were significantly higher in children with severe RSV-related hypoxia. In contrast, the GM retinol and α-tocopherol concentrations were significantly lower in children with severe disease. The GM selenium, copper, and CRP concentrations did not differ significantly among children with mild or severe RSV disease (Figure 6.3 and 6.4, Table 6.7).

None of the micronutrient concentrations tested varied by disease severity among the children with mild and severe HMPV associated infections with exception of α tocopherol. The GM serum α-tocopherol was significantly lower in children with severe presentation ($p=0.01$) (Table 6.7)

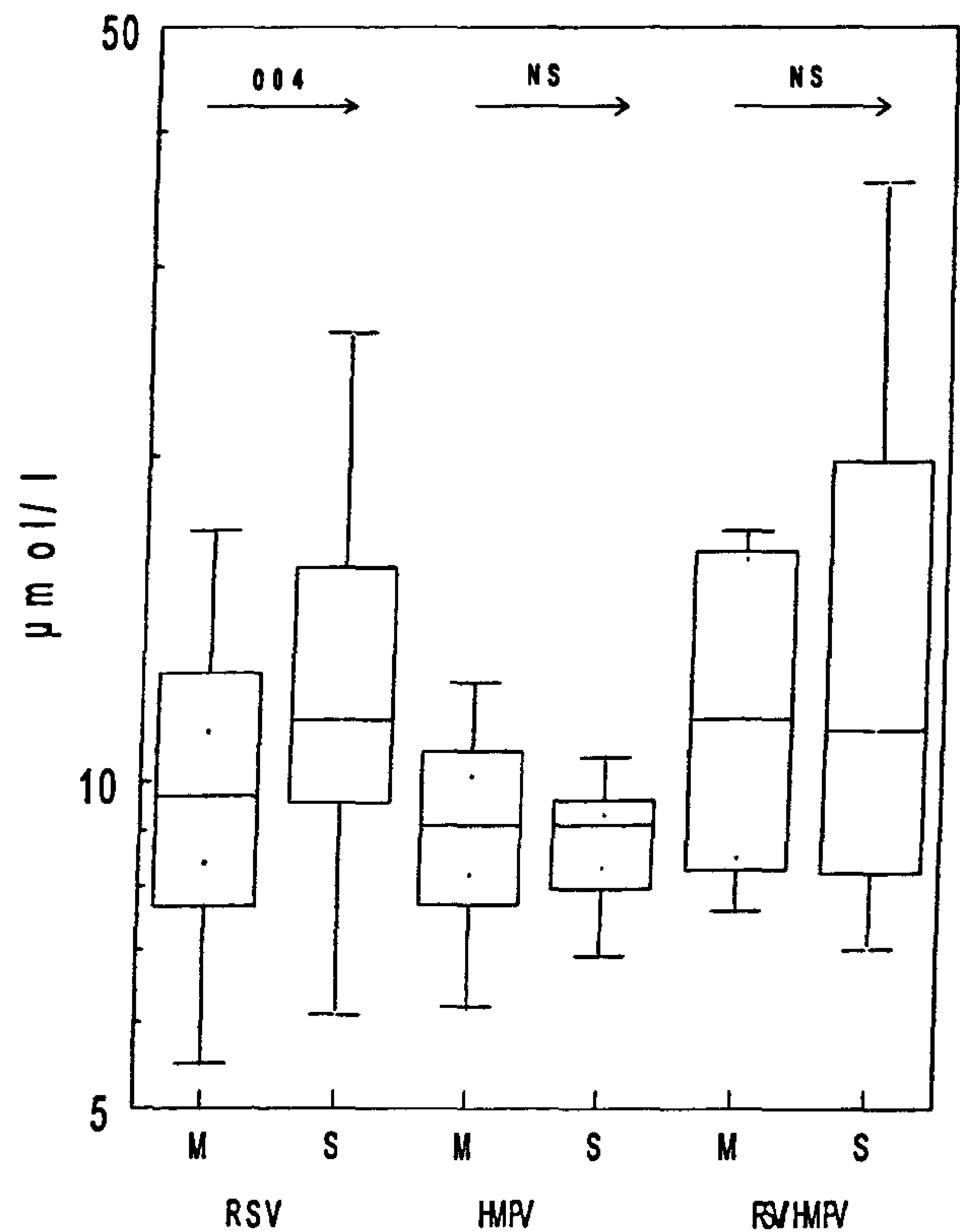
Similarly, children with RSV/HMPV coinfections had no significant differences between mild and severe disease (Figure 6.3) (Table 6.7).

Table 6.7 Micronutrient and CRP concentrations of children with RSV, HMPV and RSV/HMPV by disease severity

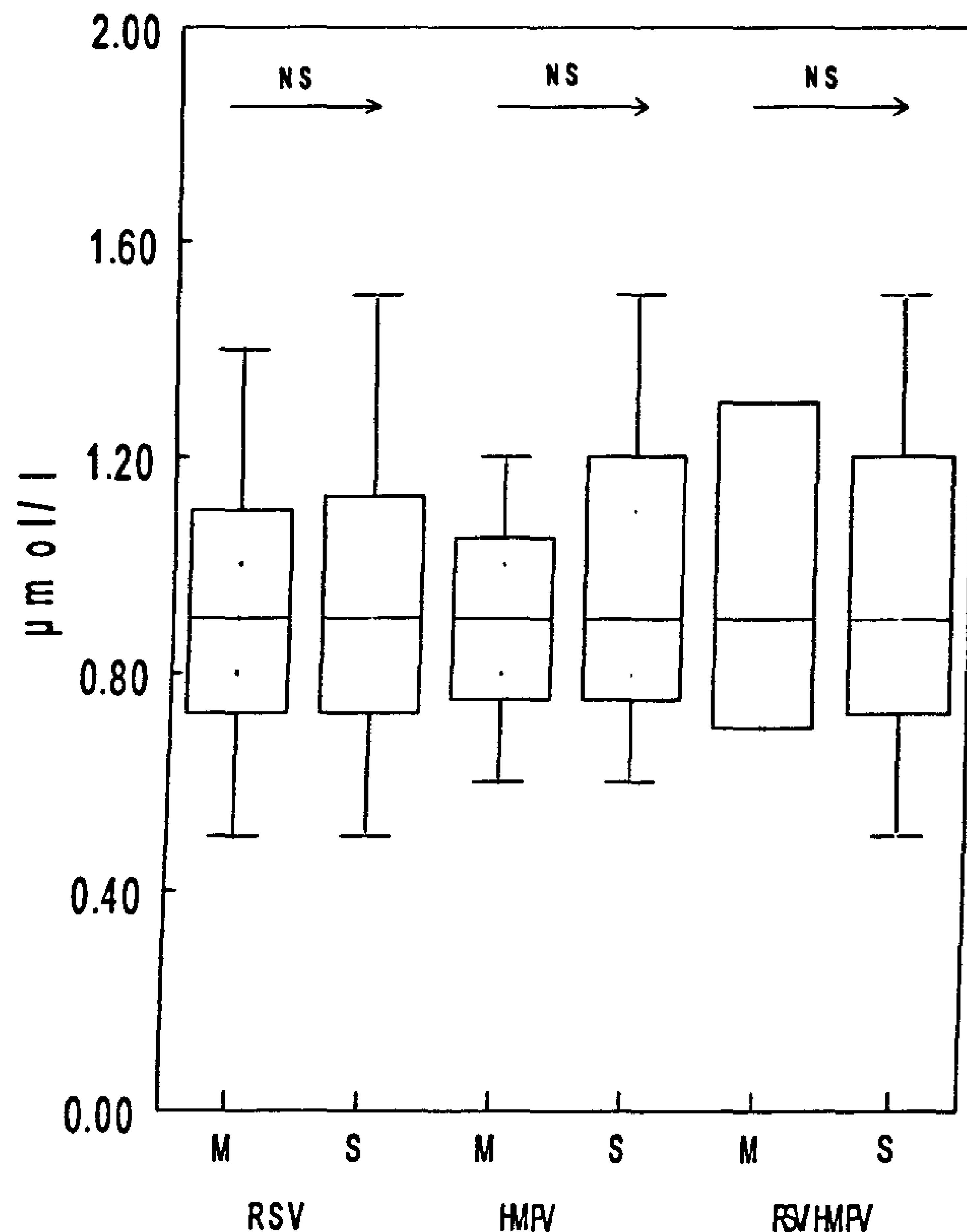
Micronutrient/ CRP GM (SE) and range	RSV		P	HMPV		P	RSV/HMPV		P
	Mild	Severe		Mild	Severe		Mild	Severe	
Zinc	9.7 (1.07)	11.4 (1.04)	**0.04**	9.1 (1.05)	9.3 (1.06)	0.7	11.4 (1.22)	11.1 (1.14)	0.9
	4.5 - 20.1	2.6 - 37.1		5.9 -12.3	6.7 - 66		8.32 - 16.7	6.8 - 36.1	
Selenium	0.9 (1.07)	0.9 (1.04)	0.9	0.9 (1.06)	0.9 (1.08)	0.3	0.9 (1.2)	0.9 (1.08)	0.9
	0.44 - 1.8	0.1 - 2.1		0.53 - 1.19	0.44 - 1.8		0.7 - 1.31	0.5 - 1.6	
Copper	18.4 (1.1)	17.3 (1.03)	0.3	23.2 (1.1)	25.1 (1.06)	0.4	19 (1.3)	18.2 (1.1)	0.8
	6.3 - 32.82	6.6 - 45		30.93 - 18.61	12.95 - 41.19		11.7 - 24.62	5.9 - 26.5	
Zinc/copper ratio	0.5 (1.11)	0.7 (1.04)	0.09	0.4 (1.11)	0.4 (1.07)	0.6	0.6 (1.55)	0.6 (1.13)	0.9
	0.22 - 1.95	0.18 -2.8		0.2 - 1.3	0.16 - 0.7		10.33 - 1.4	0.3 - 1.6	
Retinol	0.9 (1.12)	0.5 (1.08)	**0.02**	1.8 (1.11)	1.5 (1.09)	0.11	0.8 (1.5)	0.8 (1.15)	0.7
	0.2 - 2.3	0.1 - 2.78		0.6 - 2.78	0.5 - 2.78		0.33 - 1.25	0.2 - 2.78	
α-tocopherol	25.3 (1.09)	16.7 (1.07)	**0.03**	33.1 (1.08)	18.5 (1.3)	**0.01**	14.88 (2.4)	22.2 (1.11)	0.6
	12.7 -37.12	0.6 - 37.12		12.7 - 37.12	6.1 - 37.12		6.1 - 35.83	9.6 - 37.12	
CRP	14.5 (1.48)	13.4 (1.12)	0.8	7.1 (1.26)	14.7 (1.4)	0.4	7.5 (2.5)	14.9 (1.28)	0.5
	3 - 189	3 - 320		3 - 41	3 - 446		3 - 19	3 - 58	

Figure 6.3 Zinc, selenium, copper, zinc/copper ratio, retinol and α-tocopherol concentrations of children with RSV, HMPV and RSV/HMPV by disease severity

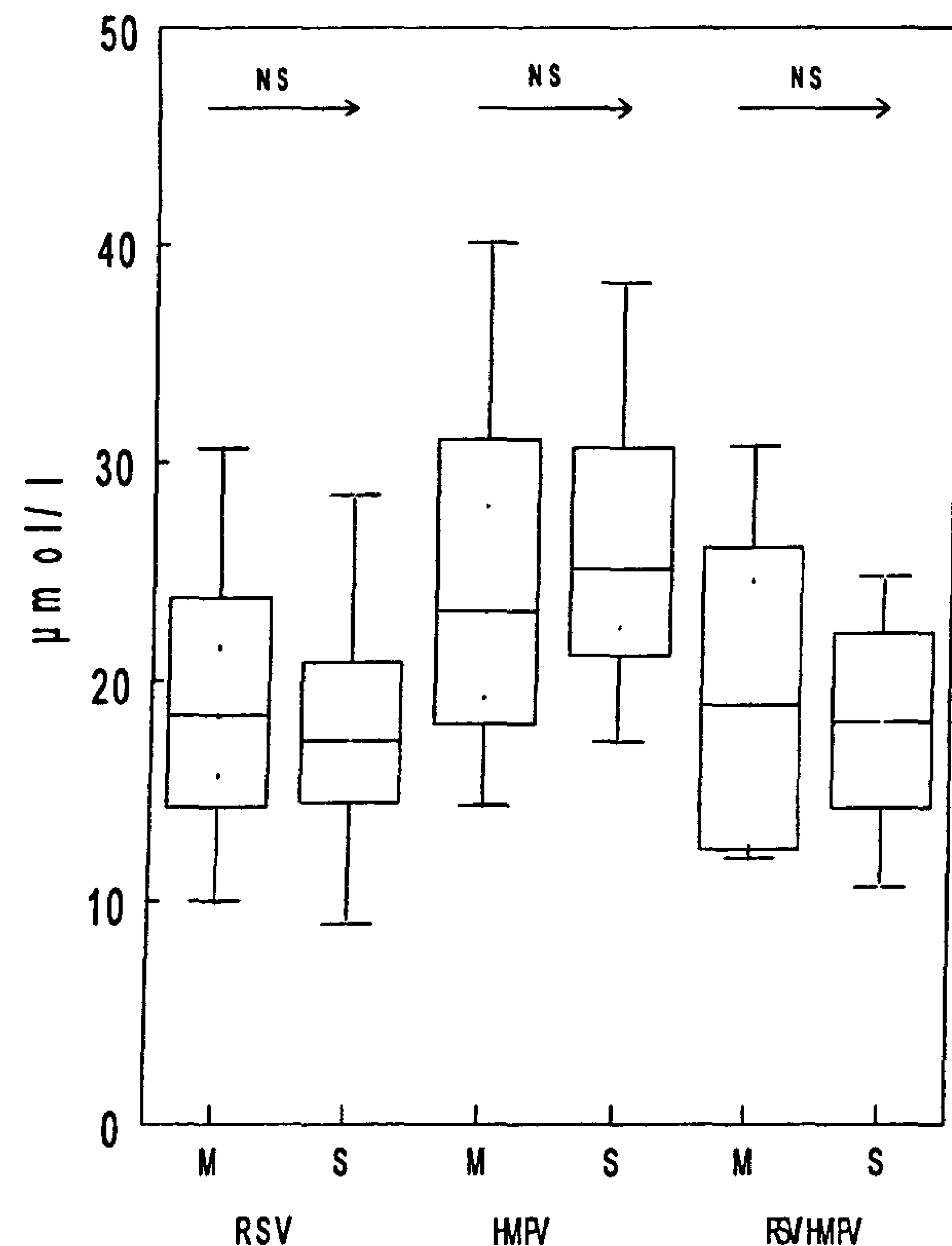
Zinc



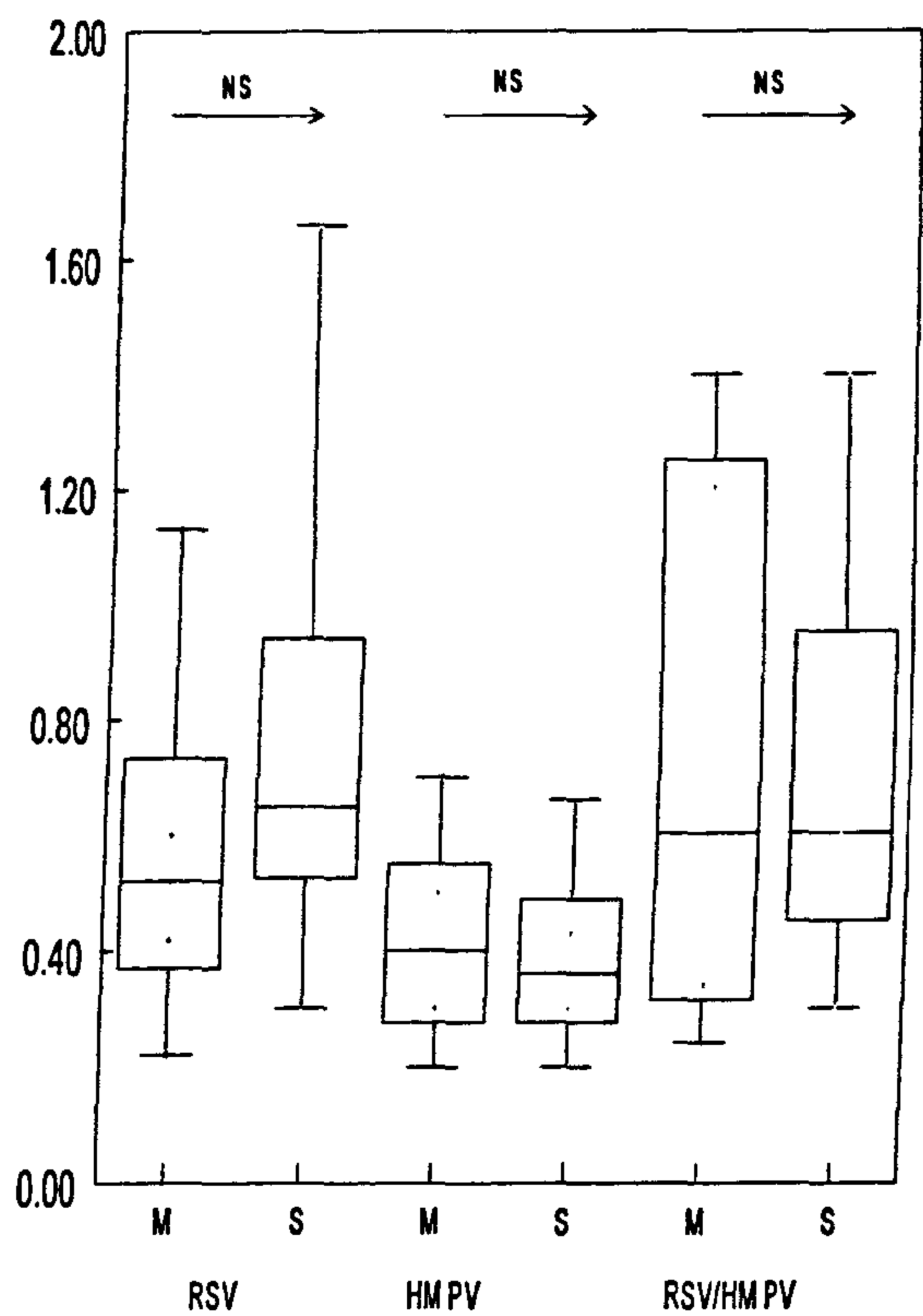
Selenium



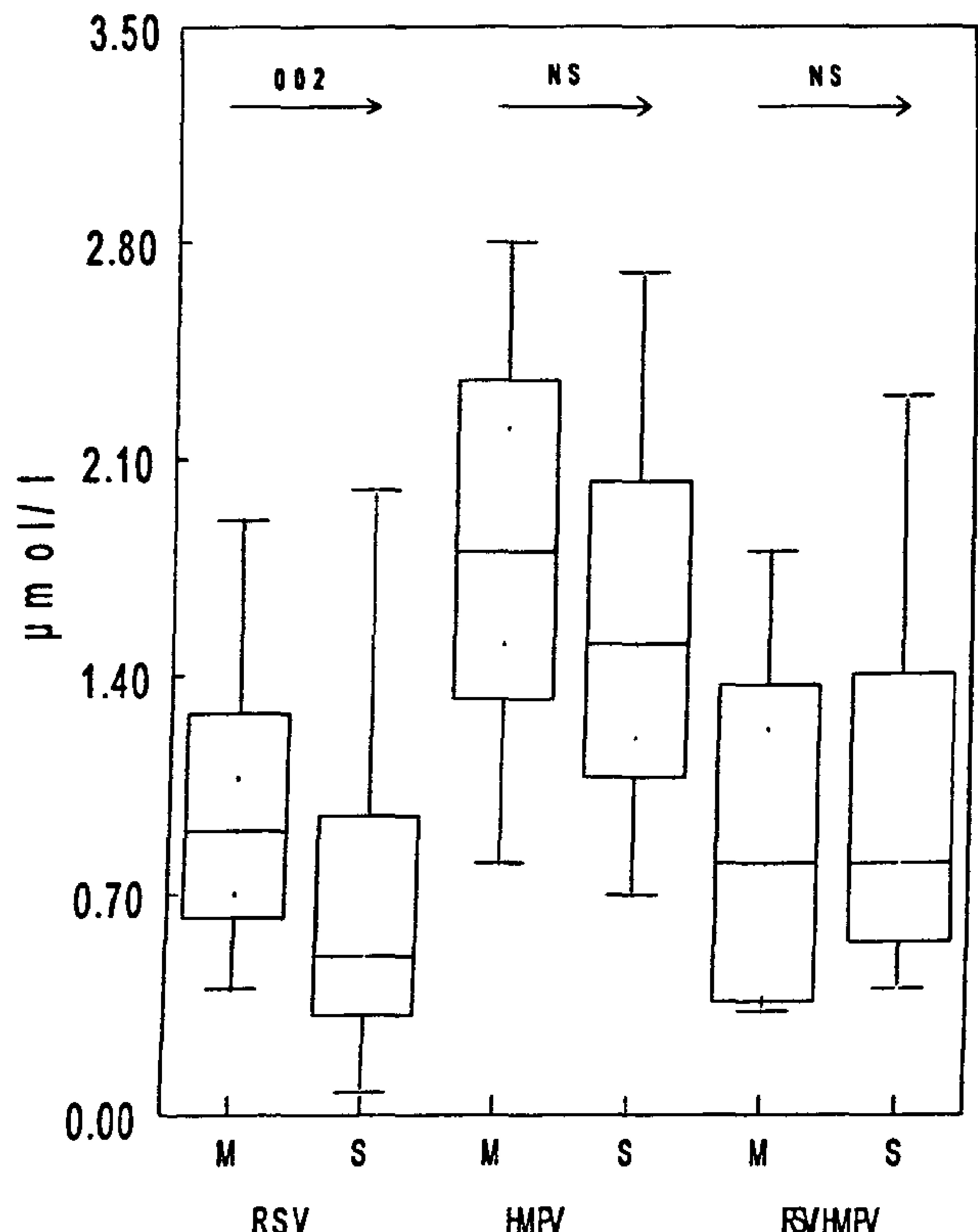
Copper



Zinc/copper ratio



Retinol



α-tocopherol

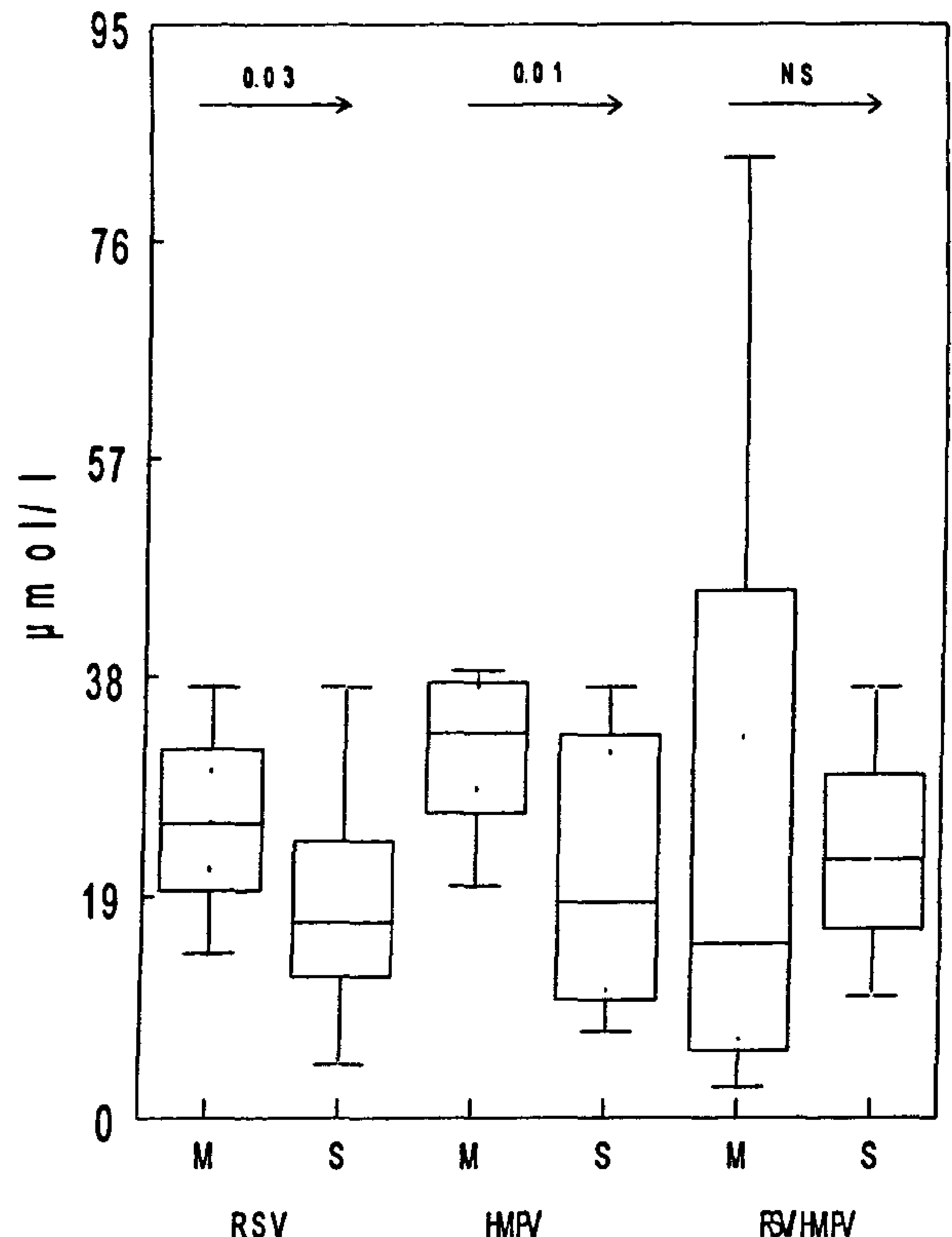
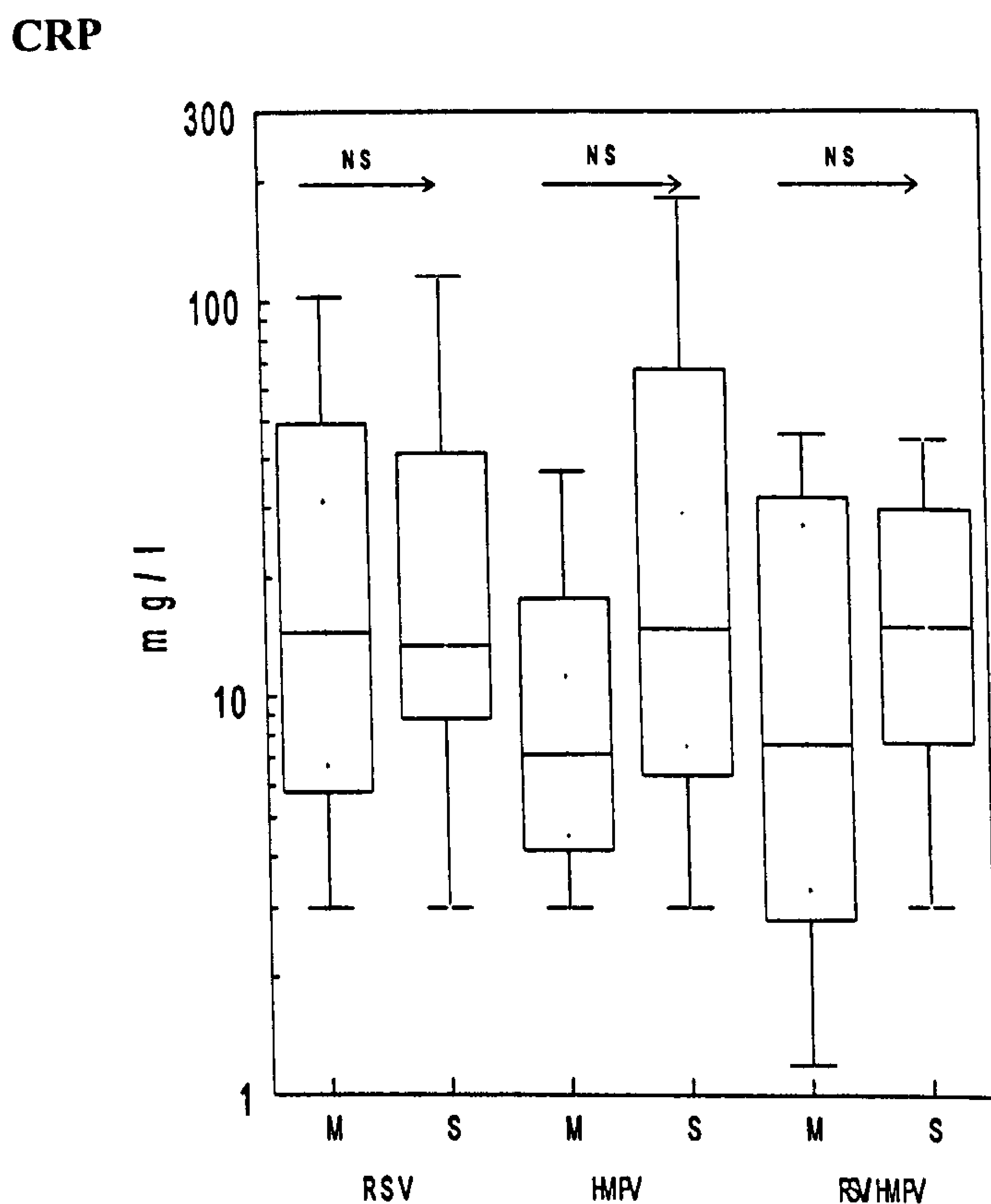


Figure 6.4 Serum CRP concentrations of children with RSV, HMPV and RSV/HMPV by disease severity



M= mild; S = severe

A further analysis of the micronutrients concentrations by RSV disease severity and CRP concentrations is shown in table 6.8 and 6.9. Table 6.10 and 6.11 describe a similar analysis for patients with HMPV. The number of patients with RSV tested by disease severity and CRP cut off shows in table 6.8.

Table 6.8 Number of patients with RSV tested for micronutrients by disease severity and CRP concentration

	Mild RSV (n =17)		Severe RSV (n =114)	
	CRP ≤ 5* n = 8	CRP >5 n = 9	CRP ≤ 5 n = 32	CRP >5 n = 82
Zinc	7 (88%)	9 (100%)	32 (100%)	80 (100%)
Selenium	7 (88%)	9 (100%)	32 (100%)	82 (100%)
Copper	7 (88%)	9 (100%)	32 (100%)	82 (100%)
Retinol	6 (75%)	9 (100%)	21 (66%)	53 (65%)
α-tocopherol	5 (63%)	5 (56%)	21 (66%)	47 (57%)

* mg/l

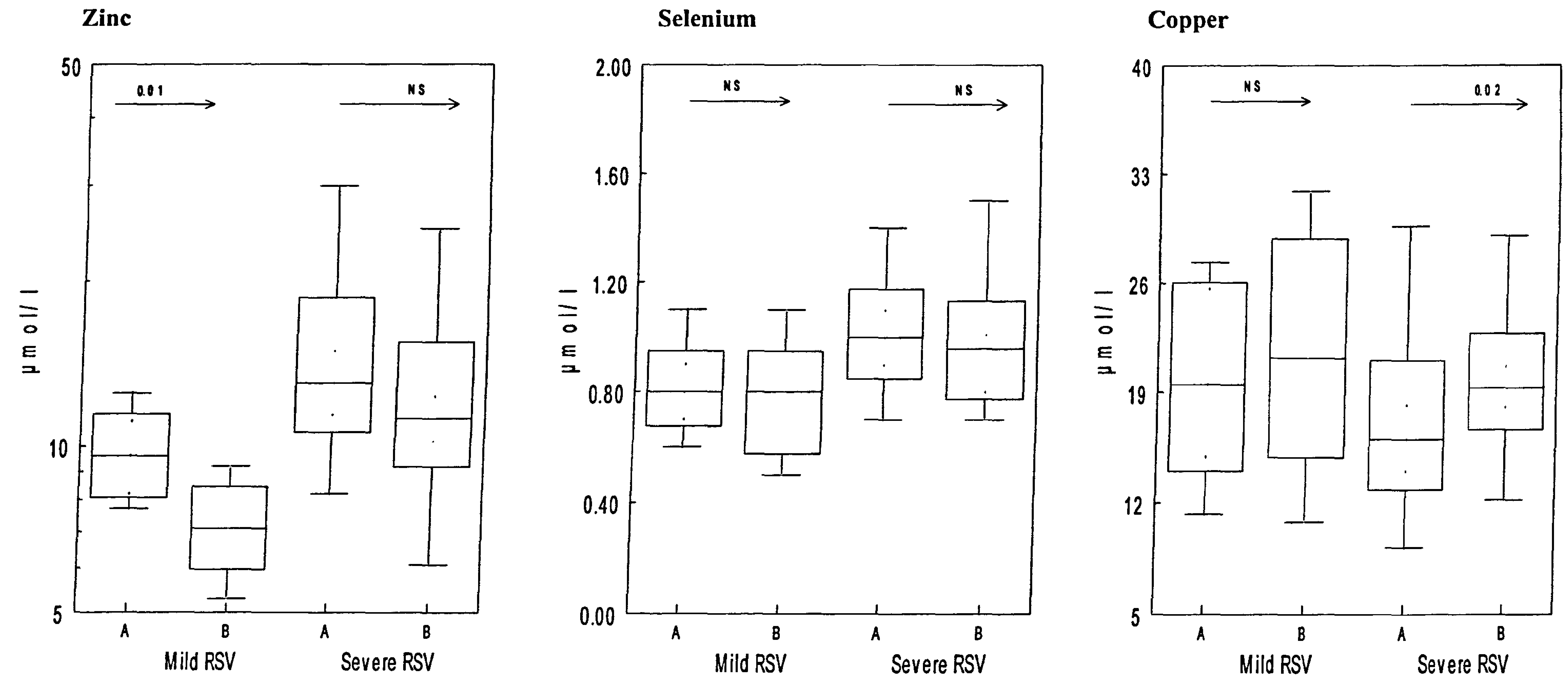
In children with RSV and mild disease presentation, the GM zinc concentrations were significantly lower in children with CRP > 5 mg/l than in children with CRP ≤ 5 mg/l . The concentrations of the remaining micronutrients were not significantly different. In children with RSV and severe disease presentation, the GM serum copper concentrations were significantly higher in children with CRP > 5 mg/l than in children with CRP ≤ 5 mg/l (p=0.005) and the zinc/copper ratios were significantly lower (0.8 vs. 0.6, p=0.002). The concentrations of the remaining micronutrients were not significantly different (Table 6.9 and Figure 6.5).

Table 6.9 Serum micronutrients concentrations in children with RSV by disease severity and CRP value

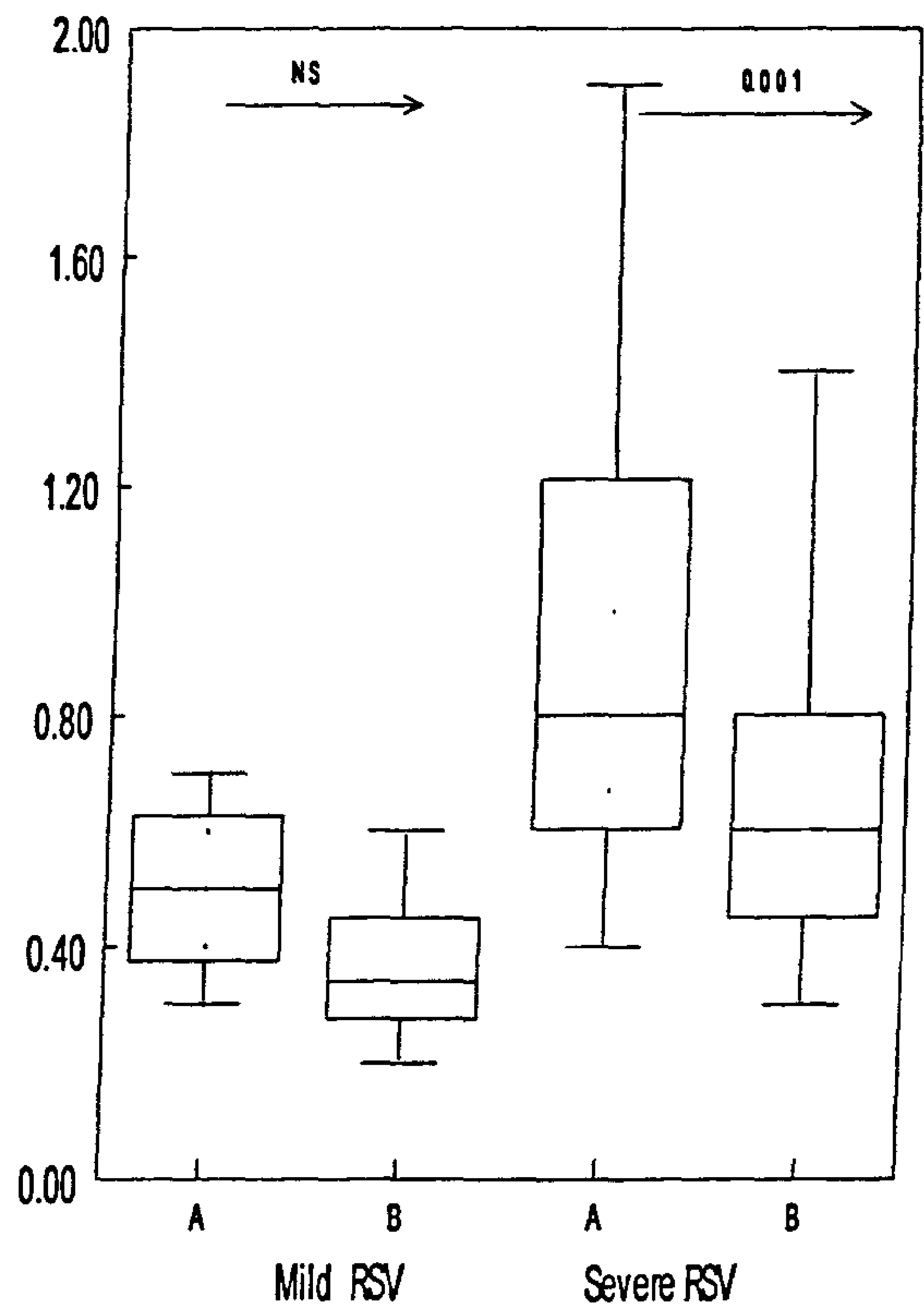
Micronutrient GM (SE) and range	RSV Mild		P	RSV Severe		P
	CRP ≤ 5*	**CRP >5**		**CRP ≤ 5**	**CRP >5**	
Zinc	9.6 (1.07) 7.68 - 13.13	7.1 (1.07) 4.5 - 10.52	**0.01**	13 (1.07) 7.5 - 31.5	11.2 (1.05) 5.1 - 37.1	0.07
Selenium	0.8 (1.08) 0.64 - 1.23	0.8 (1.1) 0.44 -1.14	0.4	1 (1.04) 0.7 - 1.5	0.96 (1.03) 0.3 - 2.1	0.4
Copper	19.5 (1.14) 9.8 - 27.27	21.2 (1.14) 10.1 - 32.82	0.5	16 (1.07) 8.2 - 36.1	19.3 (1.03) 7.5 - 45	**0.02**
Zinc/copper	0.5 (1.1) 0.3 - 0.8	0.3 (1.13) 0.2 - 0.74	0.09	0.8 (1.1) 0.3 - 2.8	0.6 (1.05) 0.18 - 2.6	**0.001**
Retinol	0.8 (1.32) 0.4 - 2.31	0.6 (1.2) 0.4 - 0.5	0.2	0.5 (1.26) 0.07 - 2.78	0.5 (1.13) 0.04 -1.9	0.7
A-tocopherol	28.2 (1.18) 14.5 - 35.24	20.4 (1.13) 12.7 - 27.14	0.09	6.6 (1.56) 0.04 - 37.12	15.1 (1.12) 0.6 - 37.12	0.9

* mg/l

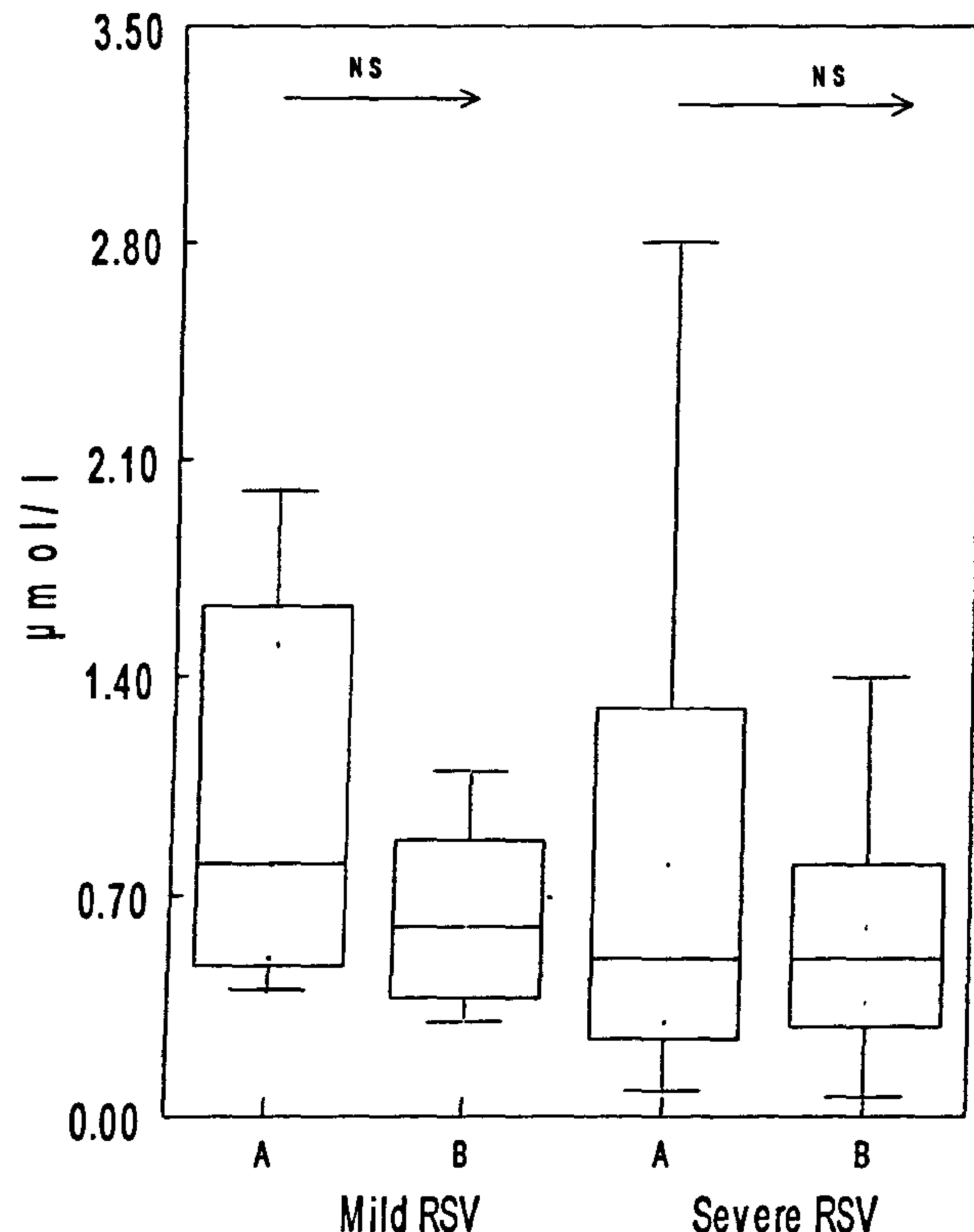
Figure 6.5 Zinc, selenium, copper, zinc/copper ratio, retinol and α-tocopherol concentrations of children with RSV by disease severity and CRP cut-offs



Zinc/copper ratio



Retinol



α-tocopherol

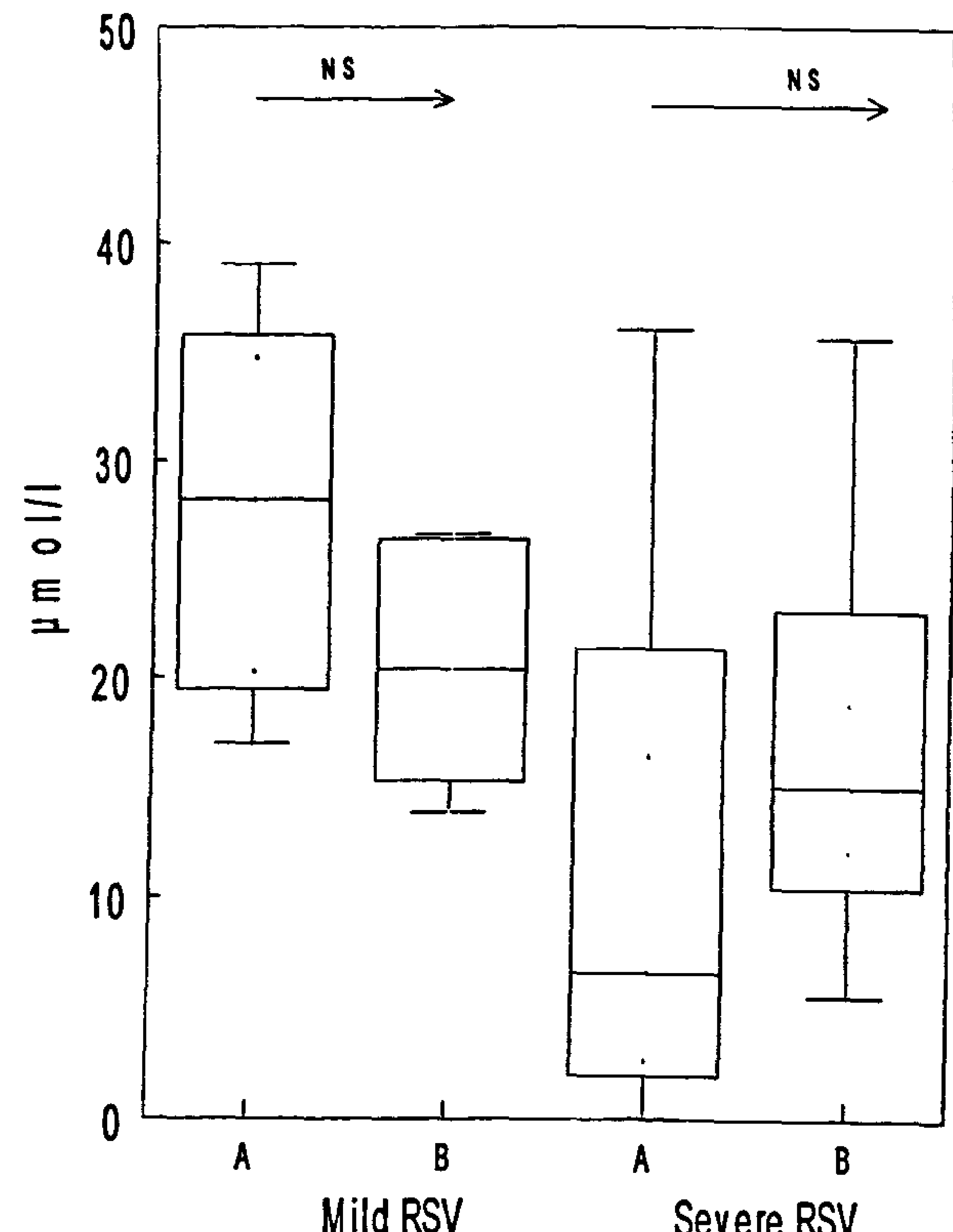


Table 6.10 shows the number of patients with HMPV tested for micronutrients by disease severity and CRP cut-offs.

Table 6.10 Number of patients with HMPV tested for serum micronutrients by disease severity and CRP cut-offs

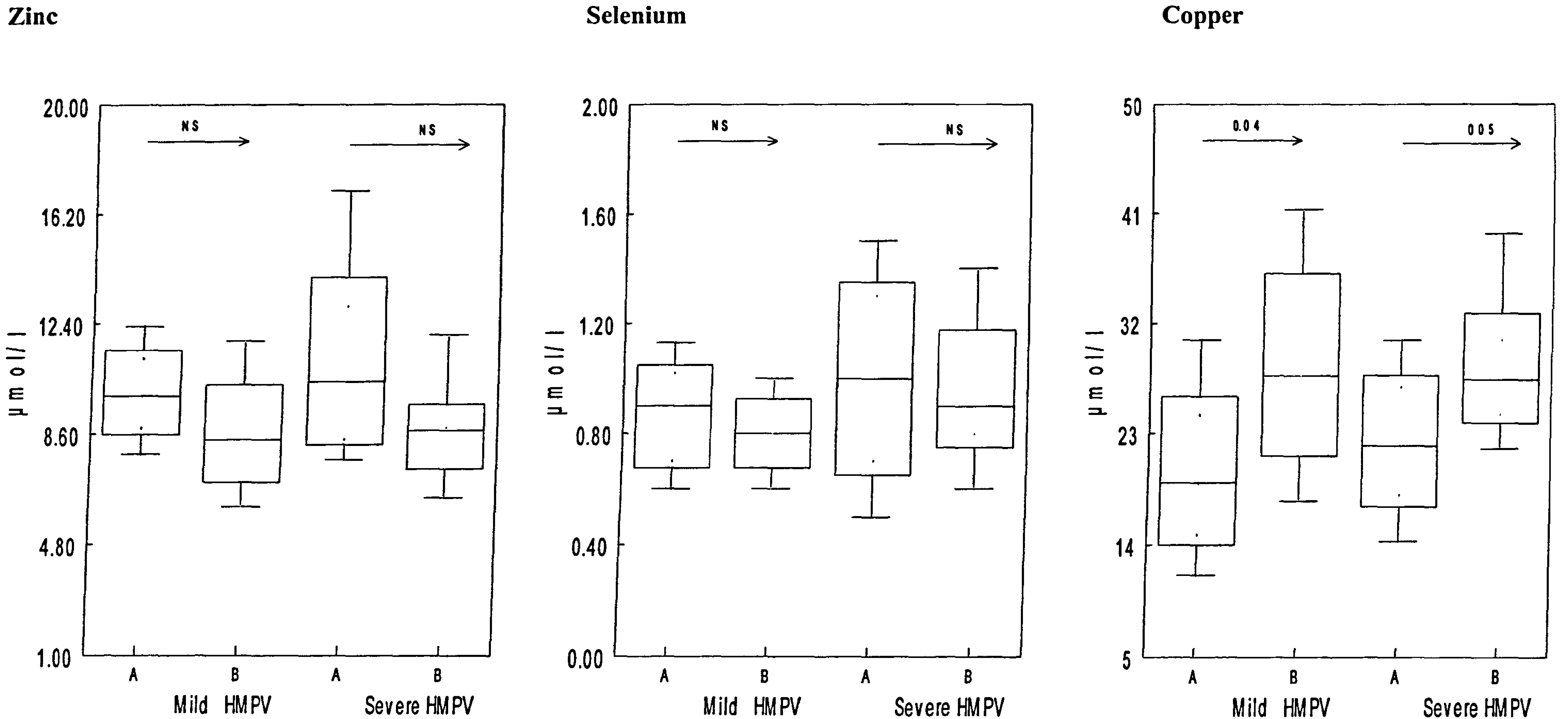
	Mild HMPV (n =17)		Severe HMPV (n = 21)	
	CRP ≤ 5 n = 8	CRP >5 n = 9	CRP ≤ 5 n = 8	CRP >5 n = 13
Zinc	8 (100%)	9 (100%)	8 (100%)	13 (100%)
Selenium	8 (100%)	9 (100%)	8 (100%)	13 (100%)
Copper	8 (100%)	9 (100%)	8 (100%)	13 (100%)
Retinol	8 (100%)	9 (100%)	8 (100%)	11 (85%)
α-tocopherol	7 (88%)	7 (78%)	3 (63%)	3 (45%)

The GM serum copper concentrations were significantly higher in children with CRP > 5 mg/l independently of the disease severity (p=0.04 and 0.05 for mild and severe respectively), resulting in lower zinc/copper ratios in children with CRP > 5 mg/l (p<0.01 for both) (Figure 6.6). The remaining micronutrient concentrations were not significantly different in children with CRP ≤ or > 5 mg/l (Table 6.11, figure 6.6).

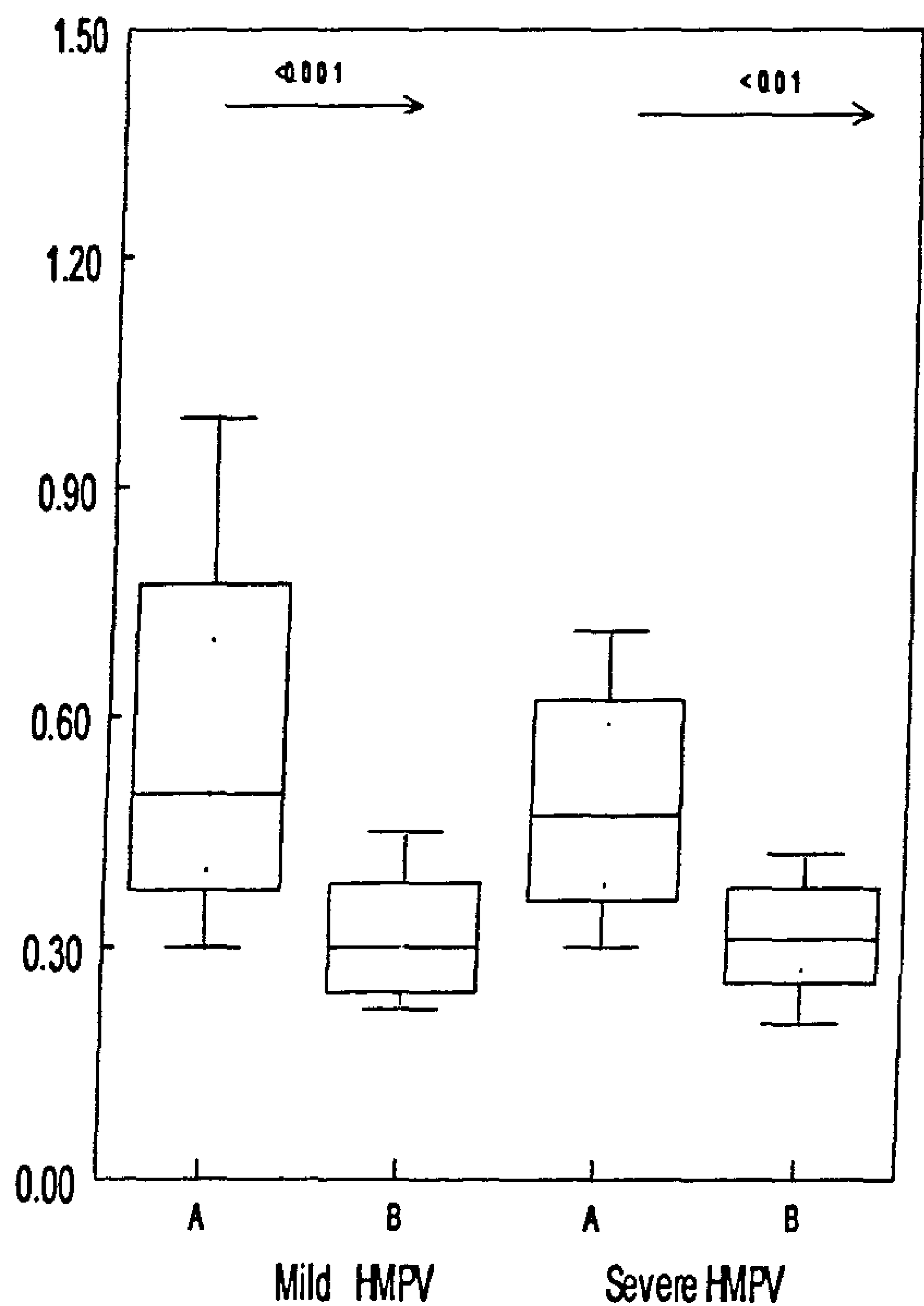
Table 6.11 Micronutrients concentrations in children with HMPV by disease severity and CRP cut-off

Micronutrient GM (SE) and range	HMPV Mild		P	HMPV Severe		P
	CRP ≤ 5	**CRP > 5**		**CRP ≤ 5**	**CRP > 5**	
Zinc	9.9 (1.06) 7.3 -12.3	8.4 (1.08) 6 - 12.3	0.12	10.4 (1.11) 7.7 - 20	8.7 (1.06) 5.7 - 14.3	0.14
Selenium	0.9 (1.08) 0.6 - 1.2	0.8 (1.08) 0.53 -1.2	0.7	1 (1.15) 0.44 - 1.5	0.9 (1.08) 0.6 - 1.8	0.6
Copper	19 (1.13) 9.9 - 32.6	27.7 (1.11) 17.4 - 42.43	**0.04**	22 (1.11) 13 - 32.8	27.4 (1.05) 22.7 - 42.2	**0.05**
Zinc/copper	0.5 (1.16) 0.28 - 1.25	0.3 (1.09) 0.2 - 0.49	**<0.001**	0.5 (1.12) 0.28 - 0.74	0.3 (1.07) 0.16 - 0.48	**<0.01**
Retinol	1.9 (1.16) 0.8 -2.78	1.7 (1.16) 9	0.5	1.3 (1.17) 0.5 - 2.25	1.7 (1.13) 0.6 - 2.78	0.1
α-tocopherol	31.6 (1.16) 12.7 - 37.12	34.6 (1.05) 25.3 - 37.12	0.7	15.6 (1.68) 6.1- 37.12	25.9 (1.4) 12.7 - 37.12	0.7

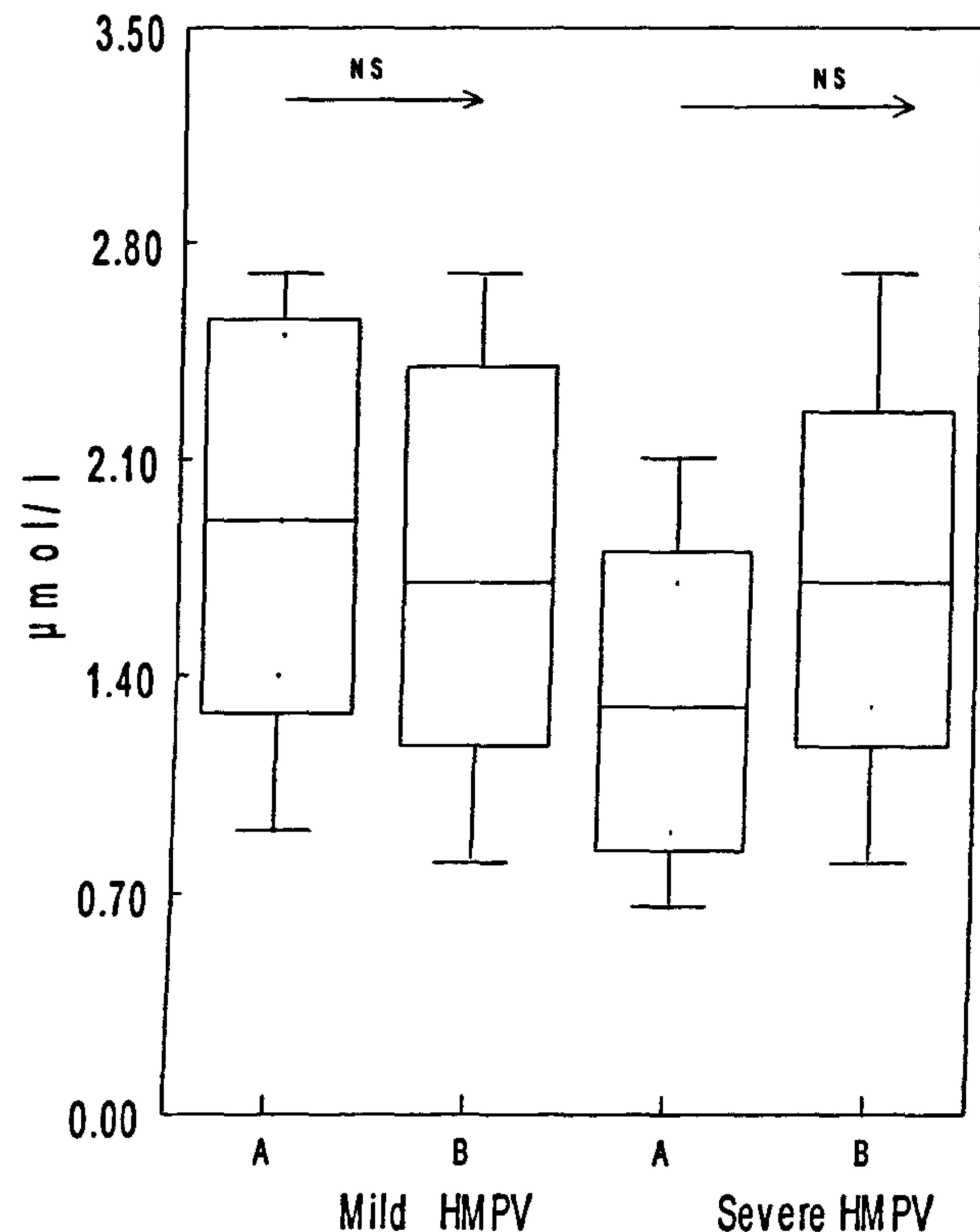
Figure 6.6 Zinc, selenium, copper, zinc/copper ratio, retinol and α-tocopherol concentrations of children with HMPV by disease severity and CRP cut-offs



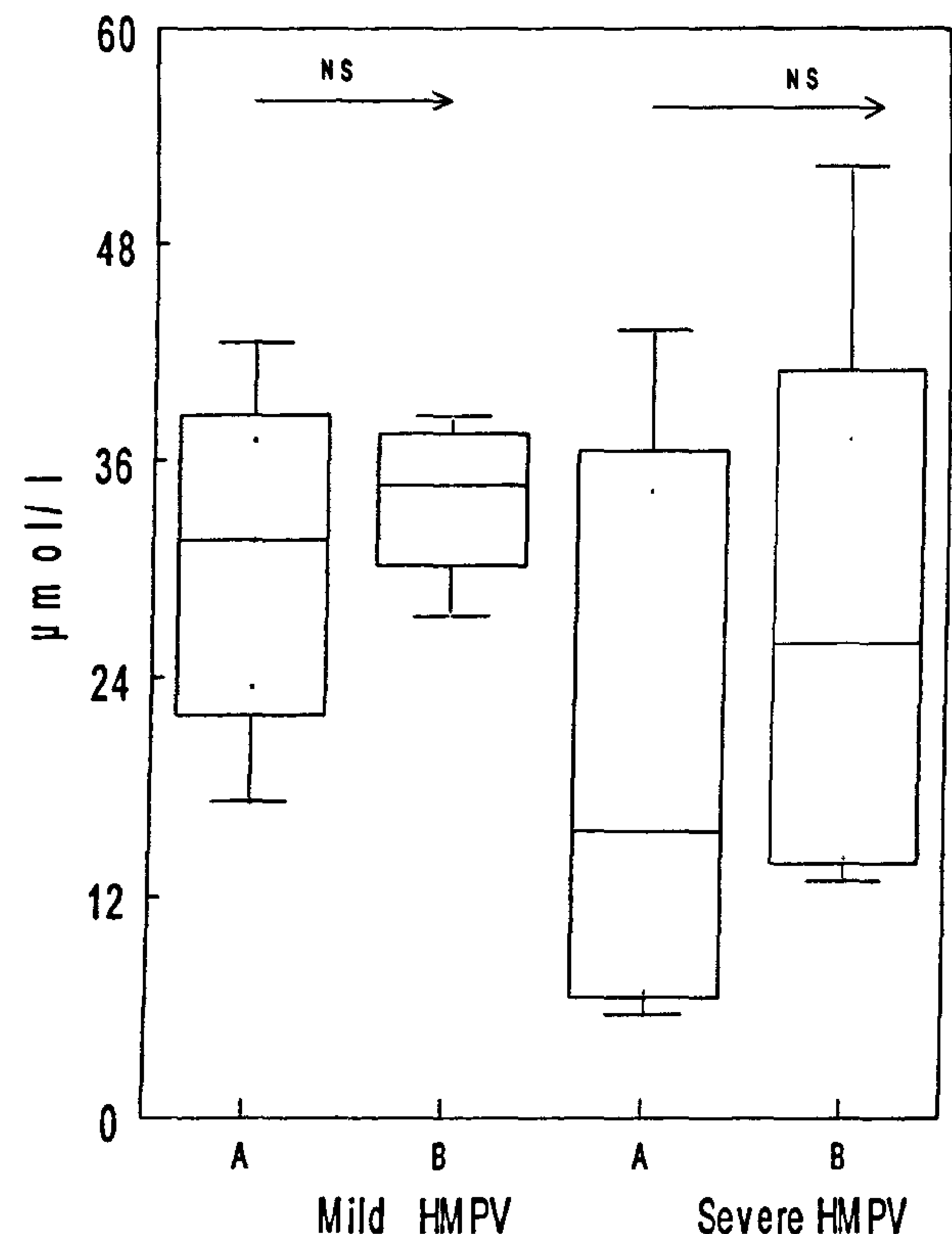
Zinc/copper ratio



Retinol



α-tocopherol



Multivariate analysis

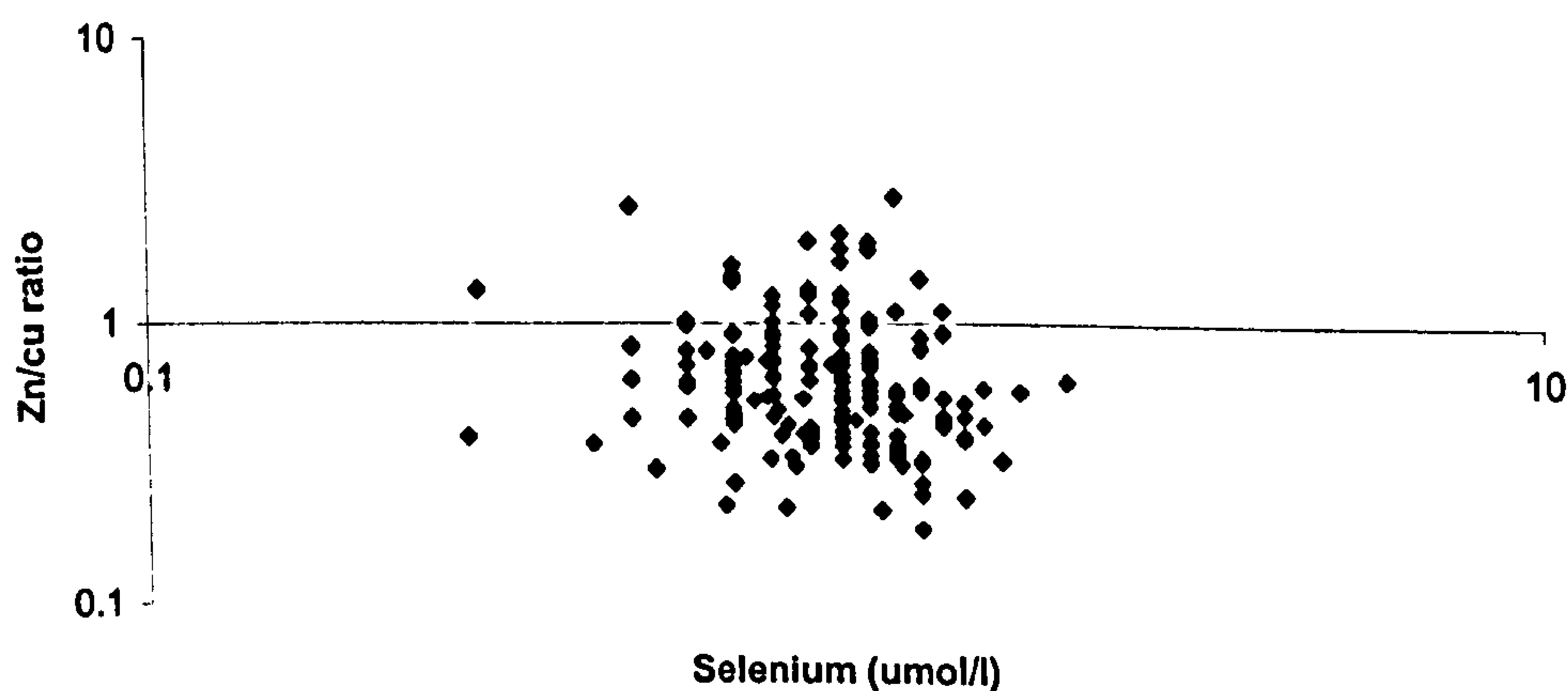
A multiple linear regression analysis was conducted to determine the relationship between micronutrients and zinc / copper ratios and CRP. Table 6.12 and figures 6.7, 6.8 and 6.9 describes these relationships.

Table 6.12 Relationship between the micronutrients and CRP in children with RSV, HMPV and HMPV/RCV co-infection

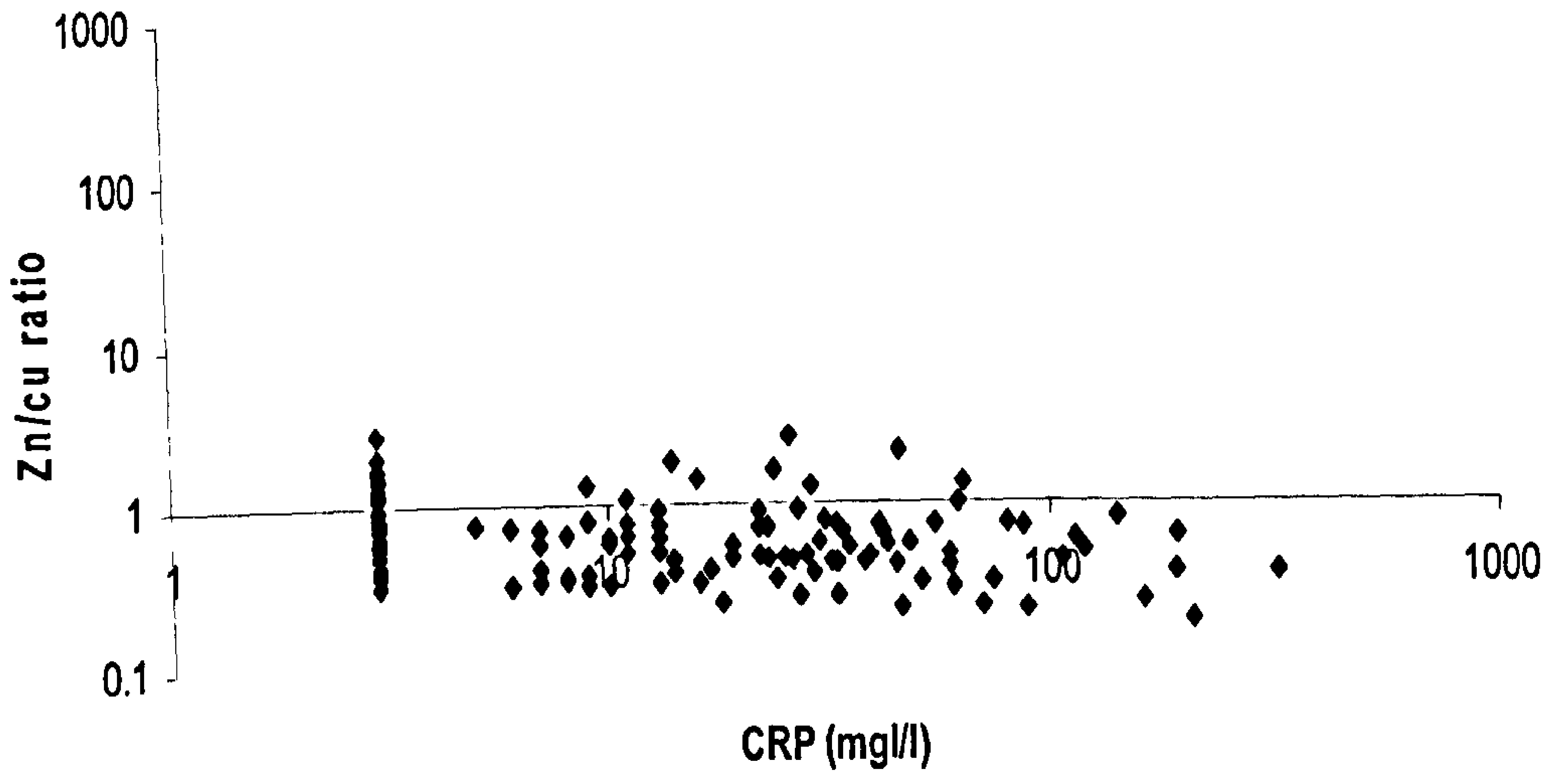
	RSV		HMPV		RSV/HMPV	
	r^2	P	r^2	P	r^2	P
Zn/Cu ratio: Selenium	0.02	**0.04**	0.01	0.6	0.04	0.3
Zn/ Cu ratio: Retinol	0	0.6	0.04	0.2	0.03	0.4
Zn/ Cu ratio: α tocopherol	0.01	0.4	0.01	0.6	0.03	0.5
CRP: zinc	0.03	0.06	0.08	0.08	0.01	0.6
CRP: copper	0.01	0.16	0.12	**0.03**	0.01	0.1
CRP: Zn/ Cu ratio	0.03	**0.03**	0.1	**0.04**	0	0.8
CRP: Selenium	0	0.5	0.03	0.3	0.01	0.6

Figure 6.7 Relationship between (a) zinc/copper ratio and selenium, (b) zinc/copper ratio and CRP (c) zinc and CRP, (d) copper and CRP in children with RSV

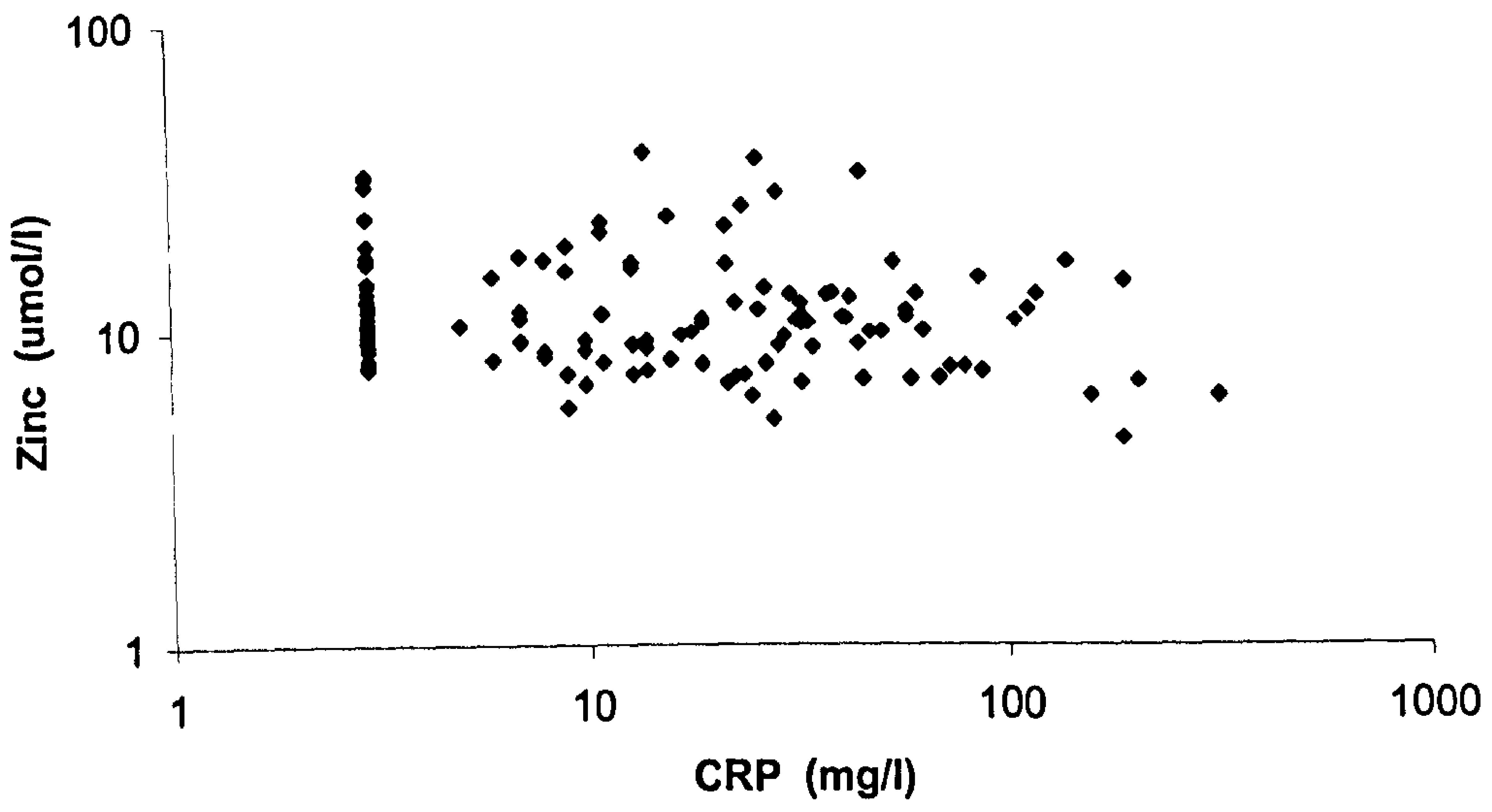
(a)



(b)



(c)



(d)

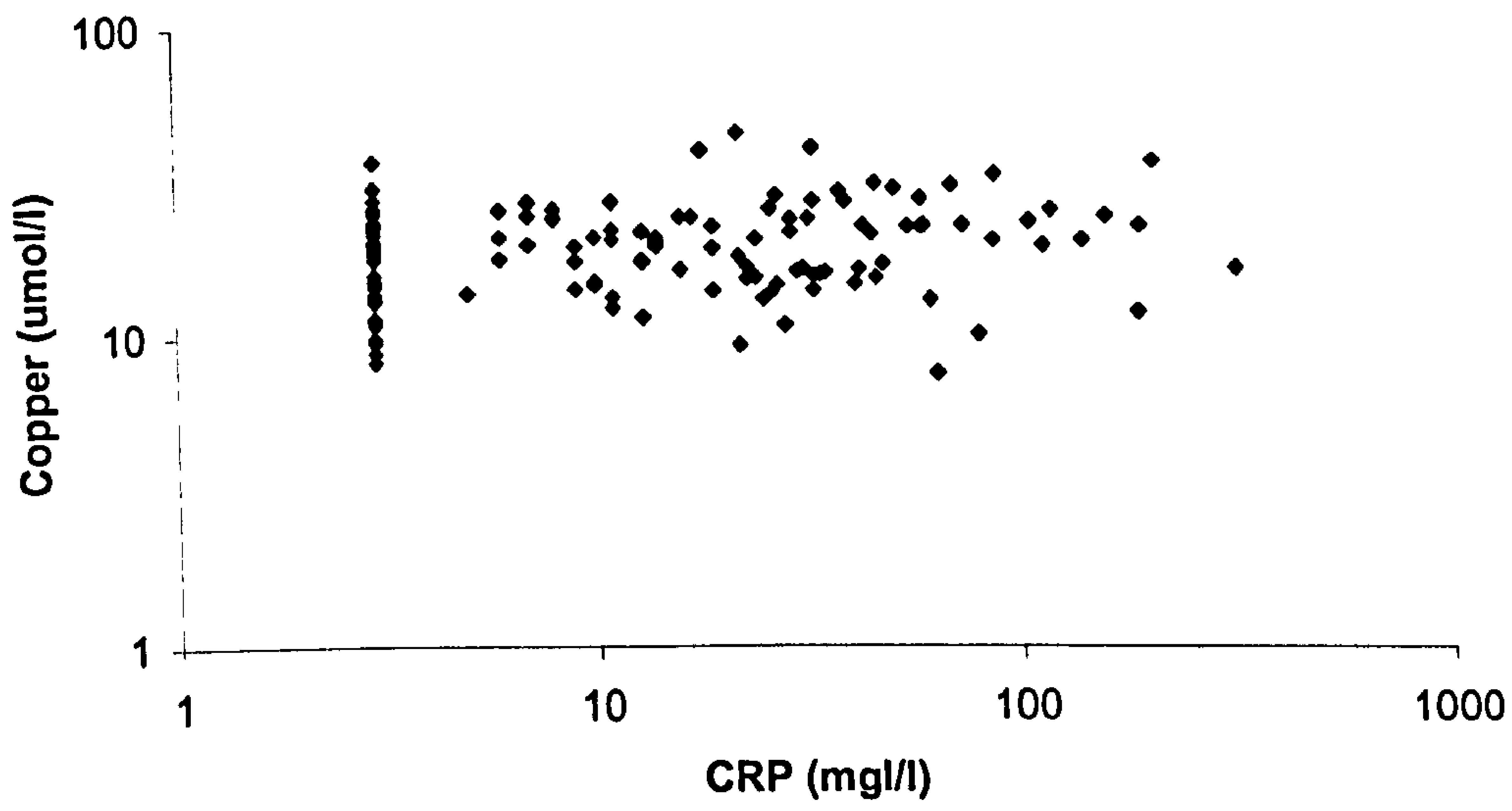
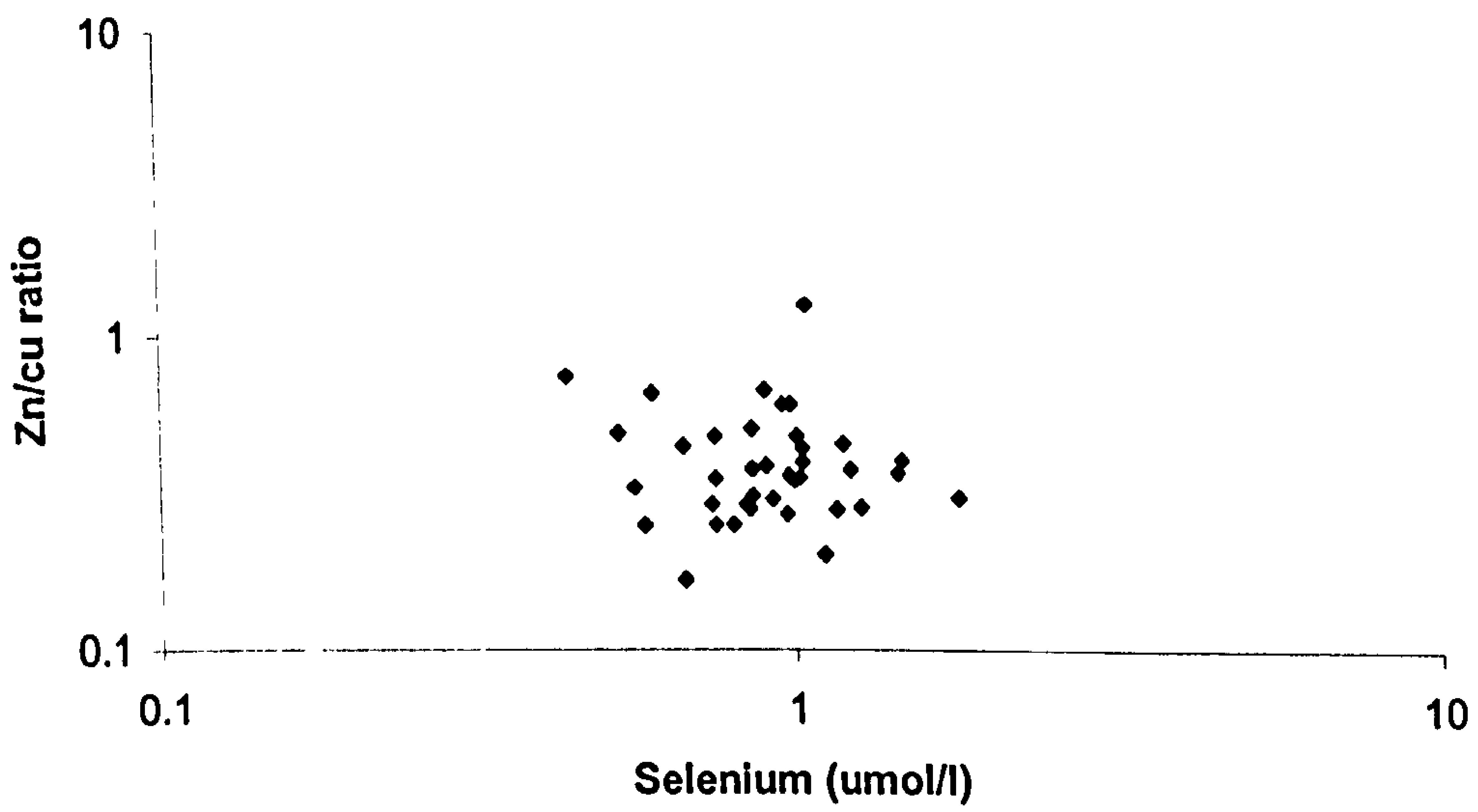
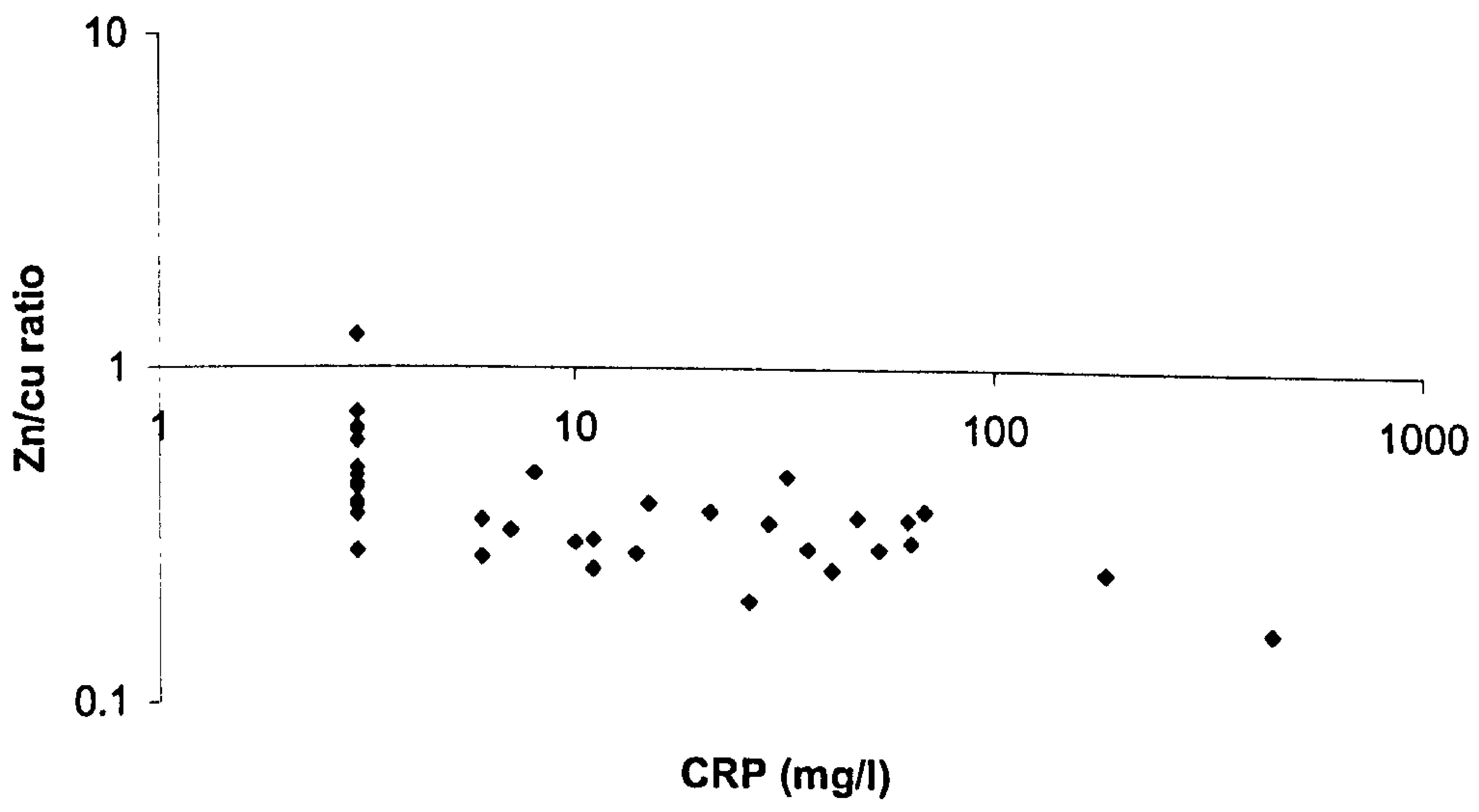


Figure 6.8 Relationship between (a) zinc/copper ratio and selenium, (b) zinc/copper ratio and CRP (c) zinc and CRP, (d) copper and CRP in children with HMPV

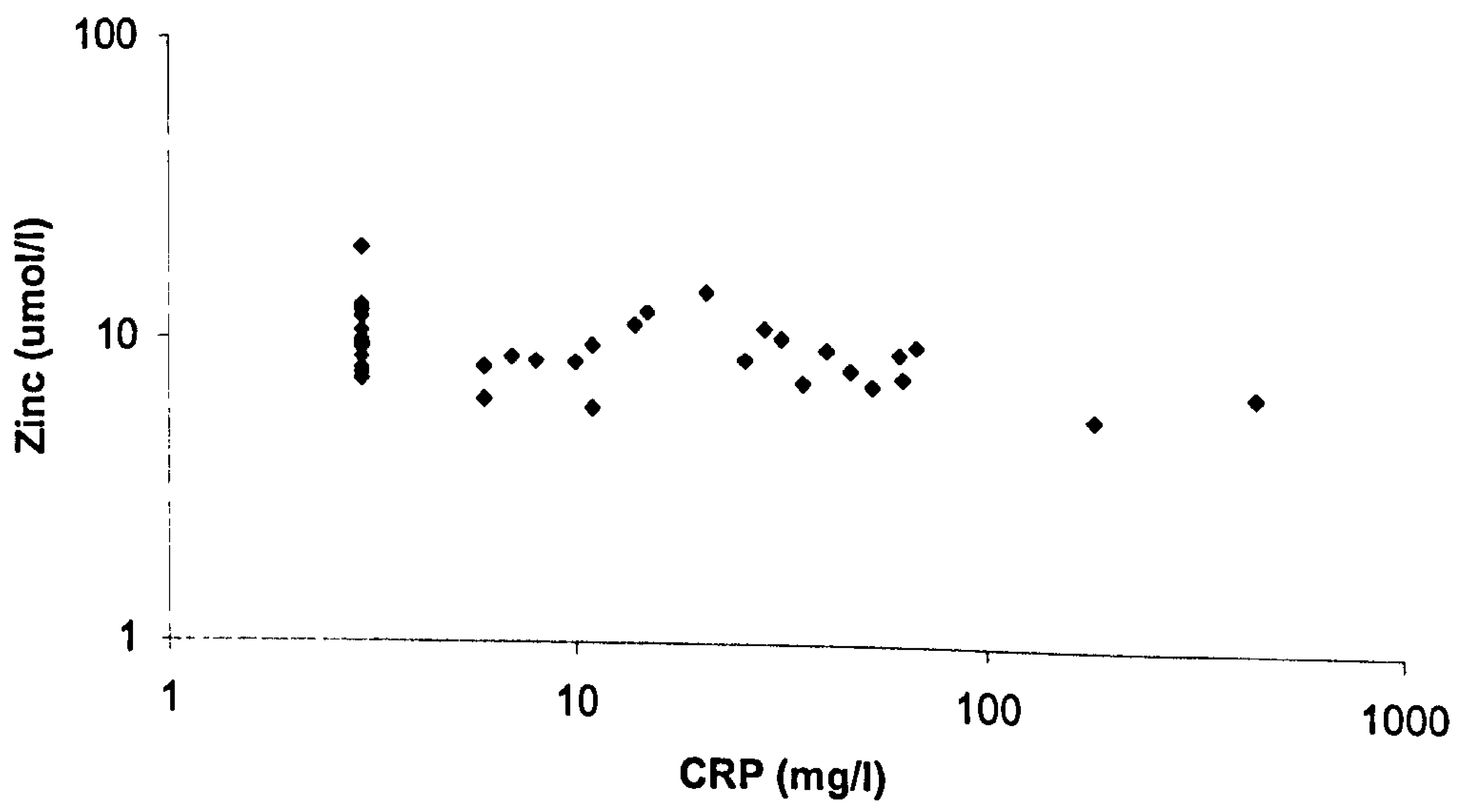
(a)



(b)



(c)



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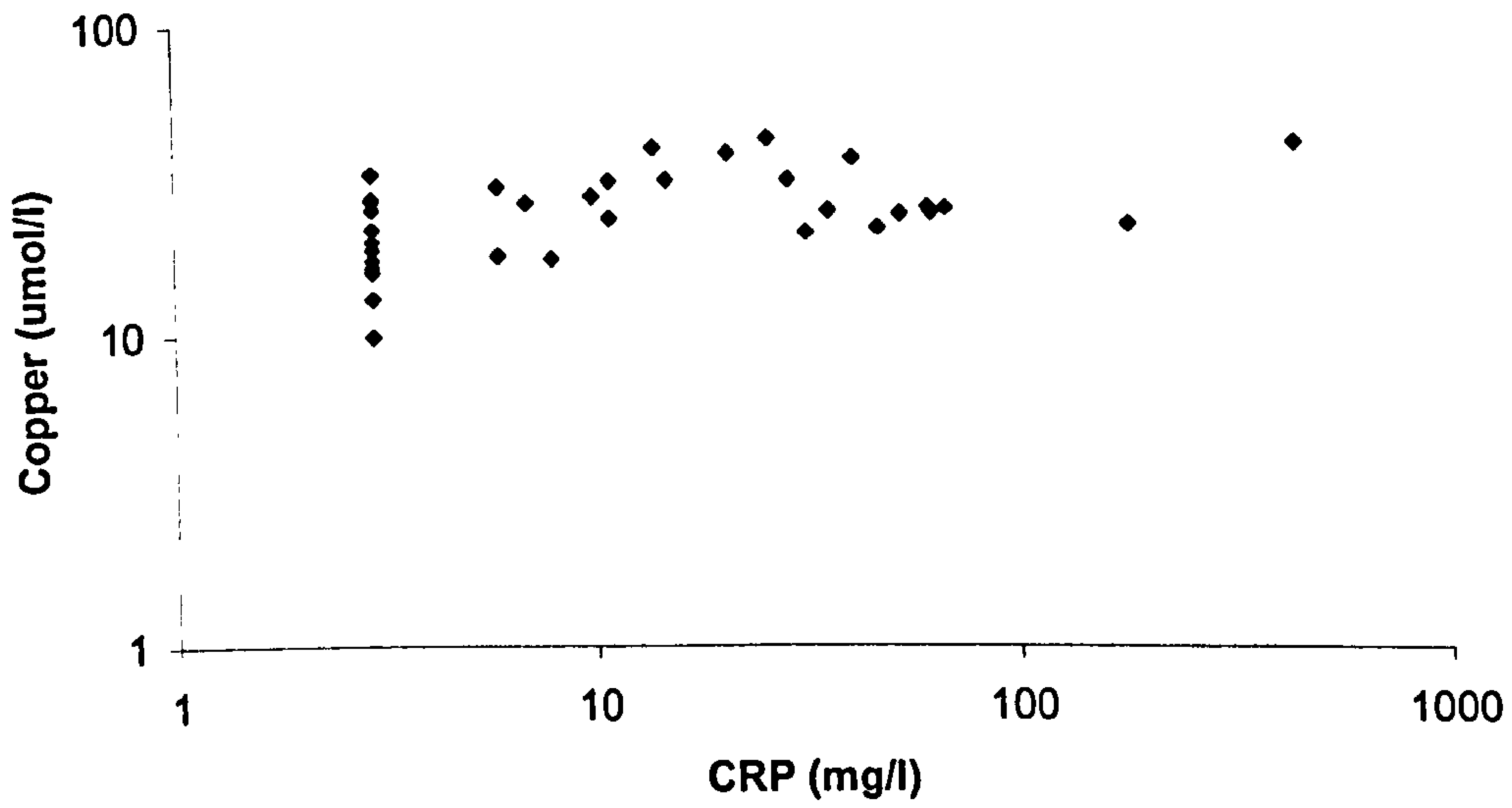
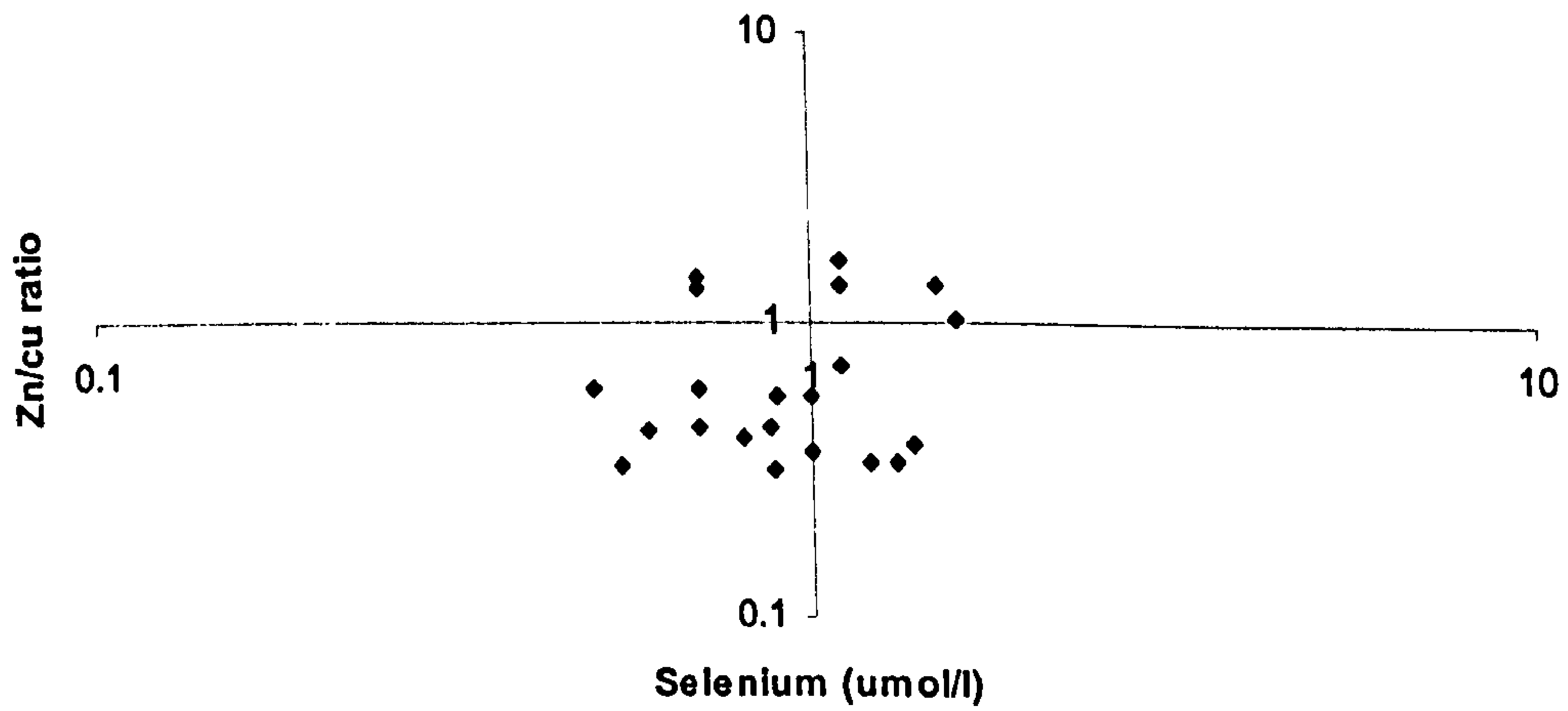
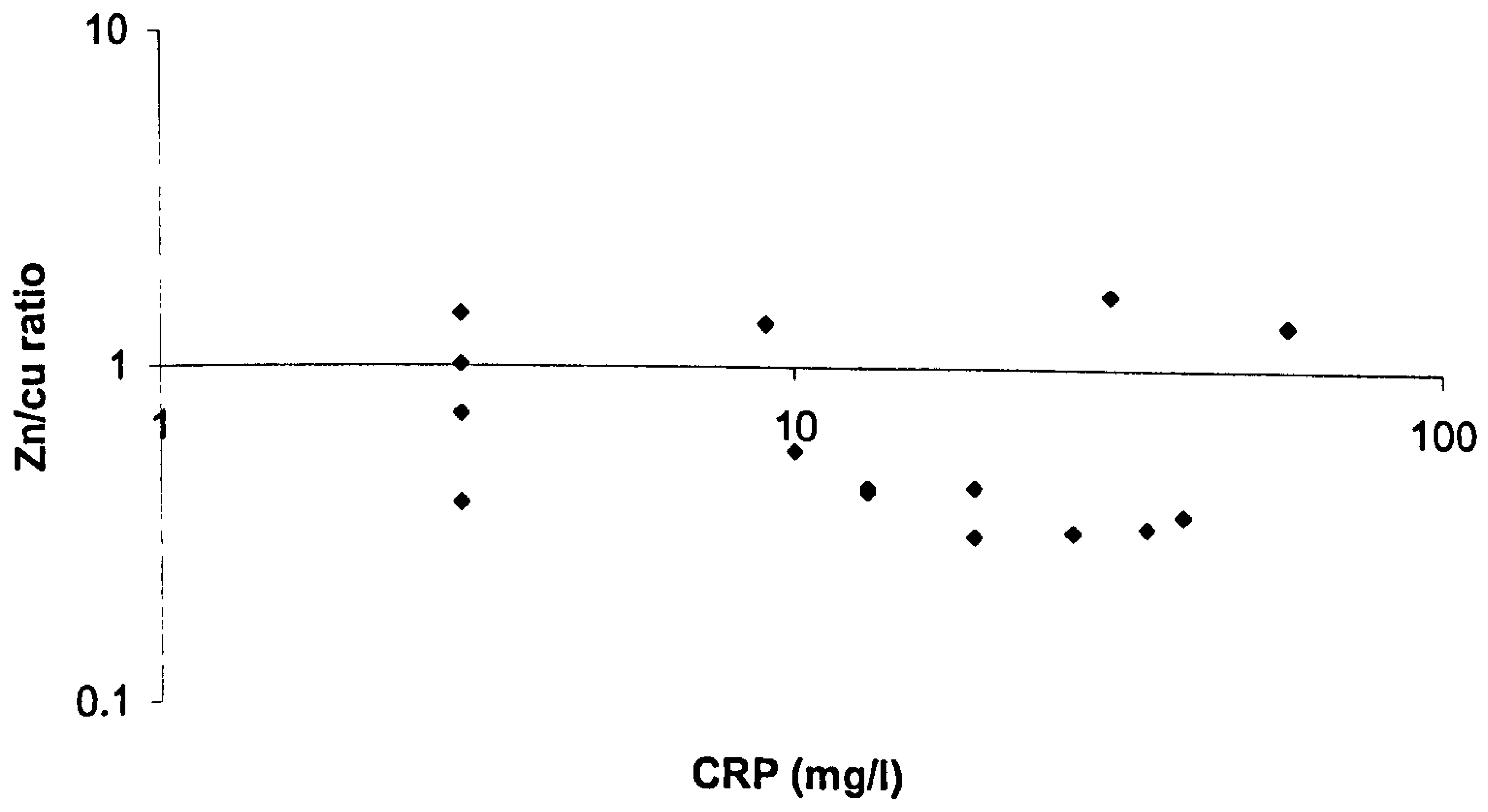


Figure 6.9 Relationship between (a) zinc/copper ratio and selenium, (b) zinc/copper ratio and CRP (c) zinc and CRP, (d) copper and CRP in children with RSV/HMPV

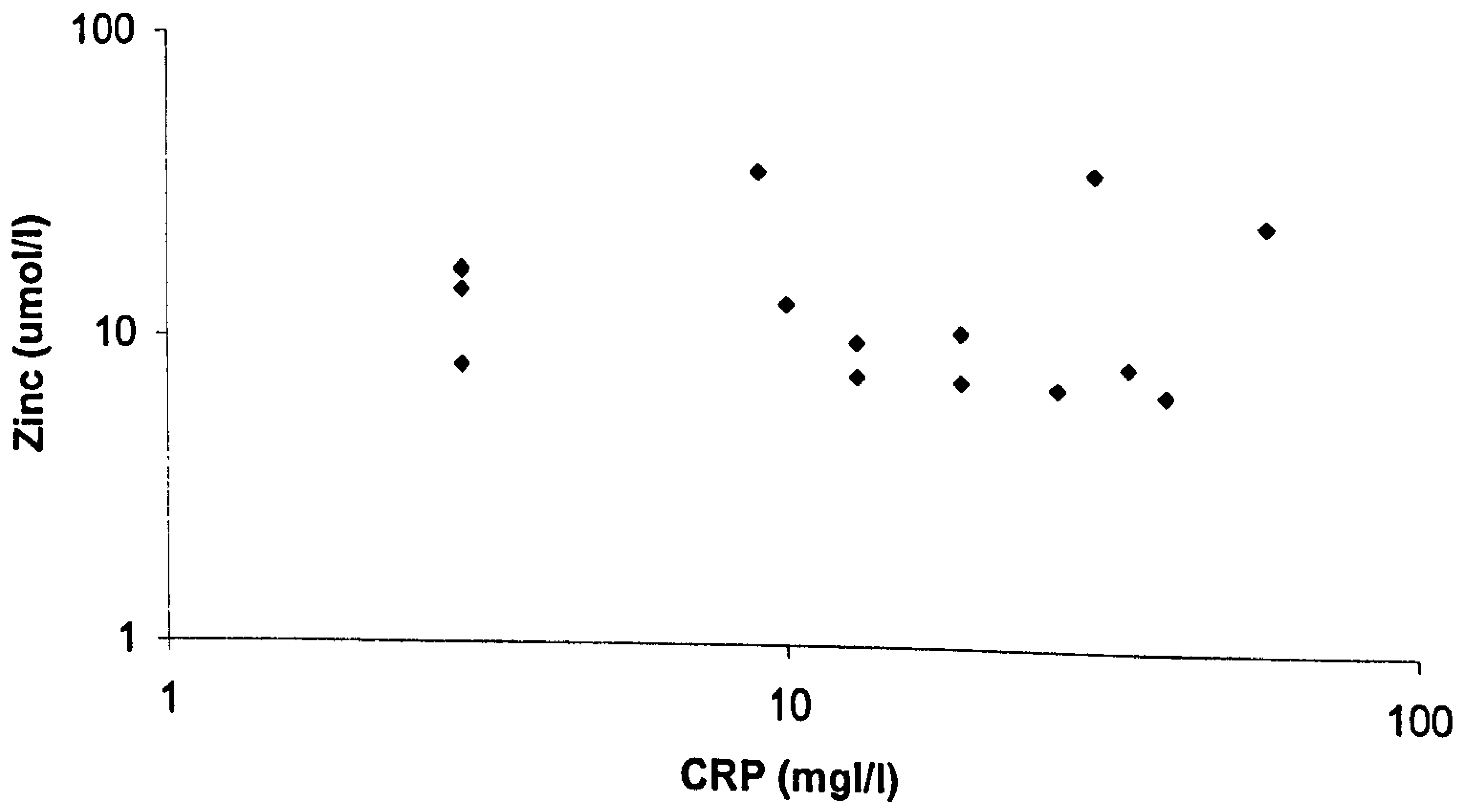
(a)



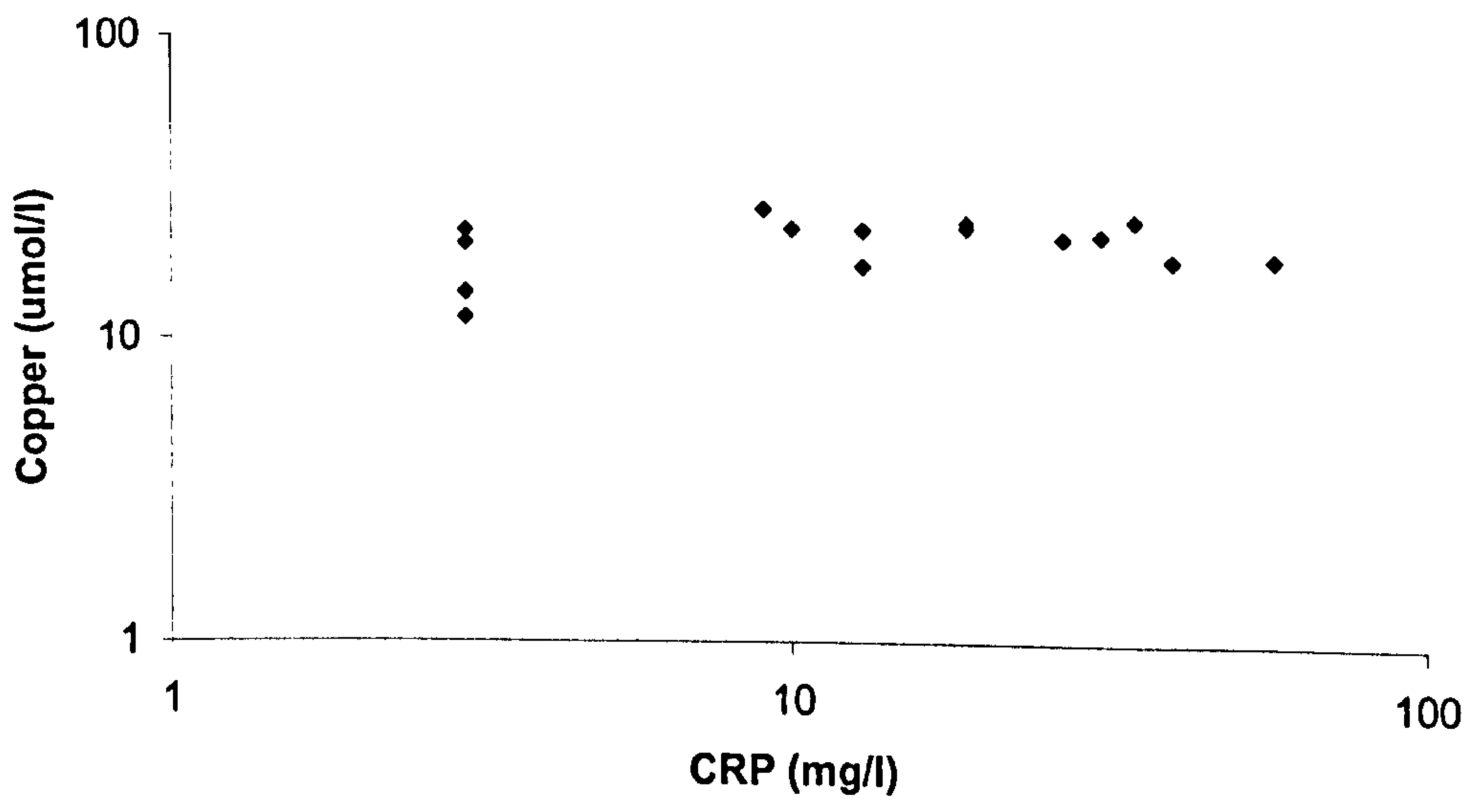
(b)



(c)



(d)



Discussion

Highly potent immunological substances are produced by the immune system in response to infection and injury. These substances include among others, cytokines and oxidant molecules, such as hydrogen peroxide and free radicals. The purpose of these products is to destroy invading organisms and damaged tissue, bringing about recovery (Noah et al., 1995). However, oxidants and cytokines can also produce damage to the healthy tissues and their excessive or inappropriate production is associated with increased mortality and morbidity after infection, trauma and inflammatory diseases (Fujita et al., 2001). Sophisticated natural defences directly and indirectly protect the host against the damaging effect of cytokines and oxidants. The nature and extent of these defences are critically determined by nutrition and as a consequence, so is the risk of illness (Marcos et al., 2003). Insufficient energy intake, macro and micronutrient defciencies impair the immune system, suppressing immune functions that are fundamental to host protection. These changes also in turn are associated with an increased risk of infections, producing physiological changes that worsen the nutritional status (Chandra, 2002).

Micronutrient concentrations by viruses

Our results demonstrate that there is an inverse relationship between zinc and copper concentrations both in children with RSV and HMPV and that, regardless of the causative virus, children with raised CRP had significantly higher copper and lower zinc concentrations than children with normal CRP. The inverse relationship between zinc and copper is likely to reflect the intimate relationship between zinc and copper. Zinc competes with copper for gastrointestinal uptake and an increased zinc intake can induce copper deficiency. This impairment of copper absorption by zinc for example, is used therapeutically in Wilson's disease (Schumann et al., 2002). The low blood concentrations of zinc in children with significant acute phase responses result from a redistribution of the mineral to the liver, mediated by an increased hepatic synthesis of metalothionein (Philcox et al., 1995). Zinc is often reduced during the acute phase of other infections, such as measles, acute diarrhoea, Tuberculosis, Leprosy, Leishmaniasis and HIV (Al-Sonboli et al., 2005). Furthermore, apparently healthy Indonesian infants with raised CRP also had lower zinc plasma concentrations than infants with normal CRP (Wieringa et al., 2002). In children, zinc supplementation improves recovery from acute diarrhoea (Al-Sonboli et al., 2005) and has small but beneficial effects in the prevention of ARI (Bhutta et al., 1999a) and measles-associated pneumonia (Mahalanabis et al., 2002). In recent studies zinc supplementation was associated with shorter duration of fever and severely ill status in children with measles

accompanied with pneumonia (Mahalanabis et al., 2004) and found to significantly decrease the incidence of pneumonia in children (Brooks et al., 2005). Our study however demonstrates that zinc and copper responses varies with the aetiological agent and it is possible that these beneficial effects may also be heterogeneous.

The high copper concentrations observed during infections have been associated with an increase in the hepatic synthesis and release of the copper-binding protein ceruloplasmin (Kreuzer et al., 1996) and previous reports have considered copper as a positive acute phase protein, which is elevated during inflammation (Periquet et al., 1995). Copper level often increases in children with raised CRP (Wieringa et al., 2002) and with severe chronic infections such as TB (Koyanagi et al., 2004).

The changes in zinc and copper we report however have not been previously documented in children with RSV or HMPV infections. Unexpectedly however a lower proportion of children with RSV than children with HMPV had low zinc levels.

A high proportion of our children also had selenium deficiencies regardless of the respiratory virus detected and surprisingly; selenium concentrations were similar in children with RSV, HMPV or RSV/HMPV. This could be due to a low selenium intake or a reduced absorption. It is known that certain diets and geographical areas are very poor in selenium. In addition, selenium intake has decreased in the last decades, as a result of water processing. This latter factor however is unlikely to be an issue in Yemen. Serum selenium concentrations were not modified by the CRP concentrations of children, which could be due to the highly prevalent deficiency of selenium in the Yemeni population which was prevalent in a high proportion of the children (81% - 89%). In other studies, selenium concentrations are reported to decrease during infections and to be lower in patients with raised CRP (Koyanagi et al., 2004). Low selenium concentrations have been linked to increased viral virulence and disease progression in children with HIV (Singhal and Austin, 2002). Children with severe malnutrition (marasmus and kwashiorkor) also have significantly lower selenium concentrations than well nourished children (Thakur et al., 2004). It has been postulated that these lower concentrations are due to the consumption of selenium as an antioxidant, as part of the process of quenching free radicals (Bates et al., 1994). Several studies have reported an inverse correlation between selenium and CRP during the acute phase of various pathological conditions. The reduction of selenium in turn induces the synthesis of CRP by the hepatocytes during the acute phase (Maehira et al., 2002, Sattar et al., 1997). In animal models, the administration of a lipopolysaccharide reduced the plasma and liver concentrations of selenium and elevated its concentrations in other organs (Maehira et al., 2002). Although there are few clinical trial to examine its role for prevention and recovery of infection, a few

reports have recently suggested that selenium supplementation might reduce the virulence of HIV (Rayman, 2000).

This thesis also found that low serum retinol and α tocopherol concentrations were more prevalent in children with RSV than in children with HMPV with a significantly higher proportion of the former children having retinol and α tocopherol deficiency. These results are in agreement with previous reports in children describing decreased concentrations of serum retinol in patients with RSV infection (Dowell et al., 1996, Neuzil et al., 1994, Quinlan and Hayani, 1996). As RSV often has a more severe disease presentation than HMPV (chapter 3), and serum retinol and α tocopherol concentrations decrease during inflammatory stresses (Strayer et al., 1998, Wieringa et al., 2002), the combination of these factors may explain lower serum retinol concentrations in children with RSV than in children with HMPV. It is not clear whether this micronutrient is important to modulate the host's response to RSV and HMPV infection and whether this effect is beneficial or detrimental.

Among children with ARI, vitamin A could be potentially detrimental due to its enhancement of the host immune response, which may induce further inflammatation of the lungs. Adverse outcomes of vitamin A supplementation have been observed among patients with ARI (Bresee et al., 1996, Stephensen et al., 1998), and a meta-analysis of five double-blind, randomised, controlled trials in 1 month – 6 years old children presenting with acute non-measles ALRI showed that there is no evidence that intervention with high-dose vitamin A improves recovery from pneumonia in developing countries (Brown and Roberts, 2004).

Despite the putative role of the oxidative stress in acute conditions such as viral infections (Schwartz, 1996), vitamin E in combination with vitamin C does not beneficially influence the course of illness in children with measles and pneumonia (Mahalanabis et al., 2005). An animal model of acute lung inflammation caused by intratracheally administered lipopolysacharides, demonstrated that an inhaled α -tocopherol aerosol may play a beneficial role in strategies to control inflammatory lung illnesses (Hybertson et al., 2005). In a double-blind, placebo-controlled trial, vitamin E supplementation significantly reduced the incidence of common colds and the number of subjects who acquired a cold among elderly nursing home residents (Meydani et al., 2004).

In our children, low plasma retinol and α tocopherol concentrations did not significantly correlate with CRP, regardless of the causative virus. In contrast, CRP concentrations were negatively correlated with serum retinol in children with shigellosis in Bangladesh (Mitrani et al., 1987). In Indonesia, the relationship between the acute phase response by measuring CRP and selected micronutrients in healthy infants participating in 6-month randomised,

supplementation trial of iron, zinc and/or β-carotene reported that vitamin A deficiency was more prevalent in infants with raised acute phase proteins (Wieringa et al., 2002).

The causes of low serum retinol concentrations may include a reduced intake or absorption. In addition, the infection can also compromise vitamin A status by increasing urinary excretion, as shown by Quinlan and Hayani in patients with RSV (Quinlan and Hayani, 1996) and febrile illnesses such as shigellosis (Mitrani et al., 1987) and measles (Semba and Bloem, 2004). Low serum retinol can also result from increased utilisation by the tissues (Fleck, 1989, Fleck et al., 1985), the reduced production of retinol-binding protein and a decrease in the release of hepatic vitamin A (Rosales et al., 1996). It is thus likely that a combination of these mechanisms operate in patients with RSV. Furthermore, although mechanisms may also operate in children with HMPV; the magnitude of their deficiency may be related to the magnitude of the inflammatory stress produced by the viruses. Since RSV produces more severe disease than HMPV, the deficiency of these vitamins may be more pronounced in the former group.

There is some evidence that low plasma concentrations of α tocopherol are associated with the acute phase response (Louw et al., 1992) and was reported to be lower in patients with TB than in healthy controls (Madebo et al., 2003).

The micronutrient concentrations of children with RSV/HMPV coinfections did not differ significantly from children with RSV with the exception of retinol, which were borderline statistically significantly higher in children with coinfections. The similarity in the pattern may be associated with the similar clinical presentation of children with RSV and RSV/HMPV coinfections. As HMPV did not seem to increase the disease severity of RSV, children with coinfection may be following the pattern of RSV. An additional possibility is that the number of children with coinfection was too small to identify significant differences.

The results of this study show that the assessment of micronutrient concentration in children with ARI could inform the development of more targeted micronutrient supplementation strategies for the prevention and treatment of ARI.

Clinical trials are needed to examine whether micronutrient supplementation improves the treatment outcome of children with bronchiolitis due to RSV or HMPV.

Micronutrients concentrations in children with RSV and HMPV by disease severity

The present study demonstrates that zinc, retinol and α-tocopherol concentrations and zinc/copper ratios are significantly lower in children with severe ARI than in children with mild ARI presentation due to RSV. The vitamin A concentration of the children are consistent with other reports that have shown that severely ill children with RSV and other infections such as shigellosis have lower serum retinol concentrations than children with mild illnesses (Dowell et al., 1996, Neuzil et al., 1994, Quinlan and Hayani, 1996, Mitrani et al., 1987). Vitamin A deficiency in HIV-1 infected individuals also contributes to an increased mortality and faster disease progression (Semba et al., 1993) and vitamin E and selenium deficiencies enhance viral virulence by genomic changes of RNA viruses such as Influenza and Coxsackie (Beck et al., 2003).

No significant differences were noted between children with mild and severe HMPV ARI with the exception of the serum concentrations of α tocopherol, which was significantly lower in children with severe presentation. Current studies suggest that vitamin E might enhance virulence in viral infections (Beck et al., 2003) although there are no published studies on vitamin E status in children with severe ARI due to HMPV.

The low concentrations of zinc, selenium, retinol and α tocopherol could result from the physiological response of the acute phase infection, from preceding deficiencies enhancing susceptibility to infection, and increased requirements during severe ARI. More investigations are needed to clarify the degree to which these factors contribute to the low micronutrient concentrations observed in severe ARI, for targeted strategies for micronutrient supplementation.

Surprisingly, not all children with severe RSV or HMPV infections had increased CRP and not all children with mild RSV or HMPV had low or normal CRP. This discrepancy could be due to other confounding variables not included in our study. For example, it is well established that children with viral infections are often co-infected with other bacteria such as *Haemophilus influenza* and *streptococcus pneumonia*. As it is well known that bacterial infections increase the serum CRP concentrations more readily than viral infections this limitation may confound our analysis. Furthermore, our classification of disease severity was based solely on the degree of hypoxia. The use of this criterion would necessarily assume that hypoxia is a relatively stable parameter. Although, hypoxia in small children could result from the presence of temporary airway blockages due to mucus and some children may have been misclassified, this is a parameter that is more objective than the clinical assessment.

The micronutrients concentrations of children with mild RSV ARI were not statistically significantly different between children with normal and raised CRP with the exception of zinc, which was significantly lower in the latter children. In children with severe RSV and in children with HMPV, regardless of disease severity, copper concentrations were significantly higher and zinc/copper ratios significantly lower in children with high CRP. These results demonstrate that serum copper correlates positively and zinc/copper ratio correlates negatively with the inflammatory stress and disease severity.

Oxidative stress may play a beneficial role in diverse processes in nature, including cell proliferation, genetic activation and expression (Spitzer, 1995) and we need a better understanding of these processes in children to better define the role of nutritional antioxidants in the prevention and treatment of acute respiratory infections.

There are no previous published studies describing micronutrient concentrations according to the CRP concentrations of children with RSV or HMPV ARI. Our results indicate that low micronutrient serum concentrations are frequent in these children, particularly in patients with high CRP levels.

Chapter 7
General discussion

In this chapter, the contribution of this work to the body of knowledge on ARI due to RSV and HMPV is reviewed according to the objectives set in chapter 2. A summary of the main findings and conclusions are presented, study limitations are acknowledged and questions for further research are suggested.

RSV and HMPV in children with respiratory infections

The present study identified RSV, HMPV and RSV/HMPV coinfection in 40%, 7% and 4% children < 2 years of age respectively. The pO_2 cut off point of ≤ 87% was chosen to classify the study population into mild and severe ARI as Sana'a is situated at a high altitude (2,200 metres above the sea level) (Duke et al., 2002). One hundred and seventy eight children (30%) were diagnosed as having mild ARI and 425 (70%) severe ARI.

RSV was the virus detected most frequently accounting for 40% of infants with ARI, mainly severe LRTI (87%), with hospitalisation rate of 31%. This frequency of RSV infection is higher to previous studies from the region, including the United Arab Emirates (29%), Saudi Arabia (29%) and Jordan (26%) (Bdour, 2001, Uduman et al., 1996, al-Hajjar et al., 1998). However this rate is still low when compared to some reports from areas in close proximity to Yemen such as, in Saudi Arabia (54%) and North Jordan (50%) (Meqdam et al., 1998, Jamjoom et al., 1993) or reports from other part of the world such as Argentina (41%) (Galiano et al., 2004). It is however difficult to compare the relative frequency of these viruses across studies, due to their differences in enrolment criteria, diagnostic methods and temporal and seasonal variations. Our study therefore confirms that RSV and HMPV are pathogens that play a major role in ARI in Yemen.

The present study revealed that group A was the predominant RSV group over the study period; it represented 82% of the samples genotyped. This is consistent with studies from the Middle East (Bdour, 2001) and some parts of Africa (Madhi et al., 2003b) and Europe (Fletcher et al., 1997). Only 208 RSV positive specimens could be genotyped and the restriction type of 6 specimens did not fit into the scheme developed by Cane et al (Cane et al., 1992). After sequencing, one of these specimens was found to be most closely related to the attenuated group B RSV strain used for vaccination of children in the early 1960s (Udem, 2001) and the other 5 were found to be group A NP2 but with two point mutations introducing a 2nd *Rsa* restriction site (Elango and Venkatesan, 1983). NP2 was the predominant genotype (46%) followed by NP4 (36%), NP3 (12%) and finally NP1 (5%). We

found that although NP2 and NP4 were seen throughout the study period, NP4 was not isolated in the beginning in October, and NP1 and NP3 were not identified during the last 2 months of the study (April and May), but peaked in March. NP2 peaked during December and January, while NP4 peaked during January and February.

Our study detected HMPV in 7% of the patients and compared to RSV, a higher proportion of the children with HMPV had mild ARI (41%). This detection rate was analogous to results of previous studies in Argentina (11%), the Netherlands (10%) and Australia (10%) (Mackay et al., 2003, van den Hoogen et al., 2001, Galiano et al., 2004). In contrast to our results, the frequency of HMPV was reported to be lower in Canada, England and USA (2%, 2% and 6% respectively) (Boivin et al., 2002, Falsey et al., 2003b, Semple et al., 2005, Stockton et al., 2002). Most of the studies however have described case series of children over a short period of time and long term studies are required to establish its real prevalence.

HMPV and RSV co-infections were detected in 4% of the children which was within the expected proportions given the relative proportions of RSV and HMPV. Twenty two of the 25 co-infected children had severe hypoxia. Group A are the most frequent RSV group found in co-infections (91%), which is in agreement with a previous report from the UK (Greensill et al., 2003). NP2 was detected a more frequently although all other genotypes were detected at least once. Some group B genotypes were only detected in children with severe disease (NP3). Although it is still uncertain whether co-infections predispose to more severe disease as reported from the UK (Greensill et al., 2003, Semple et al., 2005).

The results described that RSV and HMPV co circulated over the same period. The RSV and HMPV high incidence periods lasted from October to May. Although HMPV and RSV were detected throughout the whole study period, HMPV peaked in February and March and RSV in December and January. This is in agreement with studies from the Northern hemisphere where HMPV has been reported to peak in the first months of the year (Esper et al., 2004), while in the Southern hemisphere the peaks of HMPV have most often been reported in April and May (Cuevas et al., 2003, McAdam et al., 2004) , or in September, October and November (Galiano et al., 2004), both corresponding with the cold season.

RSV was identified more frequently in children under 6 months of age (74%) and its prevalence decreased after this age. In contrast, HMPV infection occurred more frequently in older children. A similar finding was reported from the Netherlands (van den Hoogen et al., 2003) where HMPV was less frequent in children < 2 months old than RSV. RSV and HMPV seem to have similar clinical presentation, ranging from mild to severe ARI (Peiris et al., 2003). In our setting, HMPV had a milder clinical presentation with children being less

likely to be hypoxic and with lower respiratory rates than children with RSV. Fifty one percent of the children with HMPV were clinically diagnosed as having a mild ARI in comparison to only 15% of the RSV infected children. The proportion of patients with hypoxaemia ($pO_2 \leq 87$) and in need of oxygen was higher (87%) in children with RSV than children with HMPV (59%). These findings are consistent with those of Galiano et al (2004).

In conclusion, RSV was the most frequent aetiological cause of ALRI hospitalisation in Yemen. Single RSV and HMPV infections were undistinguishable clinically but RSV resulted in a more severe clinical episode than HMPV. Both viruses had distinct seasonal profiles.

Risk factors for severe RSV, HMPV and RSV/HMPV ARI

Hospitalisation rates due to ALRI have increased steadily since 1980 (Glezen et al., 2000) and the development of new approaches for their prevention and control requires identifying and quantifying the factors that increase the risk of experiencing a serious illnesses. Although the factors that increase the risk of a child experiencing a severe ALRI are known for children with RSV infections in industrialised countries, there is a paucity of information on whether the same factors operate in developing countries. In addition, there is very limited information on the characteristics associated with severe HMPV infections and this is the first study investigating the risk factors for severe disease due to this novel virus.

Children with chronic lung and congenital heart diseases or are premature are at a higher risk of severe RSV ALRI in industrialised countries (Boyce et al., 2000, Navas et al., 1992, Bulkow et al., 2002, Kaneko et al., 2001). Premature birth and congenital heart diseases have also been associated with an increased risk for HMPV hospitalisation (Robinson et al., 2005). Our study however, did not identify any of these characteristics as risk factors and several issues may explain these findings. Most children in Yemen are born outside the hospitals and have limited access to health services. In this setting, premature babies and those with congenital malformations have a high mortality and are likely to be underrepresented among the children attending the hospital (Kaneko et al., 2001). This effect is illustrated by the overall small number of preterm infants or children with congenital heart and lung diseases enrolled in the study and a prospective cohort studies would be required to examine these associations.

Children less than 3 months are especially prone to develop severe ALRI and our findings confirm this criterion as one of the most important factors associated with disease severity for both RSV and HMPV. This is in agreement with reports from industrialised countries (Holberg et al., 1991, Kaneko et al., 2001, Lanari et al., 2002, Weigl et al., 2001).

Children with severe hypoxia were less likely to have their routine vaccines up to date and this factor may be an indirect marker of poverty and low health awareness.
Low socio-economic status has been associated with a higher attack rate of RSV infection and children in low socio-economic strata are more likely to have multiple high risk conditions (Jansson et al., 2002). Our study observed that some characterstics of the household increased the risk for severe hypoxia for both RSV and HMPV, including the use of cooking fuels other than gas, or non-governmental sources of water, the presence of pets, especially cows and donkeys, smoking within the household and having a relative with an ARI. Of these factors, using a cooking fuel other than gas was an independent risk factor for both RSV and HMPV.

The factors associated with severe RSV are in agreement with a case control study from Rochester that examined these associations and reported that parental smoking, in particular maternal smoking during pregnancy, indoor pollution and the presence of pets increased the risk of severe RSV infections (McConnochie et al., 1988). Lanari et al, also reported that environmental smoke exposure was a risk factor for severe RSV ARI (Lanari et al., 2002) and low socio-economic status was an important risk factor for severe RSV ARI in South Africa (Madhi et al., 2003b).

No significant differences were observed in the nutritional parameters of children with mild and severe ARI. This may be due to the fact that most of our population was malnourished. A Nigerian study reported that RSV was less frequently isolated from malnourished than from well-nourished children, and that malnourished children, had milder disease than well nourished children (Nwankwo et al., 1994). In the Gambia, RSV was found in 13% of well-nourished children with ALRI compared to 6% of malnourished children (Adegbola et al., 1994). Other studies from the Gambia (Weber et al., 1998b), and South Africa (Hussey et al., 2000) however have reported malnutrition was not a risk factor for the development of RSV ALRI.

There is no published information regarding the role of atopy in children with HMPV and prospective studies would be useful. Our study showed no significant differences in the medical and family history of asthma, eczema or allergies between children with mild and severe RSV or HMPV ARI. This is in agreement with Bradley et al in USA who found that levels of common allergens within the home environment had no effect of RSV severity (Bradley et al., 2005). However, Weber et al reported that Gambian mothers of children hospitalised with RSV complained of asthma more frequently than mothers of non-hospitalised children (4.2% versus 0.5%, $P = 0.05$) (Weber et al., 1999). Thus, there is still controversy of the role of atopy in the development of severe RSV infection in developing countries.

There was no significant differences in the clinical severity of children with group A or group B RSV ARI as has been reported by others (Wang et al., 1995b, Kneyber et al., 1996). However, there is some debate in the literature on whether a predominant group cause more severe clinical spectrum than others. About 50% of the reports have indicated that group A infection results in more severe disease than group B (Hall et al., 1990, Walsh et al., 1997, McConnochie et al., 1990, Taylor et al., 1989). It has also been suggested that virus variants within group A are responsible for the discrepancy observed across the studies. Similarly, to these findings, our study did not find a relationship between the N genotypes and clinical severity.

Few studies have evaluated the role of HMPV coinfection with RSV for inducing severe disease (Semple et al., 2005). Our results suggest that coinfection with the two viruses did not represent a major risk factor for disease severity, although the number of children with coinfection in our study is too small to rule out this possibility. In agreement with these results, coinfection with HMPV was reported to increase the severity of RSV illness in studies from Italy and Greece (Maggi et al., 2003, Xepapadaki et al., 2004). However, a study in an intensive care unit in the UK, 21 (70%) of 30 patients ventilated for RSV had HMPV coinfections (Greensill et al., 2003), and 9 (60%) of 15 children admitted with HMPV infections in Germany also had RSV infections (Konig et al., 2004). This raises the possibility that HMPV strains may vary with location and time. The latter studies of intensive care children however lack control groups and are difficult to interpret. In a recent case control study from the UK, dual infections with RSV and HMPV resulted in a 10 fold increase in the risk of admission to a pediatric intensive care unit for mechanical ventilation. This study however also observed that if the number of children with dual infection were compared by disease severity there were no significant differences (Semple et al., 2005) and further studies are needed.

In conclusion, the factors independently associated with an increased risk of severe RSV were age ≤ 3 months, the child not having his/her vaccines up to date, the presence of a smoker at home and using a cooking fuel other than gas. In contrast, only age ≤ 3 months, having a history of recurrent ARI and using a source of fuel other than gas were the only risk factors found to be independently associated with an increase risk of severe HMPV. Larger case control and prospective studies will be required to elucidate risk factors for HMPV.

Immunopathogenesis of RSV, HMPV and RSV/HMPV ARI

This study is the first to describe the cytokines profile of children with HMPV and to assess the immunopathogensis of RSV ARI in a developing country.

IL6 concentrations were significantly higher in children with RSV than in children with HMPV. This is in agreement with one study by Laham et al. (2004). Excess TNF- α production was reported in children with severe RSV pathology (Fujita et al., 2001) and our study demonstrated that TNF-α concentrations were significantly lower in children with RSV compared to HMPV, which may explained that the more severe disease found in children with RSV. However, its role in HMPV infections is unknown and further studies are needed. Our findings also illustrate that IL7 increases as a result of infection by RSV, HMPV or their combination. However, HMPV infections resulted in a lower IL7 concentration than RSV. Our study suggests that the early production of Th1 cytokines is weak in infants experiencing infection with RSV. In contrast, HMPV infections are associated with a strong Th1 cytokine response. Infants had increased concentrations of IL12 and INF-γ against HMPV but not RSV infections. This different response pattern could be due to a combination of factors, including differences in antigenic motifs or different properties of the virus for induction of the innate immune responses and different ages of the patients (Kurt-Jones et al., 2000).

Our results demonstrate that IL8 concentrations were higher and RANTES concentrations were lower in children with RSV compared to children with HMPV. This is in contrast to a Finnish study found that IL8 concentrations were significantly lower and RANTES concentrations significantly higher in children with RSV than in children with HMPV (Jartti et al., 2002). In conclusion, the present study revealed that RSV induces a weak Th1 immune response in comparison to HMPV and that latter exhibited a strong Th1 immune response.

The immune responses in children with RSV/HMPV coinfection did not differ from those of children with single RSV infections with the exception of INF-γ concentrations. This cytokine was present at significantly higher concentrations in children with dual infections. Similarly, children with single HMPV infections only differed from children with dual RSV/HMPV infection in the concentrations of IL6 and IL8. Similar to children with RSV, children with RSV/HMPV had higher concentrations of IL6 than children with HMPV. RSV/HMPV coinfections induced the highest concentrations of IL8. This finding would be in agreement with studies reporting that children with dual infections have more severe ARI. Although our study was too small to confirm or refute this hypothesis, this finding suggests that children with RSV/HMPV may have more severe illnesses than children with single RSV infections and that, children with RSV may have more severe disease than children with

HMPV. IL8, which has been considered as an indicator for disease severity (Bont et al., 1999a) was higher in children with RSV/HMPV than in children with RSV and this latter group in turn had higher concentrations than children with HMPV. Larger studies therefore are needed to confirm these findings.

IL6 concentrations were significantly higher in children with mild than in children with severe RSV ARI. This in agreement with previous reports that have demonstrated that high IL6 concentrations are present in children with mild URTI (Gentile et al., 2001, Noah et al., 1995). TNF-α was found to be higher in children with severe RSV ARI than in children with mild clinical presentation. This is consistent with Fujita et al, (2001) who reported that children with severe RSV pathology produced high levels of TNF-α (Fujita et al., 2001). IL7 concentrations were higher in children with mild than severe RSV ARI. This finding supports the hypothesis that IL7 may play a protective role from severe clinical illness as suggested for other viruses, such as CMV (Lu et al., 2005) and HSV-1 infections (Wiryana et al., 1997).

Our study did not identify significant differences in IL12 concentrations between children with mild and severe RSV ARI. This in agreement with other recent reports (Garofalo et al., 2004).
Yemeni infants with clinically severe RSV infection had significantly higher INF-γ concentrations than those with mild disease. This is consistent with a predominantly Th1 immune response, with more inflammation in the lungs. Our infants with severe illnesses however did not exhibit a more pronounced RSV-specific Th2 response than infants with mild infection. This is in agreement with previous studies that have reported that INF-γ concentrations were higher in children with severe RSV disease (Bendelja et al., 2000, Tripp et al., 2002).

IL10 concentrations were higher in children with severe RSV associated ARI than in children with milder disease. These differences were however not statistically significant and our data do not support the hypothesis that severe RSV infections are associated with Th2 responses in naturally acquired RSV bronchiolitis. In our study, however IL8 and RANTES concentrations did not correlate with severity. Our data are in agreement with others who reported similar findings (Laham et al., 2004, Noah et al., 2002, Oh et al., 2002).

Children with mild and severe HMPV did not show significant differences in the concentrations of any of the cytokines and chemokines. These findings however are consistent with the results of Laham et al who did not observe differences in series of patients with upper and lower ARI (Laham et al., 2004). The number of children with mild and severe

RSV/HMPV however, was small and the lack of significance could also be due to the small power to detect these differences.

Children with RSV, with the exception of IL7 which was higher and IL10, which was lower in children with older age, did not show significant differences in the cytokine and chemokine concentrations by age (≤ 3 and > 3 months).

Interestingly, there were no significant differences in the cytokine and chemokine concentrations by age (≤ 3 and > 3 months) in children with HMPV ARI. There are, to date, no comparable studies in the literature. Similarly infants with dual RSV/HMPV infections had no significant differences by age, with the exception of IL12, which was higher in infants ≤ 3 months of age. This is consistent with a predominant Th1 immunological response. This different response may be due to the mechanisms used by HMPV to stimulate the innate immune response with a shift from Th2 (IL10) in single RSV infection to Th1 responses (IL12) in young infants with co-infections. Further studies to evaluate this response however are needed.

In summary, our study demonstrated that there is an increased type 2 immune response (IL10) in infants ≤ 3 months of age with RSV, whereas there is an increased type 1 immune response (IL12) in infants ≤ 3 months of age who have RSV/HMPV coinfectious.

Gender on the other hand was not associated with differences in the cytokine and chemokine concentrations. Nevertheless, male gender is associated with increased disease severity and further studies are needed.

In summary, our results suggest that RSV and HMPV infections in infants differ significantly in regard to the types of immune responses elicited.

Antioxidant status of children with RSV, HMPV and RSV/HMPV co-infection

This is the first time that the micronutrients (zinc, copper, selenium, retinol and α tocopherol) of children with RSV, HMPV and RSV/HMPV coinfections are reported and is the first analysis of their association with the inflammatory stress and disease severity caused by these pathogens. Our results show that children with RSV have higher concentration of zinc in plasma than children with HMPV. In addition, there is an inverse relationship between zinc and copper concentrations in children with RSV and HMPV. Zinc concentrations were significantly higher and copper concentrations significantly lower in children with RSV than in children with HMPV resulting in significantly higher zinc/copper ratios in children with RSV.

Low serum concentrations of retinol and α tocopherol were more pronounced in children with RSV than in children with HMPV, with a significantly higher proportion of children having retinol and α tocopherol deficiencies among children with RSV than in children with HMPV. Our results are in agreement with previous studies reporting decreased serum retinol levels of in children with RSV (Dowell et al., 1996, Neuzil et al., 1994, Quinlan and Hayani, 1996).

Selenium concentrations were similar in children with RSV, HMPV and RSV/HMPV although concentrations in most children were low suggesting widespread selenium deficiency in the population. Retinol concentrations of children with RSV/HMPV coinfection were bordeline statistically significantly higher than in children with RSV and serum copper, and retinol concentrations were lower than in children with HMPV.

Serum zinc concentrations were lower in children with raised CRP regardless of the virus recorded, but were only statistically significant in children with HMPV. Similarly, copper concentrations were significantly higher in children with raised CRP. These changes are consistent with role of copper as a positive acute phase protein, increasing during inflammation processes (Periquet et al., 1995). Zinc/copper ratios therefore were significantly lower in children in children with RSV or HMPV with raised CRP but were not statistically significant in children with RSV/HMPV coinfection. In agreement with these findings, zinc concentrations were significantly higher and copper lower in children with severe RSV hypoxia than in children with mild hypoxia and the zinc/copper ratios, were higher in children with severe hypoxia. These differences however were not statistically significant.

Both retinol and α-tocopherol concentrations were significantly lower in children with severe hypoxia associated with RSV ARI than in children with mild hypoxia and children with

severe hypoxia associated with HMPV also had lower α tocopherol than children with mild hypoxia. Again there are no published studies for comparison.

In conclusion, zinc concentration and zinc/copper ratio were associated with CRP concentrations and disease severity. These associations were only statically significant for CRP. Retinol concentrations were associated with disease severity in RSV infections and together with α tocopherol concentrations both were associated with severe hypoxia due to HMPV. Our results regarding retinol were in agreement with previous reports demonstrating and association between disease severity in children with RSV (Dowell et al., 1996, Neuzil et al., 1994, Quinlan and Hayani, 1996). This is the first description of micronutrient status in HMPV infection and however, there are no published studies to compare status of other micronutrients by disease severity.

Limitations of the study and need for further research

The main limitation of this study is that it was based in a tertiary hospital resulting in the under representation of children with mild ARI. In addition, the cross sectional nature of the study makes most of the information difficult to interpret. Most children in Yemen are born outside the hospitals and have very limited access to health services. These factors have an effect on the study of risk factors. For examples children who are very premature or have severe congenital malformation have a significantly higher mortality than children with similar characteristics in industrialised countries. This may result in a cohort effect, with an attrition of children at risk and children at high risk being under represented among those attending the hospital. This effect is illustrated by the small number of preterm infants and infants with chronic lung disease in our study. This selection bias could also result in an inadequate power to identify some risk factors in hospitalised children.

More longitudinal and comprehensive studies are needed to establish the prevalence and the seasonal variation of RSV and HMPV and their genotypes in developing countries.

As this study is the first one to explore some aspects of HMPV infection, larger case control and prospective studies are required to corroborate the clinical spectrum and the disease burden of this infection and to elucidate risk factors for the development of severe disease.

A better understanding of the immunological mechanisms and of the interactions between RSV and HMPV and the host immune cells is needed to inform the development of preventive and curative strategies. This may facilitate the development of vaccines and public

health interventions such as the reduction of smoke pollution in the household, segregating humans from animals and better water proviosn.

Micronutrient supplementation may decrease the risk for developing severe RSV and HMPV disease and there is a need to initiate studies to investigate their potential role in children with bronchiolitis.

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Appendix 1

Operational Term and definitions

Case Definition

The following cases definition will be used:

Mild ARI: Child with upper respiratory tract infection alone, complaining of cough with absence of crackles or wheezing on auscultation, oxygen saturation ≥ 88% in room air.

Severe ARI: The child has cough, respiratory distress, and crackles with or without wheezing on auscultation, with oxygen saturation < 88% in room air.

Fast Breathing

It is defined as an elevated respiratory rate (RR) above the expected age-specific value on one minute estimation. Children <2 months of age will be considered to have fast breathing if the RR >60 breaths/min, 2-12 month of age if RR >50 breaths/min, and those >12 months of age if RR >40 breaths /minute (WHO, 1990).

Wheezing

Defined as high-pitched respiratory noise during g expiration.

Recurrent wheezing

The child is considered to have recurrent wheezing if there are more than two episodes of wheeze prior to the current episode.

Resolution of the ALRTI episode

An ALRTI will be considered as resolved if the child has at least 24 hours with a RR below the age-specific value and no lower chest indrawing and an oxygen saturation >88%.

Hyperinflation of the lungs

In poster-anterior (P-A) chest x-ray: increased radiolucency of the lungs and downward displacement of the diaphragm to the posterior ninth rib or lower, sometimes severe enough to reverse the normal upward bowing of the hemidaphragm.

Chronic lung disease

These include the following:

1. Bronchopulmonary dysplasia (BPD).
2. Congenital abnormalities.
3. Chronic aspiration.
4. Recurrent aspiration.
5. Cystic fibrosis.

Bronchopulmonarydysplasia

Is a pulmonary disease in the first week of life requiring continued oxygen supplementation or pulmonary disease at 28 days of life with a carbon dioxide pressure <42 mmHg in room air.

Immunocompromised: include those with:

- Chemotherapy treatment for cancer.
- With solid organ or bone marrow transplants.
- Congenital immunodeficiency.
- Corticosteriods for longer than one month before their RSV infection.

Appendix 2

Screening Form

To enrol the child:

The child age < 24 months	Y/N
The child has cough less than 7 days	Y/N
The legal guardian agree to provide consent	Y/N
Difficulty of breathing	Y/N
Fast breathing	Y/N
$pO_2 < 88\%$	Y/N

Appendix 3

Consent form

a. Information leaflet for parents

The purpose of this information leaflet is to summarise the main points of the medical study in which your child is taking part. Please read it carefully and feel free to ask any questions about it to Dr. Nagla'a Al-sonboli who is going to be in charge of the conduct of the study.

Your child has a chest infection. This is a very common disease in childhood frequently caused by a small germ. This infection may come in a mild form the child may develop runny nose, cough and some times the infection becomes severe with fever, difficulty in breathing and the child lip's may become blue, and have difficulty for feeding.

We are currently studying the causative agents of this infection and the factors, which may lead to make your child develop the severe rather than the mild form of this disease.

We need to compare children who have the severe form of infection with children who have mild form of infection. This is the way that we know if there are certain factors in your child's body, such as deficiency in his immunity or nutrition or genetic factors affecting his immunity, or if certain diseases like congenital heart disease or chronic lung disease or the age of your child make severe disease more likely.

If you agree to participate, we need to take a small sample of mucus from your child nasopharynex through a very small tube and we need also to have a small amount of blood from his arm. We can study these samples in order to identify which of these factors affect the heath of your child.

You can refuse to take part in the study, or withdraw your child at any time, and it will not affect the good relationship you have with the doctors. You do not have to give a reason for not letting your child in the study and we shall carry on giving your child the best care that we can provide.

We thank you very much for your help and co-operation in this study. We want you to know that when we finish analysing the results we will make sure doctors will be informed which may help to improve the care of children with chest infection

b. Informed consent form

I/ We give permission for my / our child ....

[Patient's name (s) and surname (s)]

To be included in the study of []. The purpose and nature of the study is to determine the factors that affect the deveopment of severe chest infection in children. This has been explained to me by .... and I /We

[Doctor's name(s)]and surname(s)]]

understand that my /our child participation in the study is entirely voluntary and that I / we have the right to withdraw my / our child at any time without stating a reason and without prejudice to his / her treatment. I / we have also read the explanatory document [Information leaflet for parents], or have the document been read to me / us, and I / we have the right to request more information both in relation to my / our child and the study from the supervising physician, Dr Nagla'a Al-sonboli.

Date ...../....../.......

....

Full name of the parent or legal guardian Signature

....

Full name of the witness Signature

Appendix 4

Data Entry form

Serial No:______________

Name____________________________

Age:_________months

Sex: F/M

Address________________________________ Tel: (if present_________)

Place of enrolment: ☐

(A&E=1, OPD=2, ward=3)

Admitted Y/N

Date of Admission: / / / time of admission: a.m/p.m

Date of Discharge: / / / time of addmission: am/p.m

Duration of hospital stay: -------------- days

Present History:

C/O: Complaint

if yes, days

Cough	Y/N	☐
Fever	Y/N	☐
Difficulty of breathing	Y/N	☐
Tachypneoa	Y/N	☐
Wheeze	Y/N	☐

Blue lips Y/N ☐

Unable to drink Y/N ☐

Difficult to awake Y/N ☐

Apnoea Y/N ☐

Skin rash Y/N ☐

If yes, specify________________________________

Breast Fed Y/N

If yes, (Exclusive=1, partial=2)____________

When did you first introduce weaning food? age __________months

Vaccinated to date? Y/N

BCG	Polio	DPT	Measles	Hepatitis B	Hib
☐	☐	☐	☐	☐	☐

Is your child on medication currently? Y/N

If yes, what is his/her current medication?

Antibiotics	Antipyretics	Anti-cough	Bronchodilators	Steroids
☐	☐	☐	☐	☐

Past history: of

Recurrent respiratory infections Y/N

Eczema Y/N

Recent wheeze Y/N

Recurrent wheeze Y/N

Asthma (diag. By doctor) Y/N

Congenital heart disease Y/N

If yes, specify ______________________________

Is it associated with pulmonary hypertension Y/N

Chronic Lung disease Y/N

If yes, for how long ______________________________

Immune deficiency Y/N

If yes, specify ______________________________

Hay fever Y/N

Rickets

What is the gestational age of your child when was born__________months

Family history: of

Hay fever Y/N

Asthma Y/N

Allergies Y/N

If yes, specify______________________

Eczema Y/N

Congenital heart disease Y/N

TB Y/N

Smoking Y/N

If yes, specify______________________

Is there other family member has respiratory infection at the present time Y/N

If yes, specify______________________

Social history:

How many rooms do you have at your house? ______________

How many adult people live in the child's house: ______________

How many children live in the child's house: ______________

What type of water do you use? ____________________

Government pipes ☐ Private sources ☐ Mix of both ☐

What type of fuel you use for cooking?

Gas ☐ Wood ☐ Electricity ☐ Petrol ☐

Are there any pets indoors? Y/N, if yes specify____________________

Is there animal contact outdoors? Y/N, if yes

specify_________________________

On examination

Dysmorphic features Y/N

If yes, known syndrome ______________________________

BW:---------- Kg

Length/Ht--------cm

State of consciousness: ☐ Alert =1, Drowsy=2, Unconscious=3

Respiratory rate___________/min,

Hear rate_______/min Temperature: ________ °C

Oxygen saturation: ___________%

Cyanosis Y/N

Chest indrawing Y/N

Pallor Y/N

Dehydration Y/N degree ☐

Wheeze Y/N

Crackles Y/N

Heart murmurs Y/N

☐

Hepatomegaly Y/N cm

Splenomegaly Y/N cm

Signs of Rickets Y/N

If yes, which of the following signs:

Anterior fontanel size__________cm

Crainiotabes Y/N

Wrist broadening Y/N

Rosary beads Y/N

Harrison sulccus Y/N

Deformed chest Y/N

Lt wrest x-ray changes Y/N/ND

Specify___________________________

Signs of Vitamin A deficiency Y/N

Xerophthalmia Y/N

Pitot's spots Y/N

Chest X ray findings

Chest x-ray taken Y/N

Hyperinflation Y/N

Consolidation Y/N

Others Y/N

Specify____________________________________

Clinical diagnosis

Mild Upper respiratory tract infection Y/N

Moderate Bronchiolitis Y/N

Severe Bronchiolitis Y/N

Other, specify _____________________________

Investigations

Hb_______gm/dl, Not done ☐

WBC__________, N____________- L________ Band form__________

E________, M__________, B________________

ESR_______________, Not done: ☐

CRP____________, Not done: ☐

Blood culture Y/N if positive, ___________________________

Appendix 5

Follow up form

	Admission	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Date & time						
Place of follow up (A&E=1, Ward=2, PICO=3, Nursery=4)						
Respiratory rate						
Temperature						
Oxygen saturation						
Consciousness (Y/N), degree						
Difficulty of breathing (Y/N)						
Apnoea (Y/N), duration						
Cyanosis (Y/N)						
Chest indrawing (Y/N)						
Inability to drink (Y/N)						
Difficulty to awake (Y/N)						
Dehydration (Y/N), degree						
Wheeze (Y/N)						
Crackles (Y/ N)						
Hepatomegaly (cm) (Y/N)						
Splenomegaly (cm) (Y/N)						
Other problems						
On oxygen? (Y/N)						
Ventilated? (Y/N)						
N/G T? (Y/N)						
If breast milk given (expressed) or substitute?						
IVF? (Y/N), Why?						
On antibiotics? (Y/N)						
If yes, why?						
Outcome: Discharge improve, still in the hospital, DAMA, Died						
If died, cause of death						

Appendix 6
Management during hospitalisation

a. General supportive therapy during hospitalisation:

1. Oxygen therapy

Will be given to all children by nasal prongs or nasal catheter at a flow rate of 1-2 litres/ min, humidified with bubble humidifier. Once the oxygen saturation is consistently >88 % in room air for at least 3 hours oxygen therapy will be discontinued.

2. Fever

If the axillary temperature is 38.2C or more, Paracetamol 15 mg/kg body weight, every 6 hours can be given as necessary.

3. Dehydration

a. Severe Dehydration/signs of shock:

Will be treated with (1) oxygen. And (2) insertion of IV line and Ringer's lactate or 0.9 saline 20ml/kg IV as fast as possible. Heart rate, capillary refill, peripheral perfusion, BP will be monitored (every 15 min. if there is some improvement in the heart rate, capillary refill and pulse volume at the end of infusion repeat 20 ml/kg as fast as possible will be given. If there is no improvement, further management and inotropic therapy will be site specific

b. Some dehydration

Will be treated with 0.45% saline 50-100 ml/kg over 12 hours. It will be given concurrently with normal daily fluid requirement. If the child is able to take orally, oral rehydration salt solution will be used.

Standard fluid therapy

In patients with stable circulation fluid will initially be given by intravenous route for first 48 hours if the patient is too ill to feed orally or unable to feed well. During this period child will be fully monitored for adequacy of hydration.

Patients with normal hydration (no signs of dehydration) will be given 0.18 % in 5% glucose as normal maintenance fluid requirement at the following rate:

- 4ml/kg/hout for the first 10 kg body weight.
- 2ml/kg/hour for next 10 kg (10-20kg) body weight.
- 1 ml/kg/hour for remainder of body weight (>20.1 kg)

Fluid therapy will be aimed at maintaining normal hydration status. Oral fluids (breast feeding in a previously breast fed infant) will be started when the child is able to feed and protect the airway. Once the child is on oral feeds –intake will be allowed ad lib, governed by the child himself/herself.

4. Control of seizures

- Diazepam 0.3 mg/kg. IV if seizure not aborted repeated after 5 minutes.
- Phenytoin 20 mg/kg. IV diluted in saline, at a rate not exceeding 0.75 mg/kg /min.
- If seizure not controlled. 25% glucose solution 1 ml/kg IV and
- Diazepam 0.3 mg/kg, IV (third dose).

If still uncontrolled after 20 minutes, it will be treated as status epilepticus with diazepam infusion 0.01mg/kg/min. stepped up by 0.005 mg /kg/min every 10-15 min until seizure is controlled or until maximum infusion rate of 0.06 mg/kg/min has been reach. Infusion tapered off over next 12 hours after control of seizures
Antibiotic Therapy:

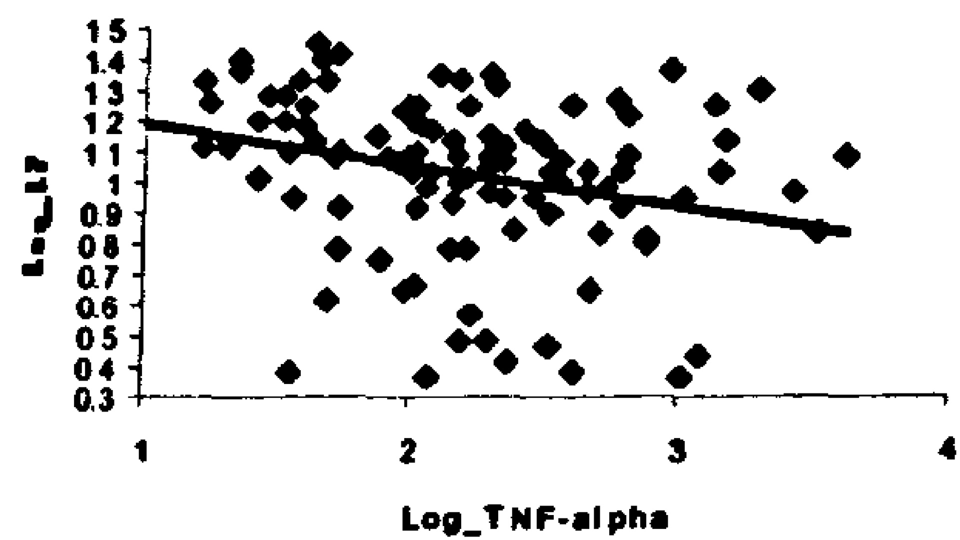
b. Antibiotics

Although no place for antibiotics in bronchiolits treatment but bacterial infections or mixed viral bacterial infection can't be excluded so we may need to give antibiotic. In this case Crystalline penicilline (benzyle pencillin) 50,000 iu /kg IV/IM every 6 hours and Gentamycin 7.5 mg/kg, IV/IM every 24 hour will be given.

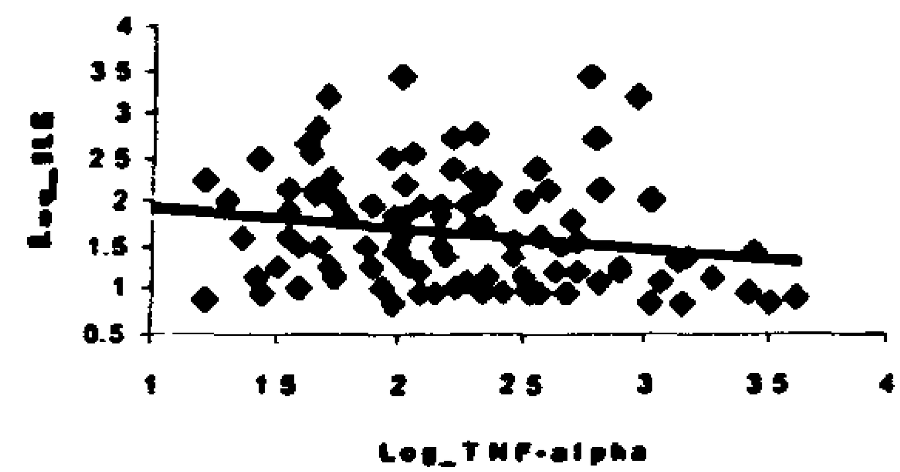
Appendix 7

Correlations of cytokines and chemokines

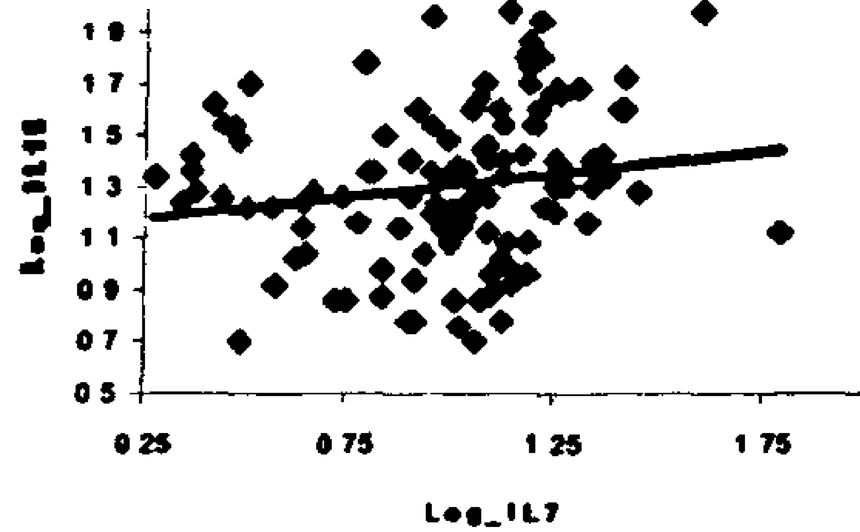
IL7 and TNF-α concentrations



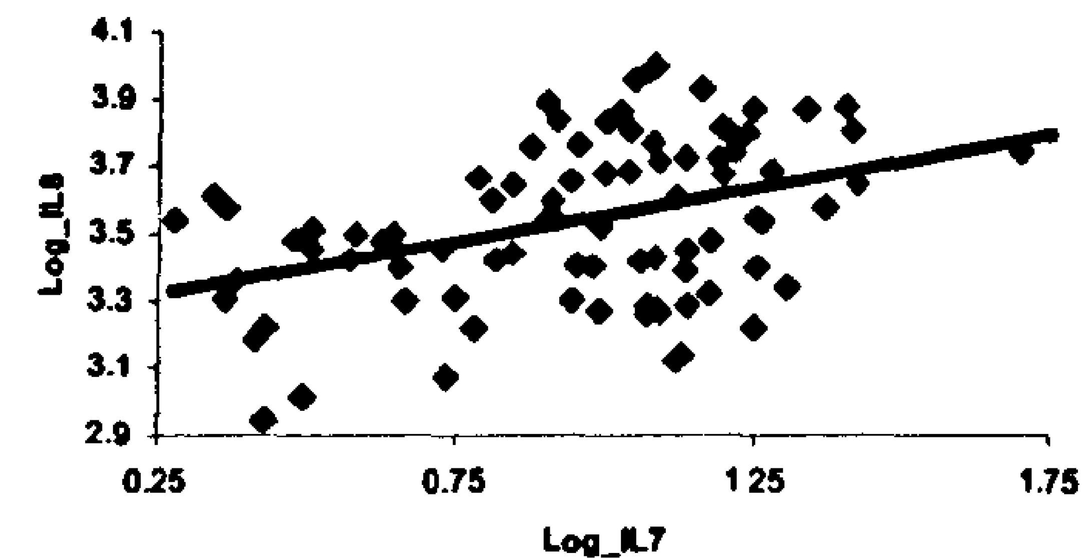
IL6 and TNF-α concentrations



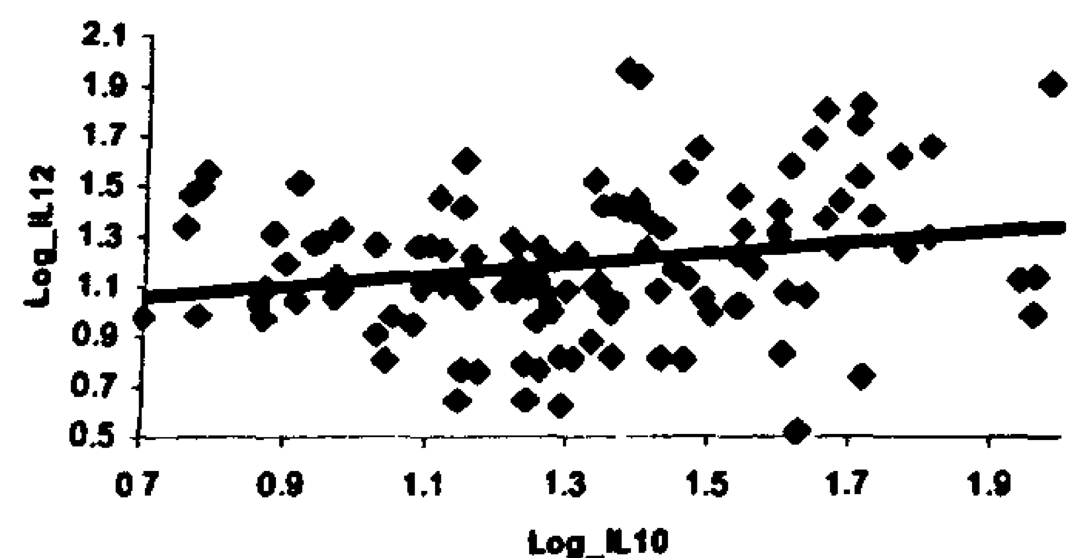
IL10 and IL7 concentrations



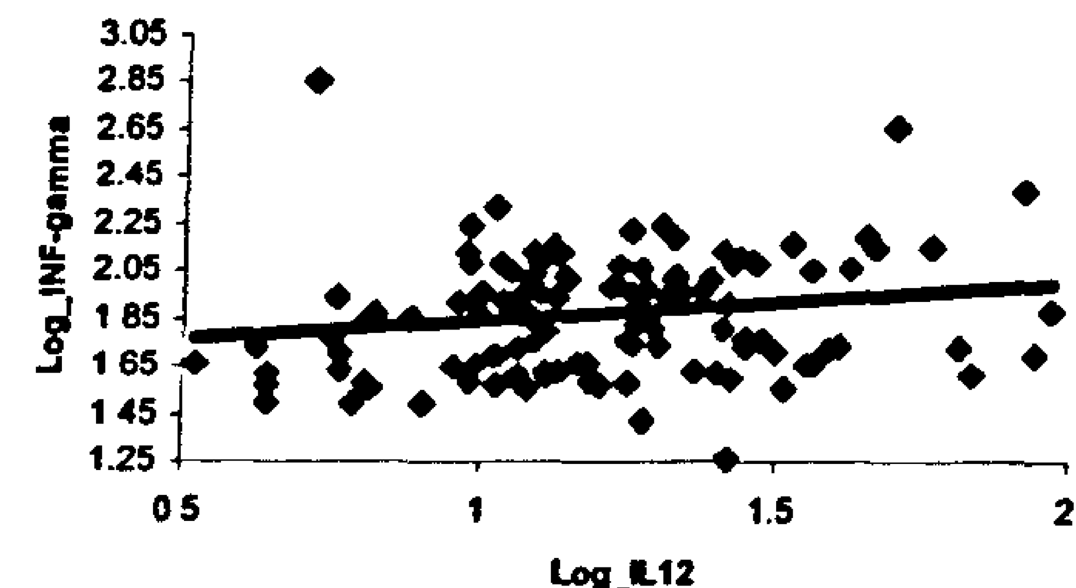
IL8 and IL7 concentrations



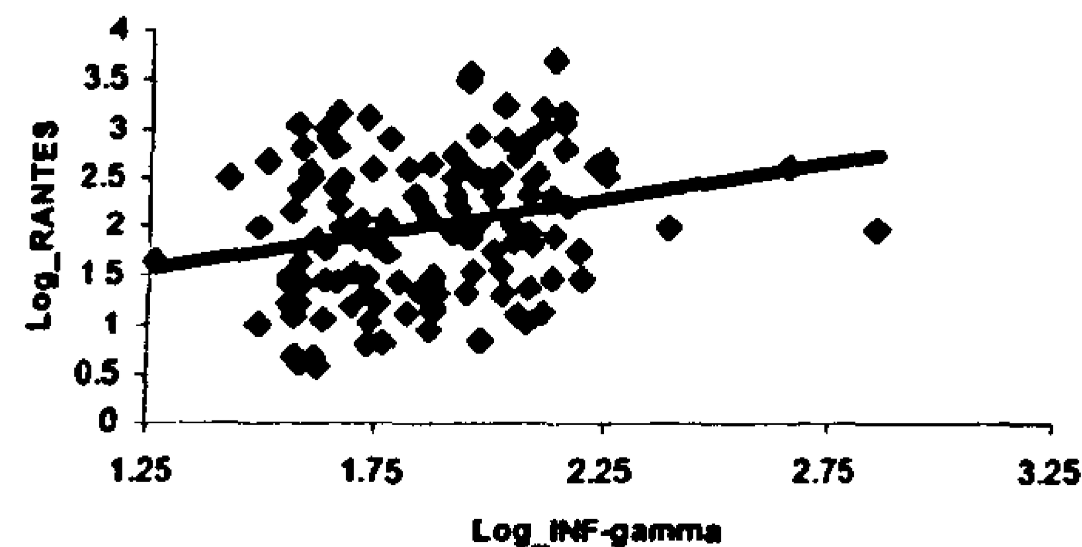
IL12 and IL10 concentrations



INF-γ and IL12 concentrations



RANTES and INF-γ concentrations



Appendix 8

Logistic regression analysis of the risk factors for Severe RSV and HMPV disease

A. Logistic regression analysis of the risk factors for severe RSV disease

1)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤ 3 months of age	2.7552	0.7504	10.1158	1.0135	0.6636	1.5273	0.1267
>3 children in the house	1.1369	0.4394	2.9414	0.1283	0.4850	0.2645	0.7914
Family member with ARI	1.7416	0.6749	4.4946	0.5548	0.4837	1.1471	0.2514
Indoor pets	1.1314	0.4001	3.1997	0.1235	0.5304	0.2328	0.8159
Mixed breastfeeding	0.9462	0.2188	4.0920	-0.0553	0.7471	-0.0740	0.9410
Rural residence	1.6562	0.3358	8.1681	0.5045	0.8142	0.6197	0.5355
Smoker at home	3.2876	1.2395	8.7203	1.1902	0.4977	2.3913	0.0168
Vaccines not up to date	3.7162	1.1404	12.1099	1.3127	0.6027	2.1779	0.0294
Non-governmental water	1.8114	0.7183	4.5679	0.5941	0.4719	1.2590	0.2080
Source of fuel other than gas	10.9393	2.1181	56.4994	2.3924	0.8377	2.8559	0.0043
CONSTANT	*	*	*	-1.0268	0.8347	-1.2301	0.2187

2)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤ 3 months of age	2.8431	1.0429	7.7504	1.0449	0.5117	2.0421	0.0411
>3 children in the house	1.1303	0.4425	2.8872	0.1225	0.4785	0.2560	0.7979
Family member with ARI	1.7375	0.6748	4.4738	0.5525	0.4825	1.1449	0.2523
Indoor pets	1.1323	0.4002	3.2038	0.1242	0.5307	0.2341	0.8149
Rural residence	1.6475	0.3363	8.0715	0.4992	0.8108	0.6157	0.5381
Smoker at home	3.2730	1.2430	8.6181	1.1857	0.4940	2.4003	0.0164
Vaccines not up to date	3.7743	1.2460	11.4327	1.3282	0.5655	2.3489	0.0188
Non-governmental water	1.8059	0.7190	4.5360	0.5911	0.4699	1.2579	0.2084
Source of fuel other than gas	10.9604	2.1251	56.5302	2.3943	0.8370	2.8606	0.0042
CONSTANT	*	*	*	-1.0751	0.5204	-2.0658	0.0388

3)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤3 months of age	2.8777	1.0610	7.8047	1.0570	0.5091	2.0763	0.0379
>3 children in the house	1.1641	0.4714	2.8744	0.1519	0.4612	0.3294	0.7419
Family member with ARI	1.7532	0.6831	4.4995	0.5615	0.4809	1.1675	0.2430
Rural residence	1.7019	0.3557	8.1419	0.5317	0.7986	0.6658	0.5055
Smoker at home	3.2419	1.2347	8.5120	1.1762	0.4925	2.3881	0.0169
Vaccines not up to date	3.7073	1.2381	11.1012	1.3103	0.5596	2.3416	0.0192
Non-governmental water	1.8100	0.7211	4.5435	0.5933	0.4696	1.2635	0.2064
Source of fuel other than gas	11.1144	2.1649	57.0610	2.4082	0.8346	2.8853	0.0039
CONSTANT	*	*	*	-1.0596	0.5157	-2.0546	0.0399

4)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤3 months of age	3.0198	1.1142	8.1843	1.1052	0.5087	2.1726	0.0298
Family member with ARI	1.8029	0.7118	4.5662	0.5894	0.4741	1.2431	0.2138
Rural residence	1.6691	0.3474	8.0186	0.5123	0.8008	0.6397	0.5224
Smoker at home	3.2238	1.2331	8.4283	1.1706	0.4903	2.3873	0.0170
Vaccines not up to date	3.9207	1.3097	11.7363	1.3663	0.5594	2.4423	0.0146
Non-governmental water	1.8364	0.7337	4.5964	0.6078	0.4681	1.2984	0.1941
Source of fuel other than gas	11.3062	2.1831	58.5543	2.4254	0.8391	2.8904	0.0038
CONSTANT	*	*	*	-1.0369	0.4712	-2.2007	0.0278

5)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤3 months of age	2.8137	1.0685	7.4090	1.0345	0.4940	2.0942	0.0362
Family member with ARI	1.8445	0.7313	4.6523	0.6122	0.4720	1.2971	0.1946
Smoker at home	3.0947	1.2027	7.9632	1.1297	0.4822	2.3426	0.0191
Vaccines not up to date	4.6355	1.7233	12.4689	1.5337	0.5049	3.0380	0.0024
Non-governmental water	1.7759	0.7140	4.4173	0.5743	0.4649	1.2354	0.2167
Source of fuel other than gas	14.0554	3.0424	64.9335	2.6430	0.7808	3.3850	0.0007
CONSTANT	*	*	*	-0.9960	0.4633	-2.1497	0.0316

6)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤ 3 months of age	2.9050	1.1143	7.5738	1.0664	0.4889	2.1812	0.0292
Family member with ARI	2.0288	0.8188	5.0271	0.7074	0.4630	1.5280	0.1265
Smoker at home	3.1726	1.2415	8.1077	1.1545	0.4787	2.4118	0.0159
Vaccines not up to date	4.7408	1.7709	12.6919	1.5562	0.5024	3.0974	0.0020
Source of fuel other than gas	14.5882	3.1750	67.0293	2.6802	0.7780	3.4449	0.0006
CONSTANT	*	*	*	-0.8456	0.4437	-1.9058	0.0567

7)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
≤ 3 months of age	3.2026	1.2486	8.2144	1.1640	0.4806	2.4220	0.0154
Smoker at home	3.5178	1.3930	8.8835	1.2578	0.4726	2.6612	0.0078
Vaccines not up to date	4.5942	1.7355	12.1622	1.5248	0.4967	3.0698	0.0021
Source of fuel other than gas	13.9712	3.0656	63.6721	2.6370	0.7739	3.4076	0.0007
CONSTANT	*	*	*	-0.4534	0.3528	-1.2851	0.1987

B. Logistic regression analysis of the risk factors for severe HMPV disease

1)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
> 2 adults per household	1.6077	0.2804	9.2167	0.4748	0.8909	0.5329	0.5941
Source of fuel other than gas	3.8831	0.3858	39.0879	1.3566	1.1782	1.1515	0.2495
Being male	4.6671	0.4755	45.8038	1.5405	1.1652	1.3221	0.1861
Family member with ARI	2.3070	0.4525	11.7630	0.8360	0.8311	1.0058	0.3145
Mixed breast feeding	3.2299	0.4878	21.3850	1.1725	0.9644	1.2157	0.2241
Indoor pets	0.7248	0.1187	4.4259	-0.3219	0.9232	-0.3487	0.7273
Smoker at home	4.0608	0.5254	31.3883	1.4014	1.0434	1.3431	0.1792
Non-governmental water	4.0379	0.5660	28.8065	1.3957	1.0025	1.3922	0.1638
≤ 3 months of age	6.9282	1.1040	43.4786	1.9356	0.9371	2.0655	0.0389
Vaccines not up to date	2.3135	0.2802	19.1044	0.8388	1.0771	0.7787	0.4362
Recurrent respiratory infections	11.2442	1.1747	107.6295	2.4199	1.1525	2.0997	0.0358
Allergies	0.5449	0.0626	4.7432	-0.6071	1.1040	-0.5499	0.5824
CONSTANT	*	*	*	-3.7147	1.4613	-2.5421	0.0110

2)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
> 2 adults per household	1.5124	0.2743	8.3379	0.4137	0.8710	0.4749	0.6348
Source of fuel other than gas	3.6204	0.3753	34.9241	1.2866	1.1564	1.1125	0.2659
Being male	4.5566	0.4752	43.6947	1.5166	1.1534	1.3148	0.1886
Family member with ARI	2.2354	0.4445	11.2426	0.8044	0.8241	0.9761	0.3290
Mixed breast feeding	3.2255	0.4839	21.5021	1.1711	0.9679	1.2099	0.2263
Smoker at home	3.7042	0.5331	25.7384	1.3095	0.9891	1.3240	0.1855
Non-governmental water	4.0655	0.5755	28.7220	1.4025	0.9975	1.4060	0.1597
≤ 3 months of age	6.7323	1.0968	41.3252	1.9069	0.9258	2.0597	0.0394
Vaccines not up to date	2.2515	0.2765	18.3336	0.8116	1.0700	0.7585	0.4482
Recurrent respiratory infections	10.1542	1.1458	89.9848	2.3179	1.1132	2.0823	0.0373
Allergies	0.5364	0.0610	4.7145	-0.6230	1.1090	-0.5617	0.5743
CONSTANT	*	*	*	-3.6371	1.4066	-2.5857	0.0097

3)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	3.7656	0.3939	36.0016	1.3259	1.1519	1.1511	0.2497
Being male	4.7208	0.5108	43.6288	1.5520	1.1346	1.3679	0.1713
Family member with ARI	2.1869	0.4365	10.9572	0.7825	0.8222	0.9517	0.3413
Mixed breast feeding	3.3894	0.5108	22.4901	1.2206	0.9655	1.2642	0.2062
Smoker at home	3.9826	0.5991	26.4737	1.3819	0.9665	1.4299	0.1528
Non-governmental water	3.5917	0.5539	23.2905	1.2786	0.9538	1.3405	0.1801
≤ 3 months of age	7.4023	1.2678	43.2208	2.0018	0.9003	2.2235	0.0262
Vaccines not up to date	2.1524	0.2715	17.0611	0.7666	1.0563	0.7257	0.4680
Recurrent respiratory infections	10.5811	1.2400	90.2909	2.3591	1.0939	2.1566	0.0310
Allergies	0.4947	0.0592	4.1352	-0.7038	1.0833	-0.6496	0.5159
CONSTANT	*	*	*	-3.4215	1.2877	-2.6570	0.0079

4)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	4.6861	0.5603	39.1954	1.5446	1.0837	1.4253	0.1541
Being male	4.9037	0.5195	46.2847	1.5900	1.1453	1.3882	0.1651
Family member with ARI	2.1763	0.4423	10.7088	0.7776	0.8130	0.9565	0.3388
Mixed breast feeding	3.2822	0.5017	21.4707	1.1885	0.9583	1.2403	0.2149
Smoker at home	4.0836	0.6147	27.1300	1.4070	0.9662	1.4562	0.1453
Non-governmental water	3.6834	0.5811	23.3471	1.3038	0.9422	1.3838	0.1664
≤ 3 months of age	7.2514	1.2632	41.6256	1.9812	0.8916	2.2220	0.0263
Vaccines not up to date	1.8066	0.2564	12.7299	0.5915	0.9962	0.5937	0.5527
Recurrent respiratory infections	10.5770	1.2613	88.6946	2.3587	1.0850	2.1739	0.0297
CONSTANT	*	*	*	-3.5391	1.2740	-2.7779	0.0055

5)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	6.4589	0.9930	42.0103	1.8655	0.9553	1.9526	0.0509
Being male	4.8731	0.5176	45.8794	1.5837	1.1440	1.3843	0.1663
Family member with ARI	2.0027	0.4234	9.4722	0.6945	0.7928	0.8760	0.3810
Mixed breast feeding	3.9110	0.6726	22.7412	1.3638	0.8982	1.5184	0.1289
Smoker at home	4.6004	0.6928	30.5504	1.5262	0.9659	1.5800	0.1141
Non-governmental water	4.1910	0.6973	25.1902	1.4329	0.9151	1.5659	0.1174
≤ 3 months of age	7.7172	1.3528	44.0230	2.0435	0.8884	2.3001	0.0214
Recurrent respiratory infections	13.2357	1.6873	103.8277	2.5829	1.0509	2.4577	0.0140
CONSTANT	*	*	*	-3.5947	1.2875	-2.7920	0.0052

6)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	7.5495	1.1847	48.1105	2.0215	0.9449	2.1393	0.0324
Being male	5.3283	0.6060	46.8487	1.6730	1.1091	1.5084	0.1315
Mixed breast feeding	3.3997	0.6254	18.4801	1.2237	0.8638	1.4166	0.1566
Smoker at home	5.0423	0.7875	32.2846	1.6179	0.9473	1.7078	0.0877
Non-governmental water	4.1492	0.7186	23.9571	1.4229	0.8946	1.5906	0.1117
≤ 3 months of age	9.2416	1.7114	49.9043	2.2237	0.8604	2.5844	0.0098
Recurrent respiratory infections	12.7422	1.6813	96.5697	2.5449	1.0334	2.4628	0.0138
CONSTANT	*	*	*	-3.3764	1.2321	-2.7403	0.0061

7)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	8.2658	1.3697	49.8803	2.1121	0.9171	2.3030	0.0213
Being male	3.4809	0.5016	24.1581	1.2473	0.9884	1.2619	0.2070
Smoker at home	4.0842	0.7321	22.7858	1.4071	0.8771	1.6044	0.1086
Non-governmental water	2.9643	0.6168	14.2460	1.0867	0.8009	1.3567	0.1749
≤ 3 months of age	11.2787	2.1890	58.1122	2.4229	0.8365	2.8966	0.0038
Recurrent respiratory infections	13.4321	1.7952	100.4992	2.5976	1.0268	2.5298	0.0114
CONSTANT	*	*	*	-2.7234	1.0246	-2.6579	0.0079

8)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	10.3834	1.6443	65.5706	2.3402	0.9403	2.4888	0.0128
Smoker at home	2.4155	0.5849	9.9751	0.8819	0.7236	1.2188	0.2229
Non-governmental water	3.0278	0.6665	13.7536	1.1078	0.7722	1.4347	0.1514
≤ 3 months of age	13.1604	2.5611	67.6246	2.5772	0.8351	3.0861	0.0020
Recurrent respiratory infections	14.4400	2.0242	103.0128	2.6700	1.0025	2.6634	0.0077
CONSTANT	*	*	*	-2.1985	0.8523	-2.5796	0.0099

9)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	10.7998	1.7498	66.6579	2.3795	0.9286	2.5625	0.0104
Non-governmental water	2.8513	0.6366	12.7707	1.0478	0.7650	1.3696	0.1708
≤ 3 months of age	12.2575	2.4759	60.6827	2.5061	0.8161	3.0709	0.0021
Recurrent respiratory infections	12.9929	1.9221	87.8290	2.5644	0.9750	2.6301	0.0085
CONSTANT	*	*	*	-1.6191	0.6661	-2.4308	0.0151

10)

Term	Odds Ratio	95%	C.I.	Coefficient	S. E.	Z-Statistic	P-Value
Source of fuel other than gas	13.1215	2.2100	77.9069	2.5742	0.9088	2.8325	0.0046
≤ 3 months of age	10.3356	2.2400	47.6907	2.3356	0.7802	2.9936	0.0028
Recurrent respiratory infections	12.9950	2.0097	84.0273	2.5646	0.9524	2.6929	0.0071
CONSTANT	*	*	*	-1.2283	0.5694	-2.1571	0.0310