A Clinical and Scientific Study to Investigate the Influence of Statins on Anastomotic Healing in Colorectal Surgery

Thesis submitted in accordance with the requirements of the

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Summary of Abbreviations Used in this Document

5-FU: Fluouracil
ACS: Americal college of surgeons
AL: Anastomotic leak
ASA: American society of anaesthesiology (in reference to pre-operative assessment of patient's health and operative risk)
BSA: Bovine serum albumin
BSG: Bioabsorbable seamguard
CCI: Charlson comorbidity index
CRP: C-Reactive protein
DMEM: Dulbecco's modified eagle medium
DMSO: dimethyl sulfoxide
DULK: Dutch leakage score
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
ERAS: Enhanced recovery after surgery
ESCP: European society of Coloproctology
HMG-CoA: 3-hydroxy-methylglutaryl co-enzyme A
HUVEC: Human umbilical vein cells
IL: Interleukin
IMA: Inferior mesenteric artery
IV: Intravenous
LBP: Lipopolysaccharide binding protein
LDL: Low density lipoprotein
MMP: Matrix metalloproteinase
MOABP: Mechanical and oral antibiotic bowel preparation
NRES: National research ethics service
NSAIDs: Non-steroidal anti-inflammatory drugs
NSQIP: National surgical quality improvement programme
PBS: Phosphate buffered saline
RCT: Randomised controlled trial
SMA: Superior mesenteric artery
TME: Total mesorectal excision
TNF: Tumour necrosis factor
VEGF: Vascular endothelial growth factor
WCC: White cell count (in reference to white blood cells)

Abstract

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Title
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Anastomotic leak remains one of the most devastating complications of colorectal surgery, as it is associated with increased morbidity and mortality, increased need for permanent stoma, increased risk of cancer recurrence, prolonged hospital admission and increased costs of healthcare. Anastomotic leak represents a failure of the complex process of tissue healing, therefore it must be regarded as a multifactorial complication, rather than as a single entity. Statins are amongst the most widely used drugs in the world, prescribed for the primary and secondary prevention of cardiovascular disease. Beyond lipid lowering, statins are known to have pleiotropic effects, which may exert a positive influence on processes fundamental to wound healing. Preliminary data from clinical studies suggests that statins may reduce the incidence of anastomotic leak.

Hypothesis and Aims of this Study
The hypothesis of this study is that statins promote processes fundamental to healthy tissue healing, and may therefore contribute to reducing the risk of anastomotic leak following colorectal surgery, and that the post-operative outcomes will be improved for patients taking statins. The aims of the study are to investigate whether statins exert an influence on colonic tissue healing. This thesis describes two studies run in parallel: a scientific study to investigate the effects of statins on primary cultured human colonic myofibroblasts, and a clinical study based on prospectively collected data from patients undergoing major colorectal surgery.

Outcomes and Conclusions
Atorvastatin, in concentrations equivalent to therapeutic doses, was shown to promote both the metabolic activity, and proliferation of primary cultured colonic myofibroblasts. Data was analysed from 113 patients in the clinical study, 38.9% of whom were taking statins at the time of the study. No difference was seen in the incidence of anastomotic leaks in patients taking statins, compared to those patients not taking statins, although the study population represented a relatively small cohort of patients with a low incidence of anastomotic leak (6 leaks out of 113 patients). The outcome from the clinical study raises the possibility that statins may represent a form of pharmacological prehabilitation, by normalising the risk of anastomotic leak in a population of patients with significant comorbidities, and a higher risk of anastomotic leak. The studies described in this thesis suggest that statins have an influence of processes fundamental to colonic tissue healing, and may contribute to reducing the risk of anastomotic leak. The potential direction for further studies to investigate this relationship is described.

Introduction

Surgery to remove a diseased section of bowel, and form an anastomosis to restore intestinal continuity is routinely carried out worldwide, to treat both benign and malignant disease. Over the past 200 years, developments in surgical technique, understanding of tissue healing, control of sepsis, and developments in anaesthesia have converted gastrointestinal anastomosis from a life-threatening procedure, to a procedure that is regarded as routine and safe.(1) Despite these improvements, impaired healing of the anastomosis, leading to anastomotic leakage (AL), remains the most feared complication of colorectal surgery, as it has been shown to increase the risk of 30 day mortality, overall mortality(2-5), need for permanent stoma(6, 7), impaired anorectal function(8, 9) and impaired quality of life(8, 10). There is also considerable evidence to suggest that AL is associated with an increased risk of cancer recurrence(3, 5, 11-13), although some authors dispute that observation(14, 15). Anastomotic leak is suspected to account for a quarter of all post-operative deaths following colorectal surgery(16, 17), and is also associated with delay to post-operative chemotherapy, prolonged hospital admission and increased healthcare costs(2). The quoted incidence of AL following colorectal surgery varies widely, with modern series reporting incidence of leak ranging from 1.8%-30%(2, 18-20). The wide range of reported incidence of leak is due to variations in definition of leak, varied approaches to the diagnosis of leak, the nature of the patient population, series reporting rectal or low rectal surgery only, and retrospective analysis of data(2, 18). As the consequences of anastomotic leak are so serious, there is considerable interest in the development of strategies to reduce the incidence of leak.

Definition of Anastomotic Leak

Although anastomotic leak is widely recognised as a serious complication, and is widely researched, there is no widely accepted definition; a systematic review of the definition of anastomotic leak identified 29 different definitions of anastomotic leak in lower gastrointestinal surgery(21). The United Kingdom Surgical Infection Study Group proposed "leak of luminal contents from a surgical join between two hollow viscera" as a definition(20), however that definition has rarely been referred to and the majority of studies refer to clinically relevant observations including peritonitis, faeculant discharge, drain discharge, abscess (including pre-sacral abscess), fever, or radiological findings in the definition(20). The International Study Group of Rectal Cancer published a universal definition: "A defect of the intestinal wall at the anastomotic site (including suture and staple lines of neorectal reservoirs) leading to a communication between the intra- and extra-luminal compartments." A clinical grading schema to describe the management strategy required following diagnosis of leak was published alongside the definition: *Grade A leaks*: no change to patient management required; *Grade B:* Require intervention, but not re-laparotomy; *Grade C*: require re-laparotomy(22). The definition was validated in a study of 2103(23).

Risk Factors for Anastomotic Leak and the Physiology of Tissue Healing

Numerous studies have attempted to establish risk factors for anastomotic leak, in order to identify risk factors that may be corrected pre-operatively, or to identify patients whose risk of anastomotic leak warrants modified surgical strategy. Patients regarded as having a high risk of leak may either undergo an operation that does not require anastomosis, or may have an anastomosis protected by a proximal,

defunctioning stoma. An awareness of factors contributing to a patient's risk of anastomotic leak also offers the advantage of allowing the patient to be involved in informed pre-operative decision making(17). Wound healing is a complex process, therefore numerous potential reasons for impaired or failed wound healing exist, and compromise to any facet of wound healing may lead to failure. Although numerous risk factors for colorectal anastomotic leak have been proposed over the past few decades, there is considerable conflict in the literature surrounding many of those risk factors. Before considering factors that may predispose to impaired anastomotic healing, it is necessary to consider the biology of wound healing, and factors that contribute to normal tissue healing in the colon.

Acute Wound Healing

Acute wound healing is described as the highly regulated process of cellular, humoural and molecular events activated at the time of injury, resulting in a time-dependent, orderly pattern of tissue repair(24). The classically described steps of wound healing are haemostasis, inflammation, fibroproliferation, and remodelling(24); abnormalities in any of those processes may lead to deficient healing. If the inflammatory response is delayed, or inadequate, wound contamination or infection may ensue, leading to abnormal signalling for progression into the fibroproliferative phase of repair – the phase during which wound strength increases very rapidly. If the fibroblast response is delayed or inhibited, the formation of early scar, and laying-down of provisional matrix will be compromised, increasing the duration that the wound is subject to mechanical load, and the length

of time that the wound strength is entirely dependent upon the material that has been used to bring about tissue apposition(24).

Following clot formation, inflammatory cells migrate to the injured tissue. Neutrophils are the first inflammatory cells to reach the wound, and act predominantly to sterilise and debride the wound in the early phase. Neutrophils have also been shown to enhance wound healing by secreting angiogenic factors VEGF, TNF-α and IL-1(25). Monocytes and macrophages then infiltrate the wound. In addition to phagocytosing injured tissue, macrophages secrete numerous autocrine and paracrine factors. Macrophages have been shown to be the only inflammatory cell type that is absolutely required in tissue healing(24). During the acute phase of inflammation, the wound essentially has no inherent strength; if this phase is prolonged, the chance of wound failure is increased(26).

Fibroblasts migrate into the healing wound within the first day of wounding, and proliferate rapidly, under the influence of growth factors, in normal wound healing they become the predominant cell type in the healing wound by day 4(25). Fibroblasts subsequently secrete collagen and other extracellular matrix (ECM) proteins, contributing to the wound ultimately gaining strength(27). During the proliferative phase of wound healing, collagen breakdown and synthesis occur concurrently, with breakdown predominating over the first 2-3 days; again emphasising the wound's dependence on the material used for the repair during early wound healing(17, 28). Wound contraction, another fundamental step in wound healing, is also mediated by fibroblast activity, as the fibroblasts attach to,

and migrate through early matrix(25). Studies comparing dermal wound healing with intestinal wound healing have shown that intestinal anastomoses heal at a much greater rate than dermal wounds, and that collagen is produced by both fibroblasts, and smooth muscle cells in the bowel(18).

In addition to an understanding of wound healing at the cellular level, it is important to consider the anatomy of the bowel in anastomotic healing. The layers of the colon, from innermost to outermost, are mucosa, submucosa, muscularis propria and serosa; each layer has a role in tissue healing.

Mucosa: The innermost layer of the colon, composed of columnar epithelial cells and mucin cells, and openings into crypts of Lieberkühn(29). The crypt bases contain stem cells, which mature into epithelial cells as they migrate towards the lumen. The lamina propria, between mucosa and muscularis propria harbours immune cells and capillaries. Neither the mucosa nor lamina propria contribute strength to the anastomosis, however the mucosa's role in the secretion of mucus, and rapid epithelialisation of the wound protect against the considerable bacterial load within the colon, thereby preventing wound complications associated with sepsis. The lamina propria is a source of vital immune and inflammatory cells(17, 28).

Submucosa: When considering anastomotic healing the submucosa is the most important layer of the bowel; a principle demonstrated by Halsted in 1887, describing "the resistance furnished to the needle on entering the submucosa"(1, 18, 30). The majority of the collagen in the bowel wall is contained within the submucosa, type I collagen predominates, although types III and V are also

present(17). Cross-linking of collagen is also fundamental to the strength that it provides – adequate nutrition and tissue oxygenation are essential for cross-linking(17, 18). The blood supply to the bowel terminates in the submucosa, forming a network of capillaries, to supply the mucosa with nutrients and oxygen(17).

Muscularis propria: Formed from an inner, circular layer, and outer longitudinal layer, the main function of the muscularis propria is peristalsis, rather than strength. This layer may become abnormal in disease states such as diverticular disease; the number of elastic fibres increases, leading to a thickened, shortened bowel. Associated oedema and inflammatory infiltration may also be present in diseased bowel. Anastomoses formed using inflamed or diseased bowel are known to be at increased risk of leak.(17)

Serosa: The thin outer layer of the bowel offers little or no strength to the anastomosis, but is essential in sealing the anastomosis. It is widely recognised that areas of bowel that lose the serosa have a high risk of leak(28). It is likely that the serosa seals small defects within the anastomosis, and therefore prevents these small defects progressing into defects which leak(17).

Blood Supply

It is well recognised that an adequate blood supply is vital to any healing tissue. This tenet is especially pertinent to intestinal anastomoses, as major vessels that supply the bowel are divided and ligated prior to forming the anastomosis, therefore blood supply from the residual vessels must be adequate to perfuse the anastomosis. In oncological surgery these vessels are ligated close to their origin, potentially limiting blood supply to a large section of bowel. The healing anastomosis requires a blood supply sufficient to deliver oxygen, inflammatory cells, nutrients and growth factors.

The bowel typically receives 20% of cardiac output, two-thirds of which is diverted to the mucosa(31). The mucosa is very sensitive to impaired perfusion, therefore susceptible to ischaemia. Ischaemic injury of the mucosa will compromise the mucosa's barrier function, and may progress, leading to full thickness ischaemia. If blood supply is restored, following a period of ischaemia, reperfusion injury may be more significant than the initial ischaemic insult(31).

Before considering anatomical factors that are relevant to anastomotic perfusion, it is also important to recognise that perfusion to the bowel is susceptible to compromise as a result of hypoperfusion arising from significant blood loss, hyperadrenergic situations ("fight or flight" response) and from cardiogenic complications, such as atrial fibrillation.

It is generally well understood that branches of the superior mesenteric artery (SMA) supply the jejunum, ileum, caecum, ascending colon and transverse colon, that branches of the inferior mesenteric artery (IMA) supply the descending colon, sigmoid colon and rectum. The marginal artery of Drummond arises as an anastomosis of the terminal branches of the SMA and IMA, this artery may be deficient or non-patent in some individuals. The area around the splenic flexure, known as Griffith's point, is regarded as a "water shed" area that is particularly susceptible to hypoperfusion during episodes of hypotension. Angiographic studies have also shown the proximal descending, and mid-descending colon to have more widely spaced, and fewer anastomotic vessels than other parts of the colon(32). The IMA is commonly ligated during left sided colonic resections; an area of controversy

surrounds preservation of the most proximal branches of that artery. Some authors advocate preserving proximal branches, in order to preserve marginal blood supply to the residual colon, therefore reduce the risk of leak, whilst others advocate ligation of the vessel at its origin to adhere to principles of oncological surgery(33-36). It is likely that, provided sufficient left sided colon is removed, high tie of the IMA is safe, and that leak risk is only increased if a high tie is associated with inadequate colonic resection before forming the colorectal anastomosis, although it is also suggested that patients at increased risk of atherosclerotic disease may benefit from preservation of proximal branches of the IMA(17, 32).

The Rectum

The rectum is worthy of special consideration from the point of view of anastomotic leaks for a number of reasons. It is widely accepted that anastomoses involving the rectum, have a higher risk of leak than ileocolic or colo-colonic anastomoses, and that leak risk increases with low rectal anastomosis(18). Serosal coverage is absent for the lower two-thirds of the rectum(37), consequently the sealing role provided by that layer is absent in low anastomoses. The majority of blood supply to the rectum is from the right and left branches of the superior rectal artery, a branch of the inferior mesenteric artery. Following ligation of that supply, the rectal stump will depend upon the middle rectal artery, a small branch of the internal iliac artery, and the inferior rectal artery, a branch of the internal pudendal artery. Angiographic studies have shown that the anastomotic blood supply arising from the middle and inferior rectal arteries is frequently limited, and that the posterior rectum is dependent upon intramural collateral vessels(17, 32, 38). Goligher identified that anastomotic dehiscence in rectal anastomoses typically occurred in the posterior

aspect of the anastomosis(39); an observation likely to be explained by the nature of the blood supply to that area. In addition to the residual blood supply to the rectal stump, the colorectal anastomosis derives a blood supply from the segment of proximal colon use in the anastomosis. The potentially sub-optimal blood supply to the rectal stump emphasises the importance of ensuring good blood supply from the proximal end of the anastomosis, and several authors have described techniques to ensure that the tissue used in the proximal edge of the anastomosis is well vascularised, and under low tension(18, 40).

The oncological management of rectal cancer has been the focus of extensive research for several decades, from the point of view of performing oncologically sound surgery, and that of performing sphincter-preserving surgery. As discussed previously, surgery to remove low rectal tumours, but preserve the sphincter complex, is associated with increased leak risk due to low rectal anatomy. The other significant advances in the management of rectal cancer that have had a significant influence on the study of colorectal anastomotic leak are total mesorectal excision and neo-adjuvant (pre-operative) therapy.

In 1982, having identified that the rectum and mesorectum had a common embryological origin, Heald introduced the concept of total mesorectal excision (TME)(41). The technique of TME involves sharp en bloc dissection of the tumour and its surrounding mesorectal tissue, with dissection in the avascular plane between the mesorectum and surrounding tissue. The technique had the advantage of incorporating the lymphatic drainage of the rectum into the resected specimen and

has been shown to confer increased disease free survival and significantly reduced local recurrence of tumour(42). TME has been widely adopted by colorectal surgeons, however in its early days the reported leak rates reached approximately 23% before returning to pre-TME era rates as surgical techniques improved to modify the leak risk (17, 43).

Adjuvant and Neo-Adjuvant Therapy

As the rectum is confined within the pelvis, rectal tumours are amenable to treatment with radiotherapy. Numerous studies over the past three to four decades have investigated the role of radiotherapy, and chemotherapy in the management of rectal cancer. The neo-adjuvant management of rectal cancer is one of the most keenly debated subjects in surgical oncology, and has been the focus of numerous clinical trials. Although opinions differ regarding the optimal pre-operative management of rectal cancer, many rectal cancer patients undergo pre-operative radiotherapy, which may be combined with chemotherapy. The effect of neo-adjuvant therapy upon leak will be discussed later in this document.

As the management of rectal cancer is such an intensively researched area, there is a vast amount of data relating to patients who have undergone treatment for rectal cancer. Data is sourced from prospectively managed registries, or from formal clinical trials. In addition to cancer-specific outcomes, data regarding post-operative complications is collected, therefore much of the data surrounding anastomotic leak is from multivariate analyses of such databases, rather than from prospective trials designed to investigate anastomotic leak specifically, and pertains to rectal anastomoses, rather than colonic anastomoses. As a consequence of the

heterogeneity of the data, the list of proposed risk factors is extensive, and there is conflict of opinion surrounding several of the identified risk factors.

Identified Risk Factors

It is appropriate to consider categories for anastomotic leak risk, as this approach may help to identify risk factors that may be modified pre-operatively, in order to reduce the risk of leak or may justify an operative strategy to reduce the risk of leak, or mitigate against the consequences of leak. The categories to consider are: anatomical, patient-specific, and perioperative.

Anatomical risk factors

The location of the anastomosis is well recognised as an influence on risk of leak; the more distal the anastomosis, the higher the risk of leak. Based on recent published series, the quoted risk of leak following ileocolic anastomosis is 2-3%, up to 10% for left sided resections, and up to 17% for low rectal anastomoses(5, 17). The increased risk of leak associated with left sided and low rectal anastomoses is likely to be due to the potential compromise to blood supply, and lack of peritoneal covering of the low rectum, as previously described. The increased volume of colonic bacteria, from proximal to distal colon, has also been proposed as an explanation of increased frequency of leak in operations involving the left side of the colon(9).

Patient-Specific Risk Factors

Several patient-related risk factors have been proposed, but again, many of these are the subject of conflicting opinion. Factors including tobacco use and high alcohol intake have been shown to increase leak risk in some studies,(20, 44, 45) numerous however other studies have failed to reproduce those findings, so these risk factors are only weakly associated with leak risk(17). Obesity and male gender have both

been associated with an increased leak risk in left-sided and rectal resections, but not in right sided resections(2, 46, 47). Isolating risk factors in cohorts of complex patients, who may have multiple risk factors, is particularly challenging, moreover it is likely that having multiple risk factors, presents a greater risk of leak. An American Society of Anaesthesia (ASA) score of 3 or more, or a Charlson Comorbidity Index (CCI) score of 3 or more are consistently shown to be associated with increased leak risk(17, 20).

Underlying Disease

The importance of forming an anastomosis with healthy tissue has been recognised for many decades(18). The majority of colorectal resections, particularly rectal resections, are carried out to manage malignant or potentially malignant disease, and in that situation the sites of transection of bowel are determined by principles of oncological surgery, which should ensure healthy tissue. Consideration of healthy tissue is particularly relevant when operating on patients with inflammatory bowel disease, or diverticular disease. Anastomoses in patients with Crohn's disease should be formed with macroscopically normal tissue, however it is not necessary to exclude Crohn's at the microscopic level before forming an anastomosis(48). In bowel affected by diverticular disease, both the longitudinal and circular muscle layers become abnormal. An increase in elastic fibres in the longitudinal layer results in thickening and relative shortening of the bowel, and the thickness of the circular layer becomes greater due to being in a chronically contractile state(49). It is also common for chronically inflamed tissue to have associated oedema. Macroscopic changes of diverticular disease – outpouches caused by herniation of the mucosa through the muscularis propria – may also lead to areas of weakness and potential

defects in the anastomosis. Sub-optimal healing, predisposing to anastomotic leak, is much more likely in thickened, oedematous tissue, or tissue with macroscopic defects, therefore bowel affected by diverticular disease should not be used in anastomoses(17).

Nutrition

The importance of nutrition in wound healing has been described above, so it is logical that malnutrition is associated with impaired wound healing. Poor nutritional status tends to be defined as weight loss ≥10% of body weight over the previous 3 months, serum albumin <35g/L, and serum total protein <55g/L. Malnutrition is widely accepted to be a risk factor for increased risk of post-operative morbidity and mortality(50), and has been shown to be associated with anastomotic leak following colonic resections, specifically right-sided colonic resections(17).

Medications

The effect of corticosteroids is especially relevant to the management of patients with inflammatory bowel disease, who have been treated medically, prior to undergoing surgery. Corticosteroids may compromise wound repair by inhibiting cellular signalling that promotes activation and migration of inflammatory cells, and by inhibiting collagen synthesis and wound contraction(27). It is therefore plausible that steroids should be regarded as a risk factor for anastomotic leak. A collection of retrospective studies failed to demonstrate an increased leak risk in association with steroids, however a recent prospectively designed study showed a convincing leak risk in association with both perioperative, and long-term corticosteroid use(17, 51). Non-steroidal anti-inflammatories (NSAIDs) have also been investigated as a potential risk factor for anastomotic leak; this is particularly relevant in the era of

enhanced recovery after surgery (ERAS), as NSAIDs are commonly used to avoid the need for opiate analgesia. A recent review of the risk of NSAIDs concluded that this class of drug does not increase the risk of anastomotic leak(52).

Neoadjuvant Therapy

Pre-operative radiotherapy, or chemoradiotherapy with 5-Fluorouracil (5-FU) or its oral pro-drug capecitabine, has become the standard of care for locally advanced rectal cancer, with the aim of reducing the risk of local recurrence. There are considerable variations in pre-operative regimes, and a review of that field is outside of the scope of this study. The effects of radiation, which may compromise tissue healing, include mucus depletion, increased apoptosis, expression and activation of proinflammatory cytokines, vascular injury, and activation of the coagulation cascade(53). Anatomical tissue planes may also lose their distinction as a result of radiation-induced injury, compromising TME surgery and post-operative haemostasis. Radiotherapy was widely accepted as a risk factor for anastomotic leak, possibly based on a study which demonstrated an association between previous abdominal or pelvic radiotherapy and anastomotic leak(16), however further studies, including a study investigating the effect over 30 years, did not find pre-operative radiotherapy to be an independent risk factor for leak(54). There is considerable conflict within the current literature regarding neo-adjuvant radiotherapy alone as a risk factor for anastomotic leak, however, the chance that it contributes to leak, particularly in the presence of other risk factors does remain(9, 17). 5-FU is known to affect cell proliferation, promote cell-cycle arrest and apoptosis, and may cause significant cytoskeletal abnormalities, and has been shown to inhibit fibroblast proliferation and collagen secretion in vitro, and has been shown to impair

experimental anastomotic healing in the rat(55-57). Side-effects of 5-FU include gastrointestinal disturbance, in addition to systemic effects such as nausea and loss of appetite, which may contribute to impaired tissue healing and impaired nutritional state pre-operatively. A randomised multicentre phase 2 trial, investigating the addition of panitumumab, an anti-epidermal growth factor receptor, compared to a standard capecitabine based chemoradiotherapy regime, showed a leak rate of 15% in the panitumumab group, compared to 4% in the capecitabine only group(58). It is likely that the effect of pre-operative systemic chemotherapy will become increasingly relevant to the risk of post-operative complications, as new agents are investigated and introduced into the pre-operative management of colon and rectal cancer.

Perioperative Factors

Prolonged operative time has been shown to be associated with increased incidence of anastomotic leak. The significance of that measure is contentious, as it could be a marker of a more challenging operation, such as removal of a large tumour, or operating deep in a male pelvis. From a clinical point of view, the benefit of recognising prolonged operative time as a risk for anastomotic leak, is that the surgeon is more likely to protect the anastomosis, and mitigate for consequences of leak, by forming a defunctioning stoma(17). Intra-operative blood loss has also been identified as an independent risk factor for anastomotic leak. Some studies quote the requirement of transfusion as a marker of leak risk, although the judgement surrounding need for transfusion is a relatively subjective measure, and might be a marker of complicated surgery, or poor surgical technique(17, 47). A review of 4340

cases showed blood loss of over 300ml to be a significant risk factor for anastomotic leak(59).

Other perioperative factors shown to increase leak risk include pre-operative diastolic blood pressure over 90mmHg, hypotension, oxygen saturation below 90% for over 5 minutes(35, 60) and administration of more than 8000ml of intravenous fluid (IV) over the 72 hour perioperative period(61). Excessive fluid administration is likely to lead to impaired tissue perfusion, and may also promote tissue oedema, both of which would compromise wound healing.

Surgical Technique and Surgical Instruments

Changes or modifications to surgical techniques are assessed for influence on multiple outcome measures, including anastomotic leak. Technological developments in colorectal surgery which have been investigated for influence upon leak, include the use of stapling devices to form the anastomosis (compared to hand-sewn anastomosis), and the increasing uptake of laparoscopic surgery, compared to open surgery. Anastomotic technique – hand-sewn versus stapled - has been evaluated in two Cochrane reviews, one review demonstrated no advantage of either technique over the other, whist a review considering ileocolic anastomoses specifically, demonstrated superiority of stapled anastomoses over hand sewn anastomoses(62, 63). Evidence from the early era of laparoscopic surgery indicates that laparoscopic surgery was associated with an increased risk of leak. That observation may be reflective of a learning curve, as more recent data shows a favourable comparison for laparoscopic surgery compared to open surgery(17, 64, 65). One specific consideration relevant to stapled anastomoses in laparoscopic

rectal surgery is the observation that 3 or more firings of the stapler, when dividing the rectum, was associated with increased leak risk in 2 studies(66, 67).

Colorectal anastomoses may be protected by forming a defunctioning stoma, typically a loop stoma, which may be a colostomy or an ileostomy, proximal to the anastomosis. The stoma should be reversed, to restore intestinal continuity, following investigations to confirm that the anastomosis is both intact, and has not strictured, and that the patient is fit for surgery. The rationale behind forming a defunctioning stoma to divert the faecal stream is that the consequences of anastomotic leak will be mitigated, and that the leak may be managed without the need for further major surgery, and that the anastomosis may ultimately be preserved. It is important to acknowledge that the formation, and subsequent reversal, of defunctioning stomas carries risks of morbidity and mortality(68), and that the presence of a stoma is associated with reduced quality of life(69), therefore the patient's risk of anastomotic leak is taken into account before making the decision to form a defunctioning stoma(20, 69). In addition to conferring benefit in the management of leaks, a number of studies have suggested that the incidence of leak is reduced by the presence of a defunctioning stoma(70-72), whilst other studies dispute that finding. The difficulty of isolating risk factors for anastomotic leak has already been discussed; the majority of the studies reporting the role of a defunctioning stoma are retrospective, and it is not always easy to establish whether the patients had similar levels of underlying risk for leak. A Cochrane review of 6 prospectively conducted trials, designed specifically to investigate the influence of defunctioning stoma on leak rate, concluded that a defunctioning stoma does reduce

the risk of leak, and the risk of needing further major surgery if a leak does occur(73). On balance, there is sufficient evidence to support a role for defunctioning stomas in the prevention of leak following anterior resection, particularly low anterior resection.

Intra-operative Assessment of the Anastomosis

Intra-operative testing of anastomotic integrity, followed by operative steps to manage signs of an unsound anastomosis, is widely endorsed. Strategies to deal with signs of compromised anastomosis include reinforcing the anastomosis with sutures, taking the anastomosis down, and refashioning it, forming a proximal stoma to defunction the distal bowel, or forming an end stoma instead of a primary anastomosis. Options for intra-operative anastomotic assessment fall into three categories: simple mechanical assessment, such as an air leak test; endoscopic assessment to visualise the anastomosis; and techniques to assess microperfusion to the tissue involved in the anastomosis. Leak-testing the anastomosis, particularly following a left sided colonic or rectal resection, is carried out by filling the pelvis with sterile saline, occluding the bowel proximal to the anastomosis, and gently introducing air into the rectum to inflate the perianastomotic bowel. Discharge of bubbles of gas indicates that the anastomosis is not sound. Intra-operative leak testing of the anastomosis, and acting appropriately if signs of leak are detected, has been shown to reduce the rate of leak risk in a number of studies(17, 74, 75). As incorporating an air leak test into the operation is a straightforward manoeuvre, and has been shown to identify deficient anastomoses, it is reasonable to suggest that leak testing should be routine practice, and does not require further investigation.

Endoscopic intraoperative inspection of the anastomosis with a flexible sigmoidoscope, allows direct visualisation of the anastomosis. Air leak testing is incorporated into endoscopic assessment, and this approach has been shown to be associated with a low anastomotic leak rate of 2.1%, in a study population of 415 patients, however that study did not have a control group(76).

Recognition and Management of Anastomotic Leak

Despite recognition of risk factors, and attempts to reduce leak rate by modifying as many risk factors as possible, anastomotic leak is accepted as an inevitable complication of colorectal surgery. Prompt recognition of anastomotic leak is essential, in order to minimise the clinical consequences of leak, prevent mortality and has the advantage of presenting options to manage the leak in the least invasive manner(6, 18). A high index of suspicion, supported by clinical observations, is essential in the diagnosis of leak; a number of authors have attempted to identify markers of leak to facilitate early diagnosis. Serum white blood cell count (WCC), serum C-reactive protein (CRP) and serum procalcitonin have all been investigated as potential markers for early identification of anastomotic leak(74, 77-81). In general, these markers tend to be significantly elevated in patients who have developed anastomotic leak, compared to those patients with a sound anastomosis, however, as the serum levels of the markers in question tend to rise with inflammatory or infective processes with causes other than anastomotic leak, an elevated level of any of the serum markers tends to lack specificity for leak. Even though the markers are shown to have a high sensitivity for anastomotic leak, their low specificity results in a low positive predictive value, therefore a normal or low

value may offer reassurance, and a high value may alert the clinician to the possibility of leak that should be investigated clinically or radiologically, but does not confirm the presence of leak. den Dulk and colleagues have attempted to create a standardised clinical tool, the DULK (Dutch leakage) score, derived from a combination of clinical observations and values of biomarkers, to increase the accuracy of the use of clinical signs and investigation results in the diagnosis of leak in its early stage(77, 78). Despite demonstrating impressive sensitivity, the DULK and modified DULK scores are compromised by a low positive predictive value. The use of such a scoring system does highlight the need for clinicians to "actively seek the leak"(78) and has the advantage of adding continuity to the monitoring of patients who are likely to be assessed by a variety of healthcare professionals during a hospital admission.

Assessment of levels of inflammatory markers in peritoneal drain fluid has also been investigated as a method of early detection of anastomotic leak. Markers that have been assessed include CRP, lipopolysaccharide binding protein (LBP), procalcitonin, interleukins 1, 6 and 10, tumour necrosis factor-α (TNF- α), matrix metalloproteinases (MMP) 8 and 9, and *E. faecalis*(74, 82-86). Assessment of peritoneal or pelvic fluid currently remains within experimental realms, however, the results of investigations into MMP and interleukin assays show elevated levels of these markers in patients who progressed to leak, indicating potential for future integration into clinical practice(74). The study to investigate concentration of *E. faecalis* in drain fluid showed that an increase in concentration was significantly associated with anastomotic leak, however, the high number of false positive tests

resulted in a positive predictive value of 30.2%(84). There is an obvious appeal to monitoring drain fluid for markers of anastomotic leak, however, as there is no proven benefit for the use of drains to either predict or prevent leak, routine drainage following colorectal surgery is not advocated, especially within ERAS protocols(86-88). Many experts would view routine drainage as a retrograde step, and would not support routine drainage simply to monitor fluid, often for several days.

Strategies to Prevent Anastomotic Leak

Although significant debate surrounds many of the previously discussed risk factors when considered in isolation, it is likely that there is an additive effect of risk factors, such that the more risk factors an individual has, the greater the risk of anastomotic leak. The majority of strategies to prevent, or minimise the incidence of leak are based upon modification of as many risk factors as possible. In reference to the risk factors already discussed, approaches to modify risk factors include smoking cessation, pre-operative nutritional support and correction of pre-operative anaemia. Factors under control of the surgeon include minimising blood loss, forming the anastomosis under low tension having ensured healthy tissue with an adequate blood supply, goal directed fluid therapy, assiduous technique and air leak testing, and decision to form a defunctioning stoma.

Biotechnology

Strategies to reduce anastomotic leak by incorporating additional elements to established perioperative and intra-operative practice remain reasonably uncommon. The role of biotechnology within medicine and surgery is increasing; the aim is to develop materials and devices to support and promoting tissue healing.

Attempts have been made to identify materials to reduce anastomotic leak, however these products remain within the developmental or experimental phase. Bioabsorbable Seamgaurd (BSG), a synthetic bioabsorbable staple line reinforcement for circular stapled anastomoses, has been investigated by a number of groups over the past few years. Results from early studies showed that the device was feasible and safe to use(89), however two recent randomised controlled trials (RCTs), with 302 and 258 patients respectively, failed to show any reduction of anastomotic leak in association with the staple line reinforcement(90, 91). The C-Seal is a thin-walled biodegradable sheath, incorporated into the lumen of the colon at the time of the anastomosis(92). The device protects the intraluminal aspect of the anastomosis, and to prevent faecal contamination of the peritoneal cavity, should leak occur. The C-Seal has been shown to be a safe and feasible option, however conclusive data to determine its efficacy is currently lacking, but should be provided when the results of a prospective multicentre trial, aiming to recruit 616 patients reports(93).

The Microbiome and Its Role in Anastomotic Healing and Anastomotic Leak

The gut microbiome is defined as the collected microorganisms, and their associated genetic material, contained within the gastrointestinal tract. Research into the gastrointestinal microbiome, and its influence on anastomotic healing, and therefore anastomotic leak, represents some of the most important recent work in this field. Some of the most influential work has been carried out by John Alverdy and colleagues(94). A comprehensive review of the gut microbiome and anastomotic leak is beyond the scope of this study; the most pertinent points are that certain microorganisms, specifically collagenase producing *Enterococcus faecalis*, are

significantly implicated in the pathophysiology of anastomotic leak(95). Establishing the principle that certain microorganisms act as drivers of leak has generated the proposal that manipulating the gut microbiome, either with antibiotics or a modified diet, may lead to a reduced risk of anastomotic leak(96). Patients with bowel disease are subject to a wide variety of factors that may influence the microbiome, including dietary restrictions, and the effects of medications including chemotherapeutic agents and immunosuppressive drugs. The relationship between the gut microbiome, its influence on tissue healing and surgical complications, and methods to manipulate the microbiome is likely to remain one of the most prominent areas of gastrointestinal surgical research for years to come.

Antibiotics represent one of the most accessible approaches to microbiome manipulation. The debate surrounding the role of pre-operative bowel preparation using antibiotics, and/or mechanical bowel preparation (MOABP), with the aim of reducing infective complications of surgery, pre-dates microbiome-focused research by several decades. Consequently, considerable variance exists in the advice issued by national and international surgical societies throughout the world. Currently, there is a trend toward the use of MOABP in North America, but much less support for this approach in Europe. This author has made a significant contribution to a recent publication outlining the rationale, and the arguments for and against for the use of pre-operative MOABP(97). It is likely that with increased understanding of the microbiome, its role in infective complications of surgery, and how it may be modified with the use of medications and diet, the role of antibiotics will become clearer(98). It may also be the case that other widely prescribed drugs, such as statins

(see below), and proton pump inhibitors (PPIs) may be found to influence the microbiome, and therefore influence tissue healing and post-operative outcomes.

Potential for Systemic Strategies to Promote Anastomotic Healing

Systemic effects of malnutrition, steroids and chemotherapy are recognised as having potential to impair healing however, to date, no systemic agents that actively promote healing have been identified. An interesting observation in a 2012 study by Singh et al. introduced the possibility that statins might be associated with a reduced risk of anastomotic leak following colorectal surgery(99). In a retrospective analysis of 269 patients, 86 of whom were taking a statin pre-operatively, the anastomotic leak rate was 1% in the statin group, compared to 7% in the group not taking statins. This observation is particularly significant, considering that patients who were taking statins had greater baseline perioperative risks, and therefore had a higher risk of anastomotic leak. The findings of this study have to be interpreted with some caution, as this was a retrospective study, designed to look at the overall effect of statins on a collection of markers in patients undergoing elective colectomy, and was not designed specifically to assess the effect of statins upon leak. The method of the study did not assess daily dose of statin, nor did the study assess patients' compliance with the medications. A more recent North American study, with data from a large, prospectively maintained surgical quality improvement collaborative investigated the relationship between pre-operative statin therapy and infectious complications of colorectal surgery(100). In this analysis of 7285 patients, of whom 34.5% were taking statins pre-operatively, there was a significantly lower incidence of post-operative sepsis, and a significantly reduced rate of anastomotic leaks following

rectal resections. As observed in the Singh et al study, the patients who were taking statins in this study were also significantly more likely to have comorbidities, and therefore a carry higher risk of post-operative complications, making the finding of a reduced incidence of infectious complications more noteworthy. Conflicting data has come from a more recent retrospective study, designed to investigate the relationship between statins and anastomotic leak risk, reported findings from 2766 patients, 19% of whom were prescribed statins perioperatively. The study identified specific statins, and prescribed dosages, and reported that patients prescribed statins perioperatively did not have a significantly different leak risk from those patients not taking a statin(101). All of these studies are compromised by retrospective methodology, and by differing definitions of leak, as the 2 studies demonstrating a beneficial effect of statins study included clinically or radiologically diagnosed leaks, whereas the second study considered only clinically significant leaks. The authors of the study that showed no significant difference also state that as the patients were not matched, it is possible that statins were normalising the risk of patients with an overall higher preoperative risk of leak(101). In the two studies that showed a reduced incidence of leak, 32% and 34.5% of patients respectively, were taking statins, compared to 19% of patients in the study that showed no difference, therefore differences in statin prescribing protocols may contribute towards the conflicting findings. Despite the compromises of the studies that reported a potentially protective effect of statins, such an observation warrants further investigation, especially as statins are used so prevalently throughout the developed world, and have been shown to have effects upon tissue healing in both

animal and human studies. The investigation of the effects of statins on colonic healing is the primary focus of the experimental work described in this study.

Statins

3-hydroxy-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, inhibit the conversion of HMG CoA to mevalonate, which subsequently inhibits the synthesis of a number of isoprenoids vital for cellular functions. The most well-recognised effect of inhibition of mevalonate synthesis is a reduction in synthesis of cholesterol(102). Statins are widely prescribed throughout the developed world, as they have been robustly demonstrated to reduce the incidence of cardiovascular disease(103). As hypercholesterolaemia is strongly associated with the risk of cardiovascular disease, it was initially believed that the beneficial effects conferred by statins were entirely due to reducing synthesis of cholesterol, particularly low density lipoprotein (LDL). Pleiotropic effects of a drug are the observed effects of the drug, beyond those for which the drug was initially intended. It is widely accepted that statins have a number of effects independent of their lipid lowering action. The pleiotropic effects of statins include stabilisation of endothelial function, decreased smooth muscle proliferation, and reduced vascular inflammation; effects which are widely believed to contribute to the demonstrated reduction in cardiovascular disease in patients who take statins(103-106)(Fig 1). Several of the pleiotropic effects of statins affect processes related to tissue healing; it is therefore plausible that statins may influence colonic tissue healing, and therefore influence the risk of anastomotic leak.

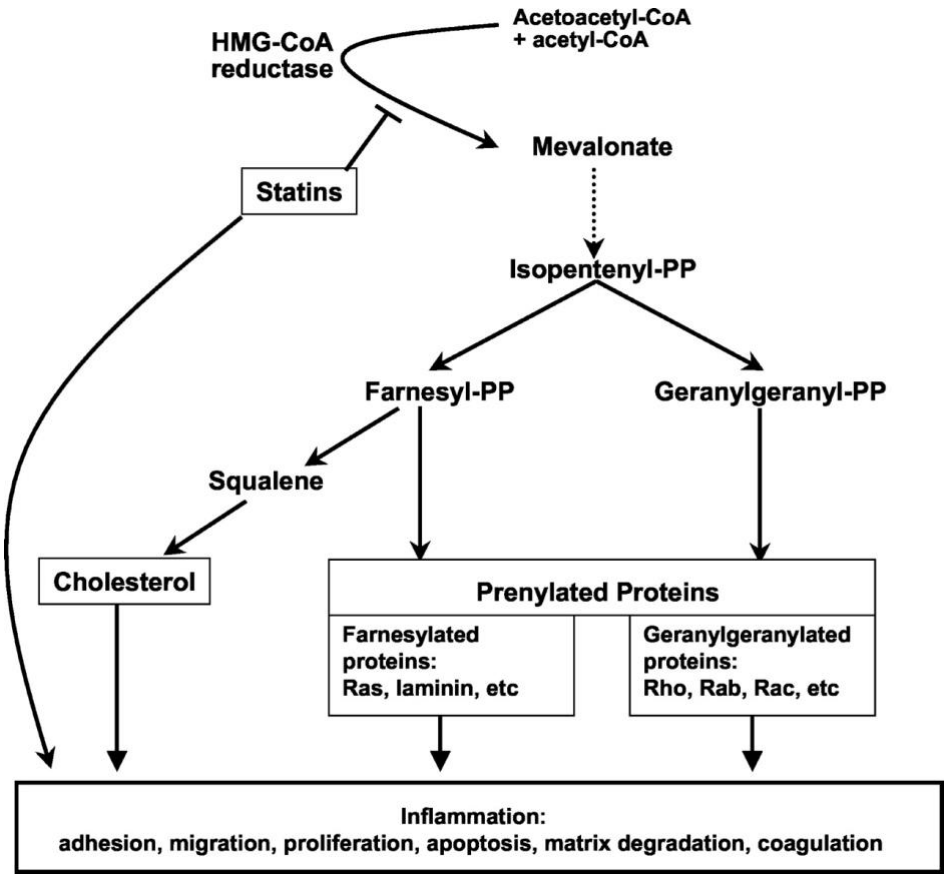


Figure 1: The mevalonate pathway and consequences of inhibition of mevalonate synthesis. Reproduced with permission of P Libby and Wolters Kluwer

The effect of statins on cells fundamental to tissue healing

Fibroblasts

As outlined earlier in this document, fibroblasts are fundamental to tissue healing, as they migrate into the wound then proliferate, under the influence of soluble growth factors and cytokines. Fibroblasts secrete proteins that form the provisional matrix, which is subsequently remodelled as the wound heals. The regulation of fibroblast proliferation, migration, and secretory function is crucial to tissue healing; insufficiency of those factors may result in failed wound repair, whilst excessive function may lead to fibrosis. Statins have been shown to affect the proliferation and migration of cardiac fibroblasts, leading to beneficial effects upon remodelling of

cardiac tissue(106). Statins have also been shown to reduce fibroblast adhesion, migration and viability of cardiac fibroblasts *in vitro*(107). Statins have also been shown to have effects upon the function of human lung fibroblasts, specifically inhibiting the secretion of matrix metalloproteinases (MMPs), proteolytic enzymes fundamental to tissue repair and remodelling(108). Although significant research has been conducted on fibroblasts derived from cardiac and pulmonary tissue, the effects of statins upon fibroblasts derived from bowel tissue have not yet been investigated, as suggestions that statins may affect tissue healing in the bowel have only been made relatively recently (99). One of the aims of this project is to investigate the effect of statins upon proliferation and function of fibroblasts derived from human colonic tissue.

The Effect of Statins upon Angiogenesis and Endothelium

The effects of statins upon angiogenesis have been extensively investigated, although none of the studies to date has been carried out on tissue derived from colon. A study on the effect of simvastatin upon proliferation, migration, sprouting and tubulogenesis, in microvascular endothelial cells derived from bovine retina demonstrated a biphasic effect on all of the observed outcomes, such that low doses promoted pro-angiogenic outcomes, whilst higher doses were found to be inhibitory with regard to angiogenesis, and the highest doses induced cell death *in vitro*(109). Atorvastatin has been shown to influence the angiogenic behaviour of human umbilical vein cells (HUVEC) with the same effect, such that low dose Atorvastatin promoted HUVEC cell migration and tube formation, but inhibited those features at higher doses(110). Both systemically administered and topically applied statins have also been shown to promote wound healing by enhancing angiogenesis in

experimental wounds in mice(111, 112). The biphasic effect on angiogenesis has also been demonstrated in liver regeneration in an experimental rat model(113). It is entirely plausible that statins would influence endothelial cell behaviour, and angiogenesis in human colonic cells, however, that work has not been carried out to date.

Animal Studies

To date, there are no reported studies describing the effects of statins upon human colonic tissue, from the point of view of wound healing, however a small number of animal studies have been reported. Statins have been shown to strengthen colorectal anastomotic wound healing in the rat, however tissue healing in the rat colon does not necessarily a provide a reliable model for the human colon, as anastomoses in the rat are much less prone to leak, even under experimental conditions specifically designed to induce anastomotic leak(114-116).

The rationale for the studies described in this thesis.

The only current evidence to indicate that statins may reduce the risk of anastomotic leak following colorectal surgery is from retrospective clinical studies. The recognised pleiotropic effects of statins, and their observed effects upon both cells and processes fundamental to tissue healing, make it plausible that statins could influence colonic tissue healing. As statins are amongst the most widely used medications in the developed world, and are generally well tolerated, promoting

tissue healing, thereby reducing the incidence of such a devastating complication as anastomotic leak, would be of considerable value.

The hypothesis of this study is that statins promote processes fundamental to healthy tissue healing, and may therefore contribute to reducing the risk of anastomotic leak following colorectal surgery, and that the post-operative outcomes will be different for patients taking statins.

This thesis presents two studies carried out in parallel. The first study was a laboratory-based study to harvest and culture myofibroblasts from human colonic tissue, and then to carry out experiments to investigate the effects of statins upon those cells. The second study was a clinically based study, using prospectively collected data from a cohort of patients undergoing major colorectal surgery in an inner-city university hospital, to establish whether there was a relationship between the pre-operative use of statins and incidence of anastomotic leak.

Laboratory Studies

Protocol for Tissue Preparation and Primary Cell Culture.

The method described below was used to culture the cells used for the experiments described later in the study. Over the course of the study, tissue was acquired from approximately 30 patients. Initially, the process was refined and developed, before cell lines were established from 5 patients using the method described below.

A full thickness section of fresh colon, of approximately 2cm in length, was acquired at time of operation, immediately after the specimen was delivered from the abdomen. The specimen was taken from the resection margin furthest from the diseased area of bowel, and therefore not required for histopathological assessment. Fat, mesenteric tissue and epiploica were dissected from the specimen in theatre. Macroscopic contamination was removed with a gauze swab, and the specimen was placed into ice cold phosphate buffered saline (PBS) with antibiotics (Penicillin, Streptomycin, Amphotericin B), and transferred to the laboratory. Specimens for cell culture were taken from patients who had no history of having taken statins.

The tissue underwent multiple washings in PBS with antibiotics, until the tissue was macroscopically free of any colonic content, and the effluent was also completely free of colonic content other than stray epithelial cells.

The specimen was placed into 70% ethanol for 3-5 seconds, then rinsed in fresh PBS 3 times, for 30 seconds per rinse.

The tissue was minced finely with size 23 scalpel blades, until it was completely homogenised.

Homogenised tissue was transferred into 50ml test tubes (3g per tube). 20 ml of collagenase II (Sigma-Aldrich; 5ml Collagenase, 15 ml PBS) was added to each test tube, and the test tubes were placed onto a rotating mixer, at 37°C, for 90 minutes.

Following incubation, the homogenised tissue was filtered through a 70μm cell strainer. The filtered fluid was centrifuged at 2000 rpm for 7 minutes, then the supernatant was aspirated and discarded.

Cell culture medium DMEM GlutaMax + (Gibco, Life Technologies), supplemented with 50ml of foetal calf serum per 500ml medium) was prepared. This medium was used for all of the myofibroblast based experiments. No additional antibiotics were added to this medium.

The cell pellet was suspended in 2 ml of culture medium DMEM+ GlutaMax (Gibco, Life Technologies), and vortexed. 14mls of the same medium were added to the test tube, which was mixed with a vortex machine for 5 seconds. Falcon T25 flasks were prepared with attachment factor (Sigma-Aldrich attachment factor solution).

The suspended cells were then transferred into the Falcon T25 flasks (4ml per flask). The flasks were incubated at 37°C, 5% CO_2 for 24 hours. Following microscopic confirmation of cell adherence to the base of the flask, the medium was aspirated, the cells were washed 3 times with PBS, and 4mls of fresh medium was applied. Isolated cells were confirmed as myofibroblasts by typical appearance under light microscopy, cell staining (see below) and by flow cytometry (data not available due to failure of now obsolete equipment).

Passaging of cells

Aseptic precautions were maintained throughout.

Cells were maintained in plasma treated tissue culture polystyrene flasks; typically, Falcon T75 (Scientific Laboratory Supplies, UK). Cell culture medium was aspirated from the tissue culture flask, and resultant adherent cells washed 3 times with PBS to remove senesced cells and residual serum proteins to prevent compromised trypsinisation.

After washing, a solution of 5g porcine trypsin, 2g EDTA in 100mL of 0.9% sodium chloride (Sigma-Aldrich, UK) diluted to a working concentration of 10% (v/v) using PBS was applied to the adherent cells. Trypsinisation was conducted at 37^{o}C for 5-10 minutes until approximately 75% of cells had detached from the base of the flask, confirmed by transmitted light microscopy. The suspension of cells and trypsin was diluted in an equal volume of cell culture medium, pre-warmed to 37^{o}C, containing 5% Foetal calf serum (v/v) (Lonza, % (v/v) to inhibit the trypsinisation reaction and therefore prevent protease induced cell damage during subsequent steps.

The diluted trypsin and cell suspension was centrifuged at 1.5x10^{3} rpm for 6 minutes at 4^{o}C to retrieve the cells. The supernatant was aspirated and the cell pellet was then resuspended in an appropriate volume of cell culture medium. Long term cell culture was facilitated by distributing the solution into the appropriate number of tissue culture vessels and diluting the suspension in a defined volume of cell culture medium. All cells were reseeded at 1/3 of the confluent cell density and incubated at 37^{o}C, in a humidified, 5%CO_2 environment. Culture medium was replaced every 3rd or 4th day by aspiration of used medium and introduction of fresh cell culture

medium, pre-warmed to 37^{o}C. The culture flasks were monitored regularly by transmitted light microscopy. When a confluent monolayer was confirmed, the passaging process was repeated in a 1:3 split.

Cell Culture Medium Containing Statin

Atorvastatin stock solution was created by dissolving 25mg of Atorvastatin calcium salt trihydrate (Sigma-Aldrich) in 2.5ml of dimethyl sulfoxide (DMSO) to give a 0.0165M stock solution of Atorvastatin, in accordance with manufacturer's instructions. The stock solution was then diluted appropriately to give a range of concentrations of Atorvastatin in culture medium. The concentrations created were 0.01µM, 0.1µM, 1µM, 10µM, 20µM to ensure a range that included accepted sub-therapeutic and supra-therapeutic serum equivalents(117). Cell culture media for control arms included DMEM GlutaMax+ with no statin, DMEM GlutaMax+ with DMSO only at the 10µM equivalent concentration (referred to as vehicle), and DMEM containing Atorvastatin 10µM and mevalonate 100µM.

Seeding Cells

3rd passage cells, cultured from a single donor, and maintained as described above, were used for the entire experiment. Confluent monolayers of cells in all T75 Falcon flasks were confirmed using phase contrast transmitted light inverted microscopy. Used medium was aspirated and cells were washed 3 times with PBS before being detached with trypsin as described above. Following detachment, cells were resuspended in 5ml medium to each flask, and flasks were inspected under phase contrast light microscopy to ensure that all cells were washed from the flask. The

suspension of detached cells was transferred to a 15ml Falcon tube and centrifuged for 5 mins at 1500 rpm at 4°C. Supernatant was aspirated. Cells were resuspended in 1ml of medium, and counted using a haemocytometer.

Cell suspensions were created, and cells were transferred to 24 well plates to populate wells with 2500 cells per well for the low seeding group, and 15000 cells per well for the high seeding group. The rationale behind the low seeding and high seeding groups was that cells in the low seeded wells would be unlikely to be limited by contact inhibition, and would be able to divide and metabolise rapidly. All cells were initially seeded in DMEM GlutaMax+ only, to ensure adequate cell adhesion to the surface of the wells, and to exclude the influence of Atorvastatin concentration in cell adhesion.

After 24 hrs incubation, cell adhesion was confirmed by phase contrast transmitted light microscopy. All medium was aspirated, the cells were washed 3 times with PBS then medium was replaced with medium for experimental conditions. Colourless DMEM was used for experimental conditions, to prevent interference with colourimetric assays. Medium for experimental conditions included: Atorvastatin 0.01, 0.1, 1, 10, 20µM, DMEM, DMSO equivalent to 10 µM (vehicle), and Atorvastatin 10µM with 100µM Mevalonate. Cells were incubated at 37˚C, 5% CO_2. Wells were populated at n=3 for all conditions. Data was collected at 4 time points: Day 1, Day 3, Day 5, Day 7.

<u>Metabolic Activity of Cells Using Alamar Blue Assay</u>

The Alamar Blue assay is a well-established assay, used to assess metabolic function and cellular health. The assay is based upon the molecule Resazurin, a nontoxic, non-

fluorescent, cell permeable redox indicator dye. Resazurin is continually converted to bright red–fluorescent Resorufin by the reduction reactions of viable, metabolically active cells. Following the reaction, the absorbance maxima of the molecule changes from 530nm to 590nm. Appropriate colourimetric techniques are used to quantify the response, and thereby indicate level of metabolic activity.

All culture medium was aspirated from each well and transferred sequentially to fresh 24 well plates, to create an identical copy of the 24 well plate containing the cells. 500µl of the aspirated medium was then replaced into its original well, to maintain the same conditions as original seeding. 50µl Alamar blue (AbD Serotec, UK) was then added to each well. The plates were incubated at 37˚C, 5% CO_2 for 4 hours. Following incubation, 100µl of medium containing Alamar Blue was aspirated from each well and transferred to black 96 well plates read using an FL_x800 microplate fluorescence reader in conjunction with the KC Junior operating platform (BIO-TEK Instruments, USA).

All wells of the 24 well plates containing the cells were washed 3 times with PBS, then immediately frozen at -85°C. These steps were repeated on repeated on days 3, 5, and 7.

CyQUANT Cell Proliferation Analysis

CyQUANT cell proliferation assay (ThermoFisher Scientific) is a non-radioactive, sensitive method to assess cell proliferation based on DNA content. The amount of DNA in each cell remains constant for a given cell line or cell type; this assay can

therefore be used to provide an accurate and simple measure of cell number. CyQUANT cell proliferation assays are more sensitive than colorimetric-based assays, and are not radioactive and do not depend upon the metabolic status of the cell.

Creation of Cell Number Standard Curve Using CyQUANT

A T75 flask, with a confluent monolayer of fibroblasts confirmed by light microscopy was selected. All medium was aspirated, and cells were washed x3 with PBS. Cells were detached with 4ml 10% trypsin, for 5 minutes, with detachment confirmed under light microscopy. 8ml of medium was added to the flask, and the cell suspension was transferred into 15ml Falcon tube, and centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated and discarded. The cell pellet was then resuspended in 1.3 ml PBS, and cells vortexed briefly. The cells were counted using a haemocytometer, showing a count of 835000 cells/ml; giving 108550 cells in total. The cell suspension was transferred to 1.5ml Eppendorf tube, and centrifuged in a microcentrifuge at 200xg for 5 minutes. The supernatant was removed and discarded without disturbing the cell pellet. The Eppendorf tube containing the cell pellet was then transferred to a freezer at -80°C for 4 hours to achieve cell lysis.

CyQUANT GR dye/cell lysis buffer was prepared in accordance with the manufacturer's instructions (1ml cell lysis buffer, 19ml nuclease free distilled water, 50µl CyQUANT stock solution dye). The tube containing the cell pellet was removed from freezer, and the pellet was allowed to thaw at room temperature. 1.0 ml CyQUANT GR dye/cell lysis buffer was added to thawed cell pellet, and the cells were resuspended by brief vortexing. A dilution series was generated within wells of 96

well plate, in 200µl volumes: n=4 for concentrations ranging from 0 to 50000 cells. The samples were protected from light and incubated at room temperature for 5 minutes. Fluorescence was measured using a FL_x800 microplate fluorescence reader in conjunction with the KC Junior operating platform (BIO-TEK Instruments, USA) with excitation and emission setting of 485/520 nm. Sensitivity was set to automatic, adjusted to high value wells. Values were plotted on a scatter chart with fluorescence on *y* axis and cell number on *x* axis. A line of best fit was added to chart, and the correlation coefficient generated.

CyQUANT Assay on Cultured Cells

The cells used were from the same cell line as those used for the calibration curve calculation. The 24 well plates which had been frozen at -80°C were thawed at room temperature, and add 200 µL of the CyQUANT GR dye/cell-lysis buffer was added to each sample well. The plates were incubated at room temperature 4 minutes, protected from light. 180 µL of CyQUANT reagent was aspirated from each well, and transferred into clean plates. Fluorescence was measured using a FL_x800 microplate fluorescence reader in conjunction with the KC Junior operating platform (BIO-TEK Instruments, USA) with excitation and emission settings of 485/520 nm. Sensitivity was set to automatic, adjusted to high value wells.

The Effect of Atorvastatin on the Metabolic Activity And Proliferation of Primary Cultured Human Colonic Myofibroblasts.

Statistical analyses

The Minitab Software (Minitab® 17.1.0) was used for the analyses. One-way ANOVA test was modelled to determine whether the mean values for metabolic activity and cell proliferation differ among the treatments (A0.01, A0.1, A1, A10, A20, Veh, Med, Meval) using Tukey Pairwise Comparisons method. It was assumed that the variance is constant across all groups and two-sided confidence interval with 95% confidence level was used for the analyses. Interval plots and Tukey confidence interval plots were constructed to present the results graphically.

Results

Cell Proliferation

Cell proliferation is quantified, and represented on the graphs, by total cell number from the CyQuant assay, having followed the calibration curve calculation method described above.

CQ 15000 (CyQUANT 15000 cells/well)

Day 1. There was no difference in cell proliferation among A0.01, A0.1, A1, A10, and controls (Veh, Med, Meval). A20 resulted in significantly less cell proliferation in comparison to A0.01 ($P<0.0001$), A0.1 ($P<0.0001$), A1 ($P=0.001$), A10 ($P=0.026$), and controls [Veh ($P<0.0001$), Med ($P<0.0001$), Meval ($P<0.0001$)].

Day 3. There was no difference in cell proliferation among A0.01, A0.1, A1, and controls (Veh, Med, Meval). A10 and A20 resulted in significantly less cell proliferation in comparison to Med as control ($P= 0.005$ and $P<0.0001$, respectively).

Day 5. A0.1 and A10 resulted in more cell proliferation in comparison with Meval as control ($P=0.007$ and $P=0.003$, respectively). A20 resulted in less cell proliferation in

comparison with A0.1 (P=0.036), A1 (P= 0.014), and Med as control (P=0.004). There was no difference in cell proliferation among other comparisons.

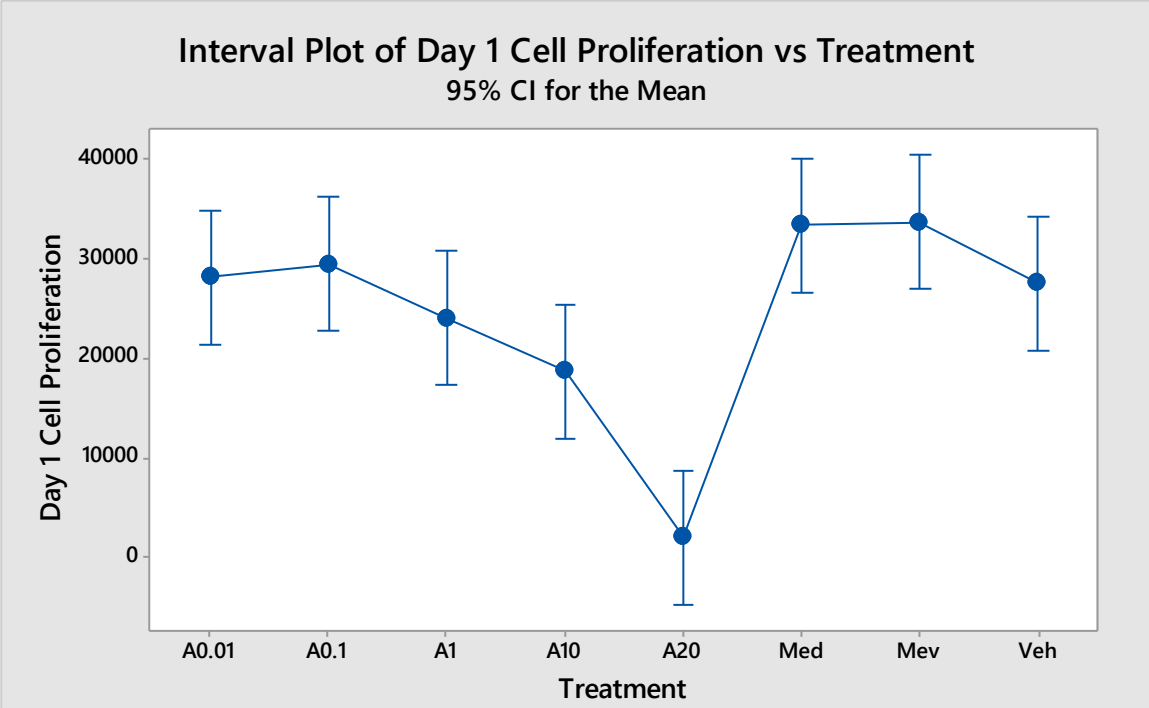
Day 7. A0.01 resulted in significantly more cell proliferation in comparison to A10 (P<0.0001), A20 (P<0.0001), and Meval as control (P<0.0001). A0.1 resulted in significantly more cell proliferation in comparison to A10 (P<0.0001), A20 (P<0.0001), and Meval as control (P<0.0001). A1 resulted in in significantly more cell proliferation in comparison to A10 (P<0.0001), A20 (P<0.0001), and Meval as control (P<0.0001). A10 resulted in significantly more cell proliferation in comparison to Med (P=0.002) and Veh as controls (P<0.0001). A20 resulted in significantly less cell proliferation in comparison to Med (P<0.0001) and Veh as controls (P<0.0001).

All figures relating to the above analysis are below.

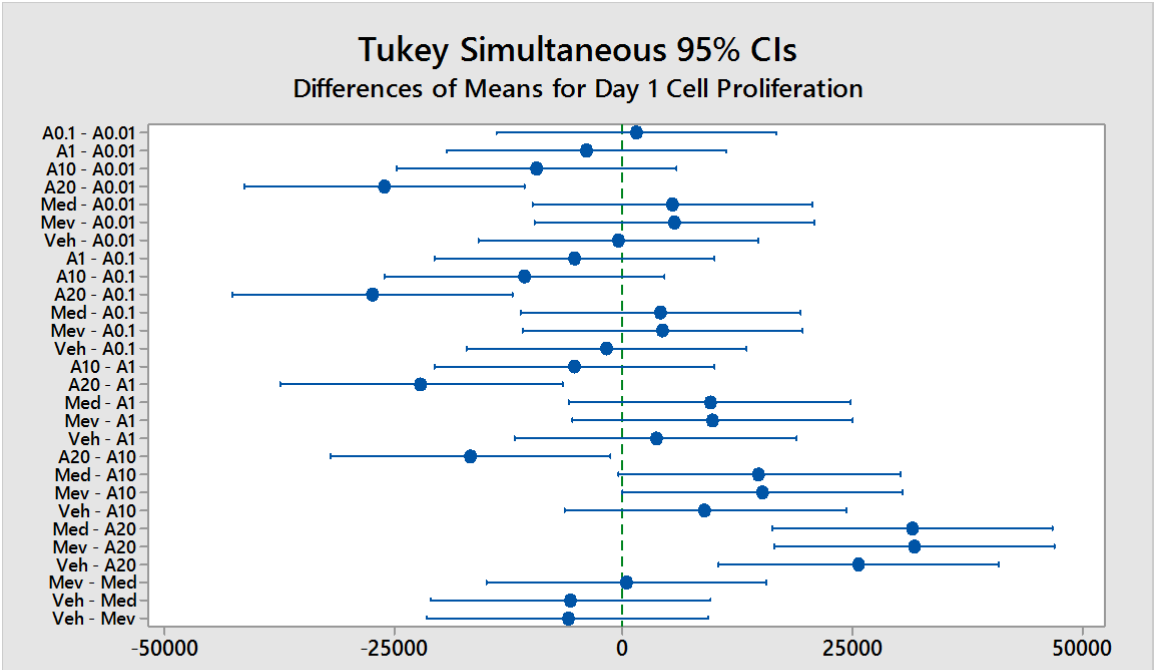
Figures to Demonstrate The Effect of Atorvastatin Concentration on Cell Proliferation Over 4 Time Points in a Densely Seeded Cell Population

CQ 15000 (CyQUANT 15000 cells/well)

Day 1

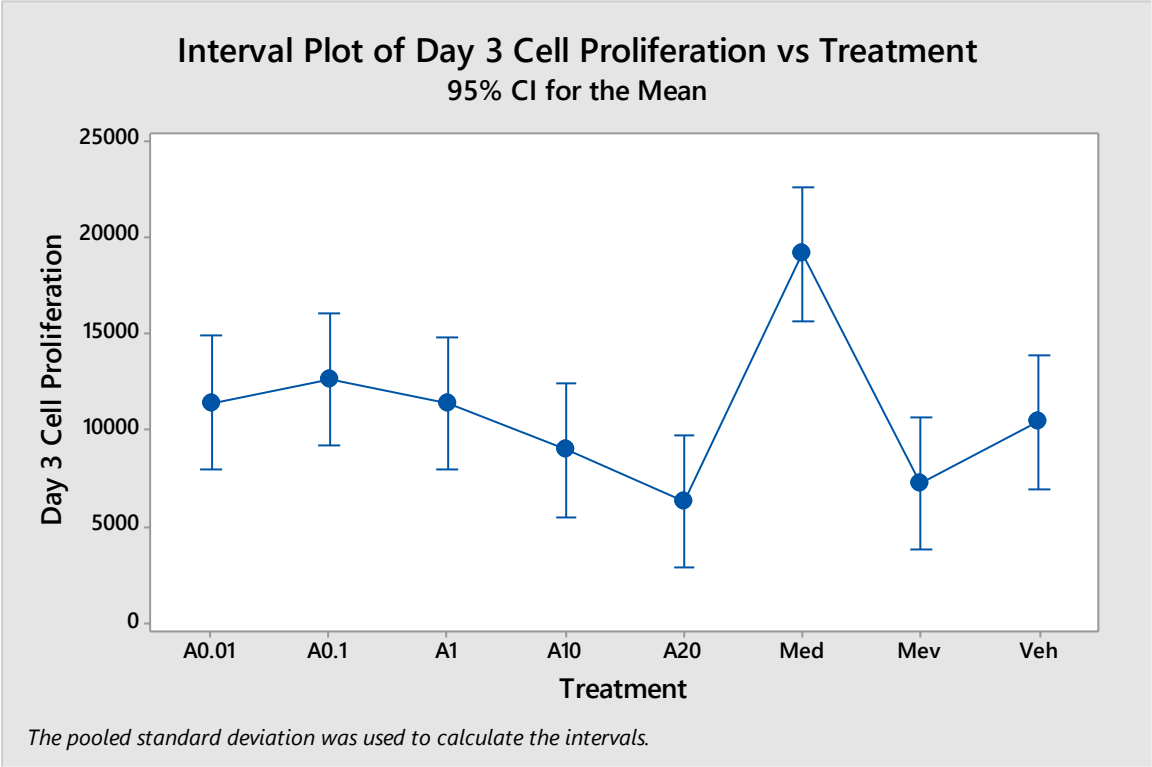


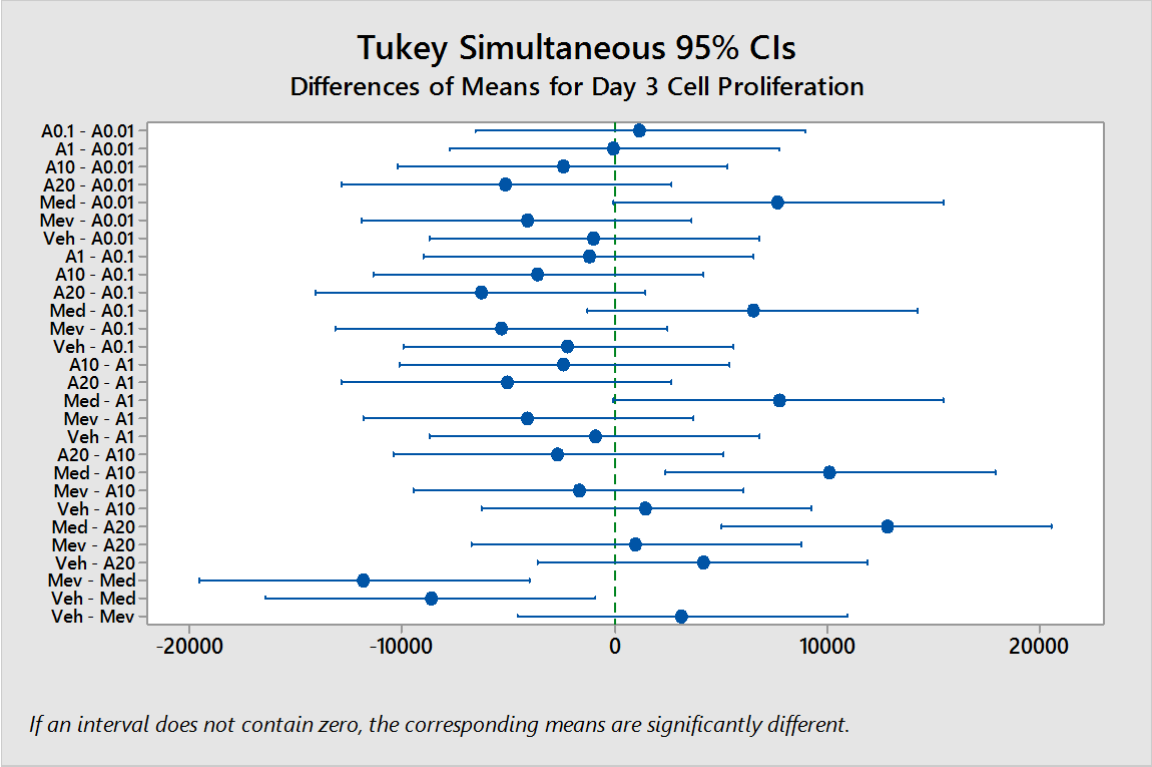
The pooled standard deviation was used to calculate the intervals.



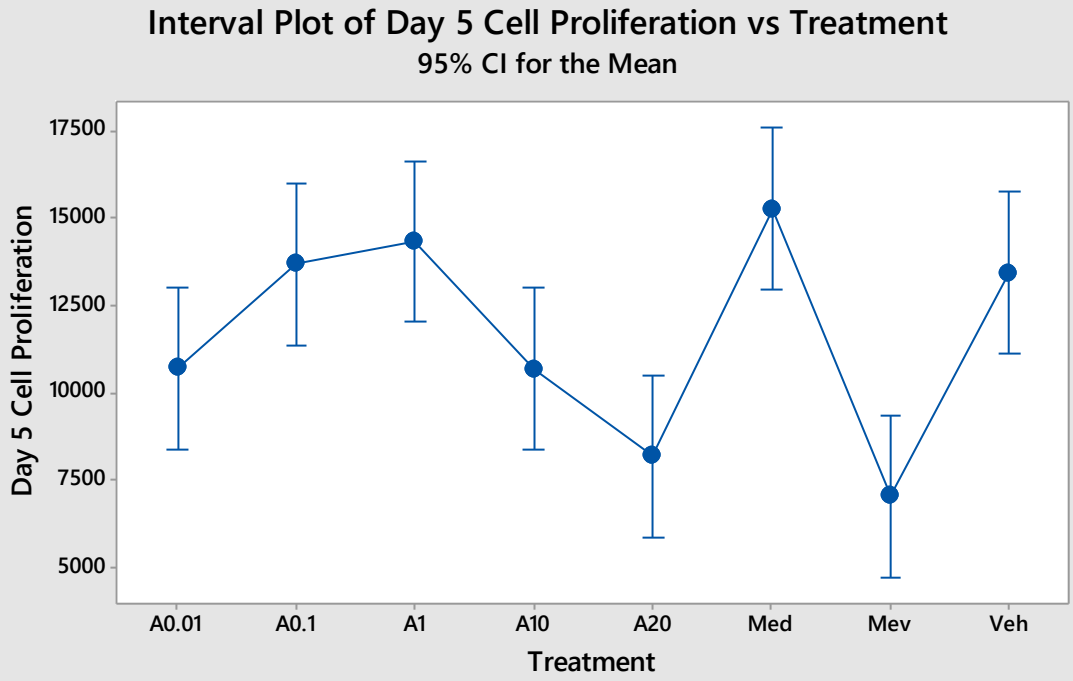
If an interval does not contain zero, the corresponding means are significantly different.

Day 3,

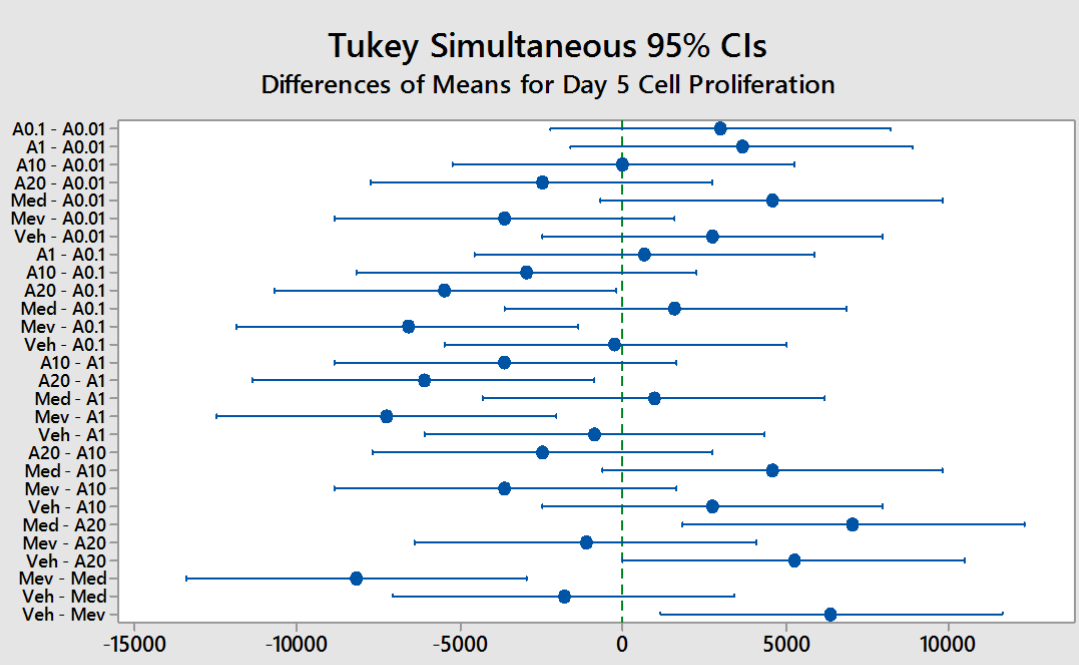

Interval Plot of Day 3 Cell Proliferation vs Treatment
95% CI for the Mean
Day 3 Cell Proliferation
25000
20000
15000
10000
5000
0
A0.01
A0.1
A1
A10
A20
Med
Mev
Veh
Treatment
The pooled standard deviation was used to calculate the intervals.


Tukey Simultaneous 95% CIs
Differences of Means for Day 3 Cell Proliferation
A0.1 - A0.01
A1 - A0.01
A10 - A0.01
A20 - A0.01
Med - A0.01
Mev - A0.01
Veh - A0.01
A1 - A0.1
A10 - A0.1
A20 - A0.1
Med - A0.1
Mev - A0.1
Veh - A0.1
A10 - A1
A20 - A1
Med - A1
Mev - A1
Veh - A1
A20 - A10
Med - A10
Mev - A10
Veh - A10
Med - A20
Mev - A20
Veh - A20
Mev - Med
Veh - Med
Veh - Mev
-20000
-10000
0
10000
20000
If an interval does not contain zero, the corresponding means are significantly different.

Day 5

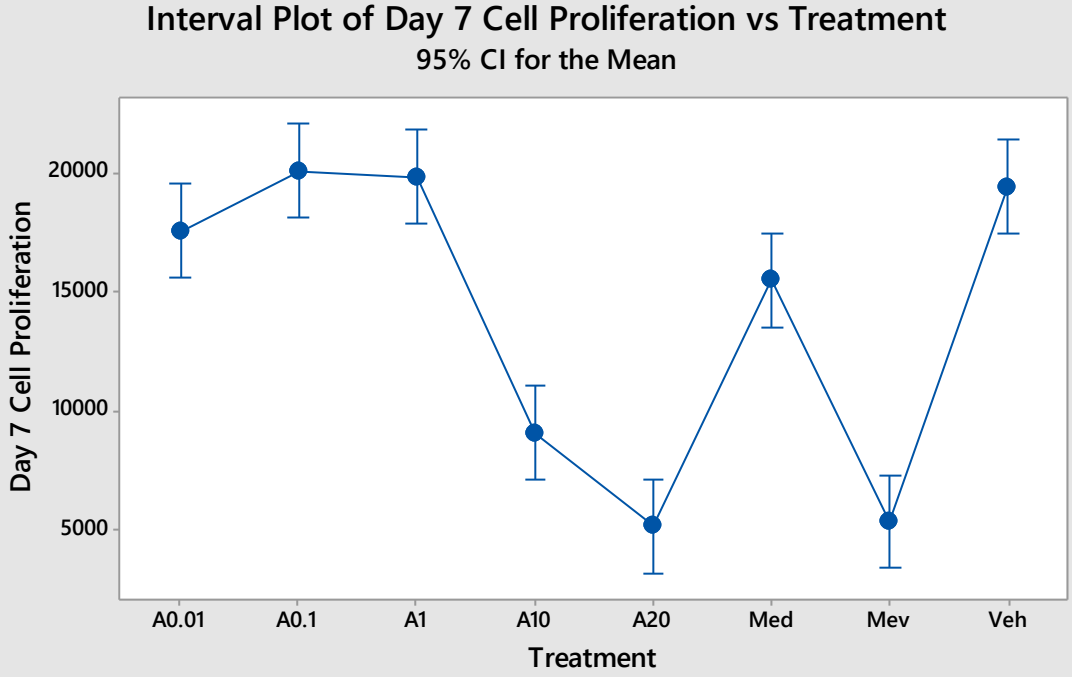


The pooled standard deviation was used to calculate the intervals.

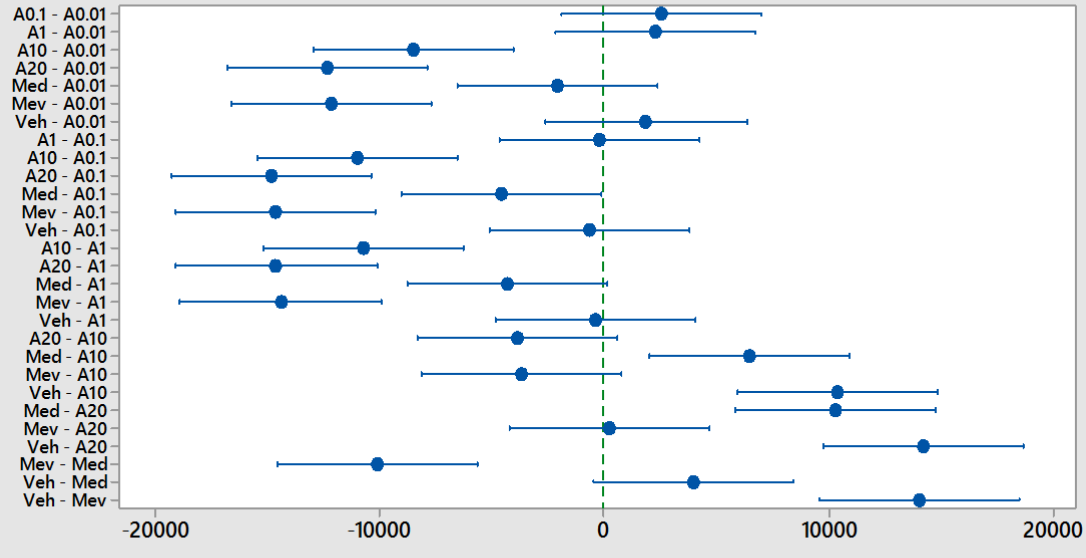


If an interval does not contain zero, the corresponding means are significantly different.

Day 7



The pooled standard deviation was used to calculate the intervals.



If an interval does not contain zero, the corresponding means are significantly different.

CQ2500 (CyQUANT 2500 cells/well)

Day 1. There was no difference in cell proliferation among A0.01, A0.1, A1, A10, A20 and Meval as control. Med as control resulted in in significantly less cell proliferation in comparison to A0.01 (P=0.005), A0.1 (P<0.0001), A1 (P=0.002), and A10 (P=0.009). A0.1 resulted in significantly more cell proliferation in comparison to Veh as control (P=0.006).

Day 3. A0.1 resulted in in significantly more cell proliferation in comparison to A20 (P=0.002) and controls [Veh (P=0.026), Med (P=0.001), Meval (P=0.013)]. A1 resulted in significantly more cell proliferation in comparison to A20 (P=0.010 and Med as control (P=0.005). There was no difference in cell proliferation among other comparisons.

Day 5. A0.01 resulted in significantly more cell proliferation in comparison to A10 (P<0.0001), A20 (P<0.0001), Med as control (P<0.0001) and Meval as control (P<0.0001). A0.1 resulted in significantly more cell proliferation in comparison to A10 (P<0.0001), A20 (P<0.0001), Med as control (P<0.0001) and Meval as control (P<0.0001). A1 resulted in significantly more cell proliferation in comparison to A10 (P=0.003), A20 (P=0.001), Med as control (P<0.0001) and Meval as control (P=0.003). Veh as control resulted in significantly more cell proliferation in comparison to A1 (P=0.011), A10 (P<0.0001), and A20 (P<0.0001). There was no difference in cell proliferation among other comparisons.

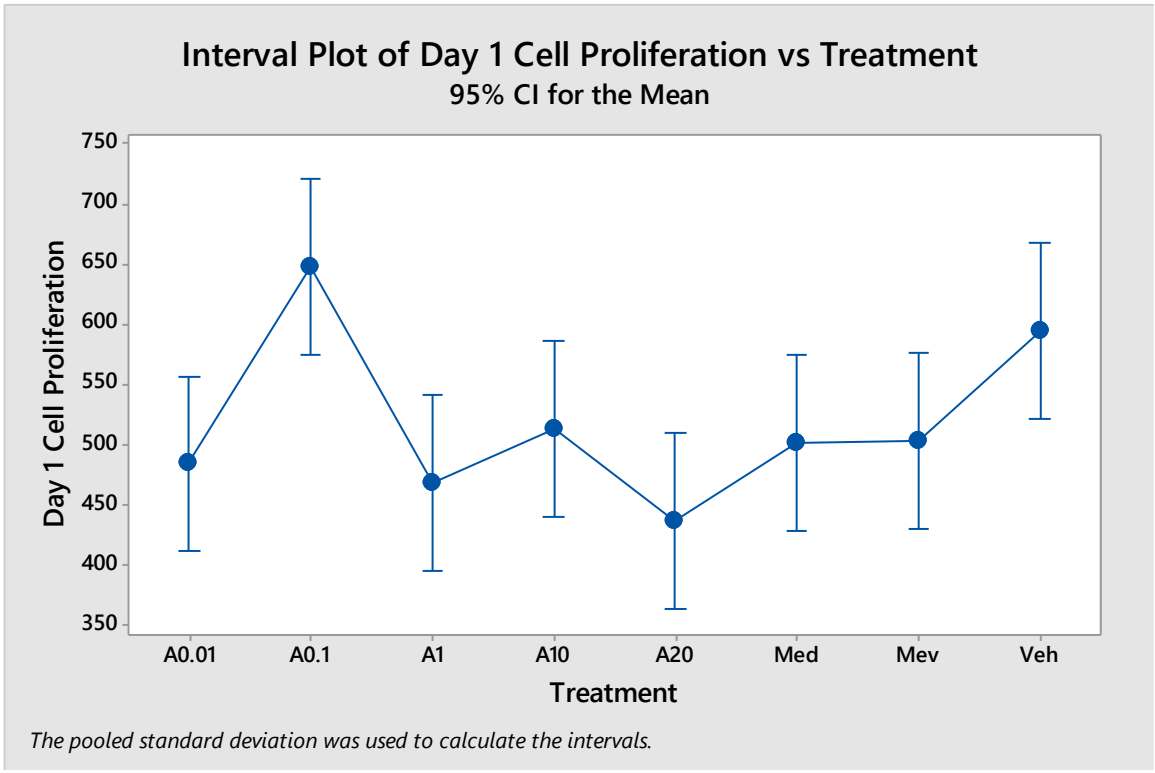
Day 7. A0.01 resulted in in significantly more cell proliferation in comparison to A20 (P=0.001) and controls [Veh (P=0.006), Med (P<0.0001), Meval (P<0.0001)]. A0.1 resulted in in significantly more cell proliferation in comparison to Med as control (P=0.002) and Meval as control (P=0.006). A1 resulted in significantly more cell

proliferation in comparison to A20 (P=0.005) and controls [Veh (P=0.026), Med (P<0.0001), Meval (P<0.0001]. A10 resulted in significantly more cell proliferation in comparison to Med as control (P=0.009) and Meval as control (P=0.029). There was no difference in cell proliferation among other comparisons.

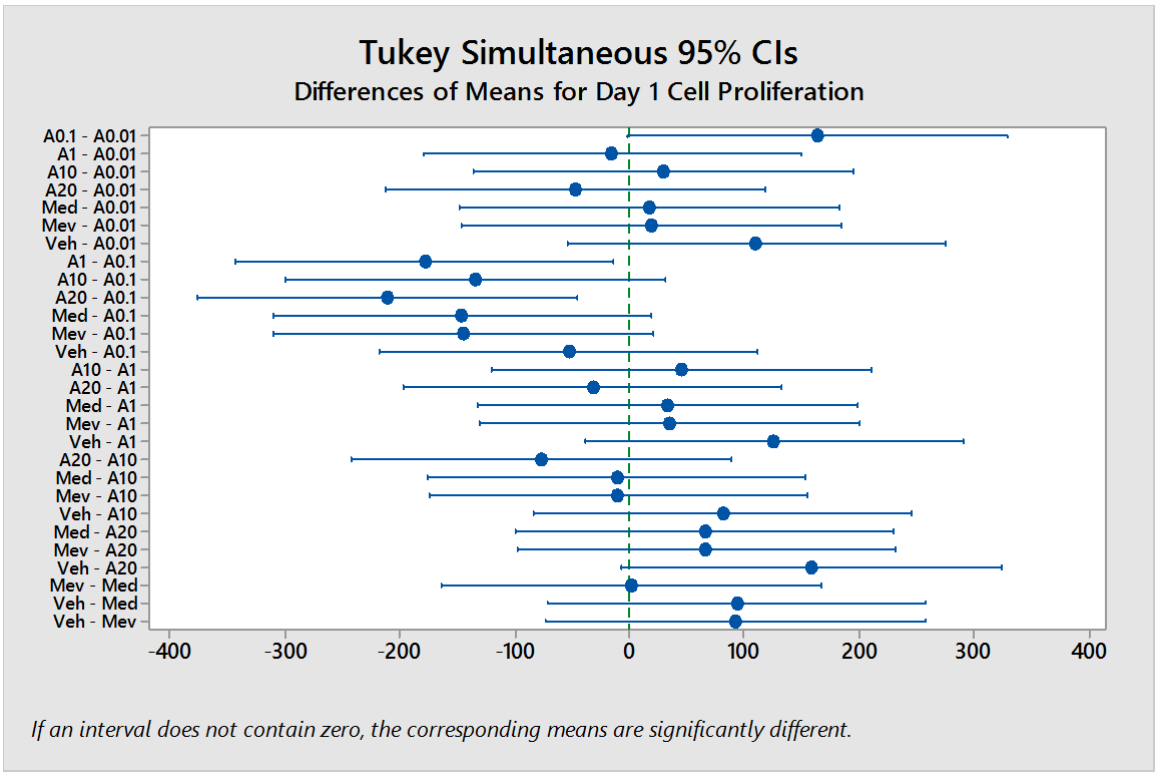
Figures to Demonstrate The Effect of Atorvastatin Concentration on Cell Proliferation Over 4 Time Points in a Sparsely Seeded Cell Population

CQ2500 (CyQUANT 2500 cells/well)

Day 1

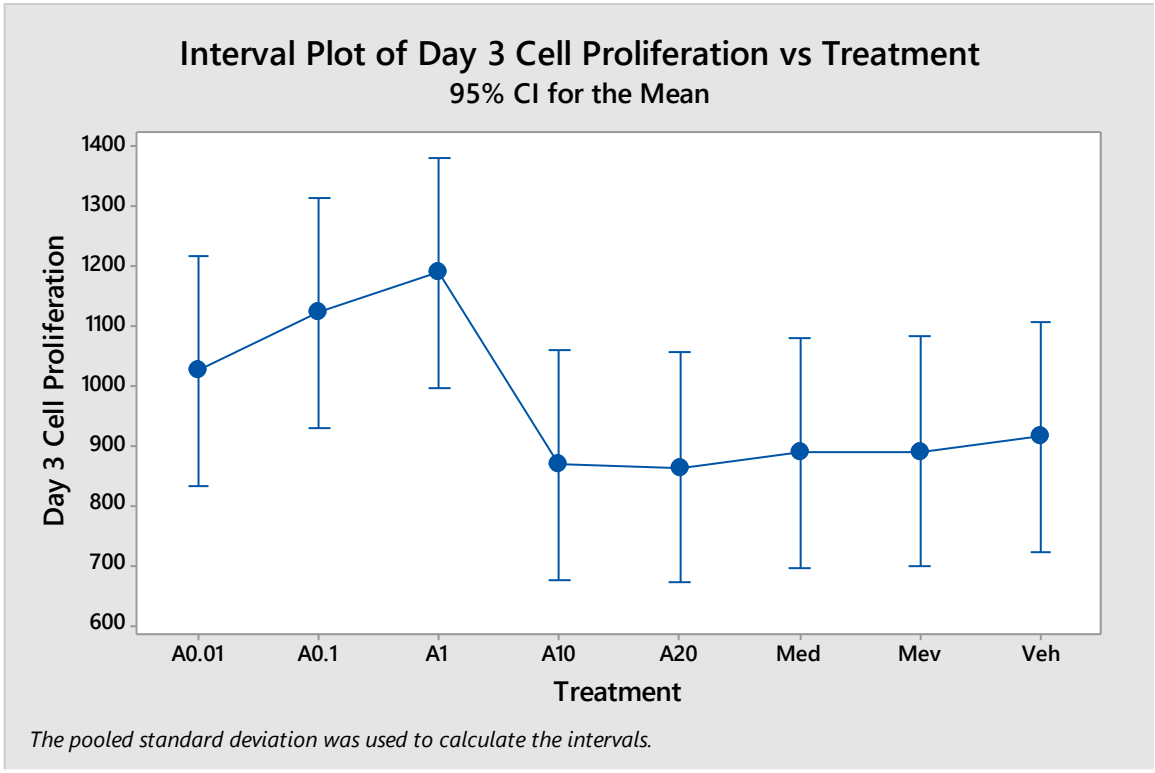


The pooled standard deviation was used to calculate the intervals.

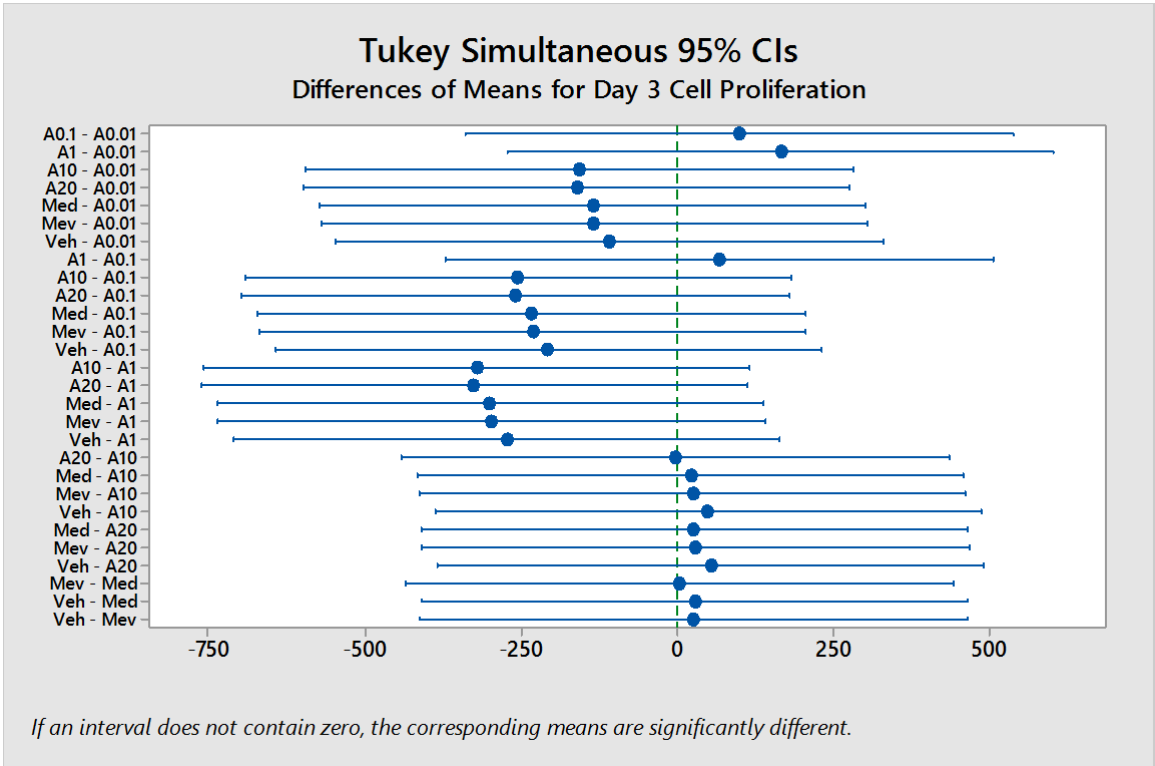


If an interval does not contain zero, the corresponding means are significantly different.

Day 3

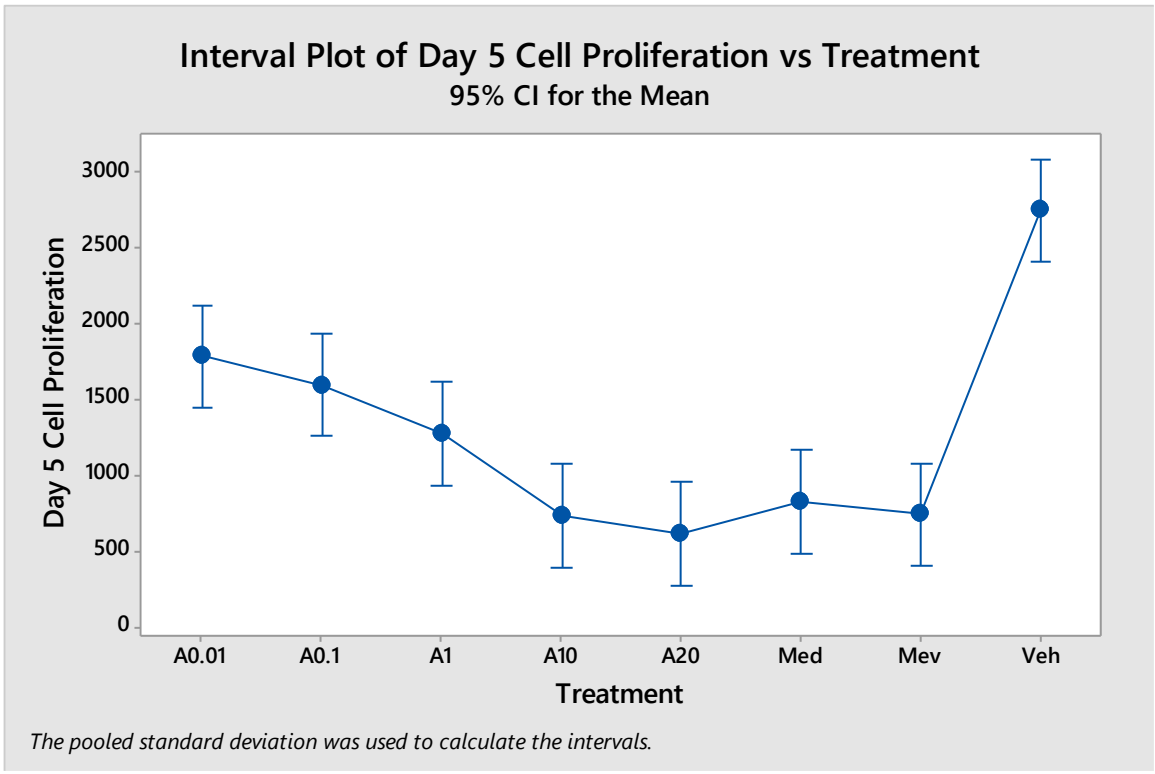


The pooled standard deviation was used to calculate the intervals.

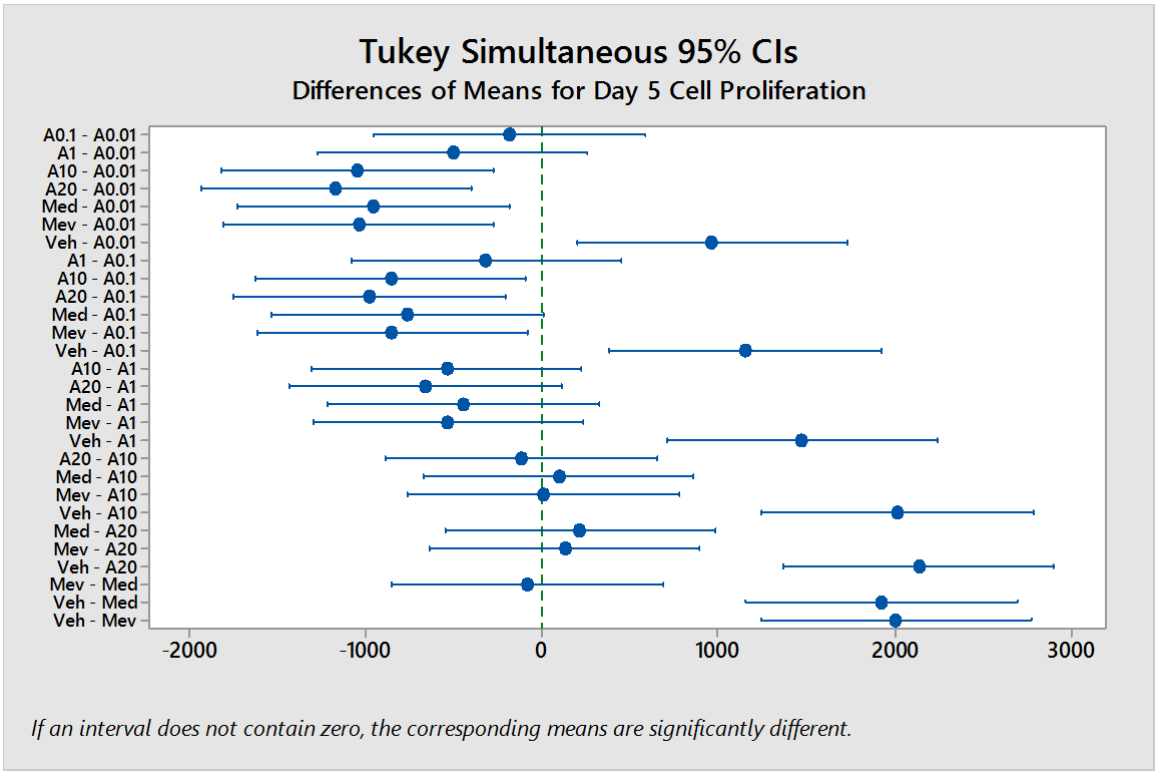


If an interval does not contain zero, the corresponding means are significantly different.

Day 5

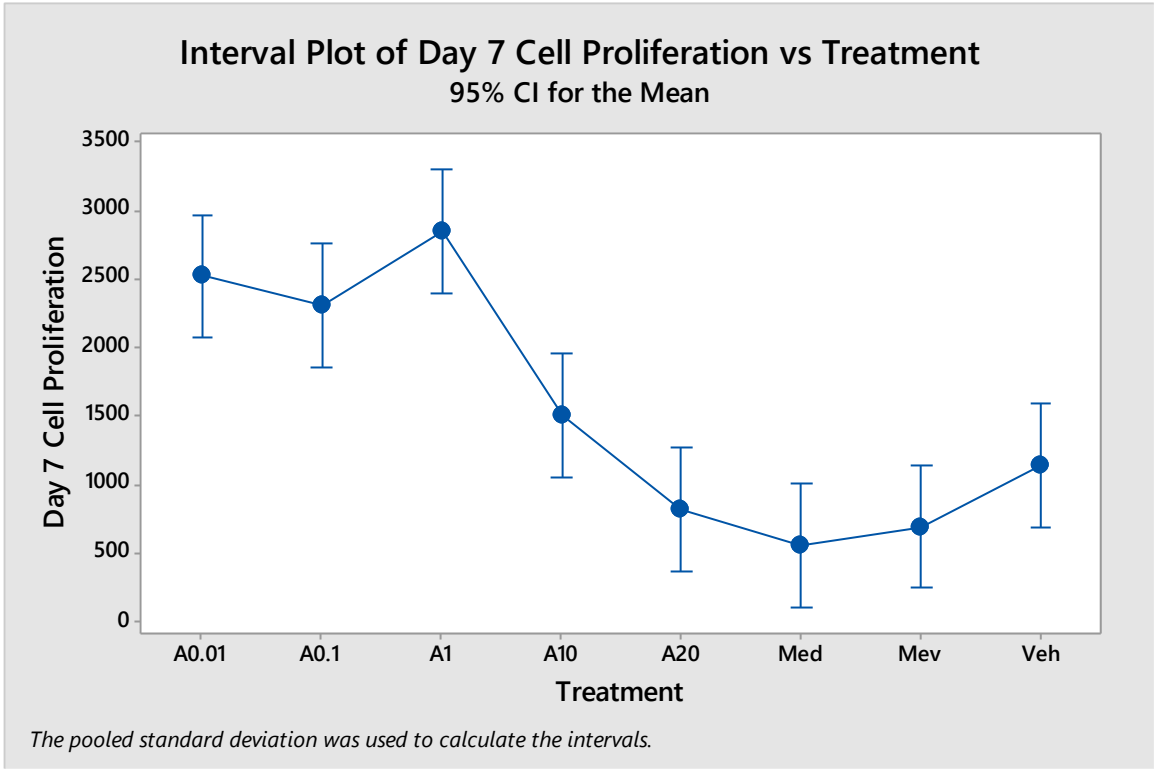


The pooled standard deviation was used to calculate the intervals.

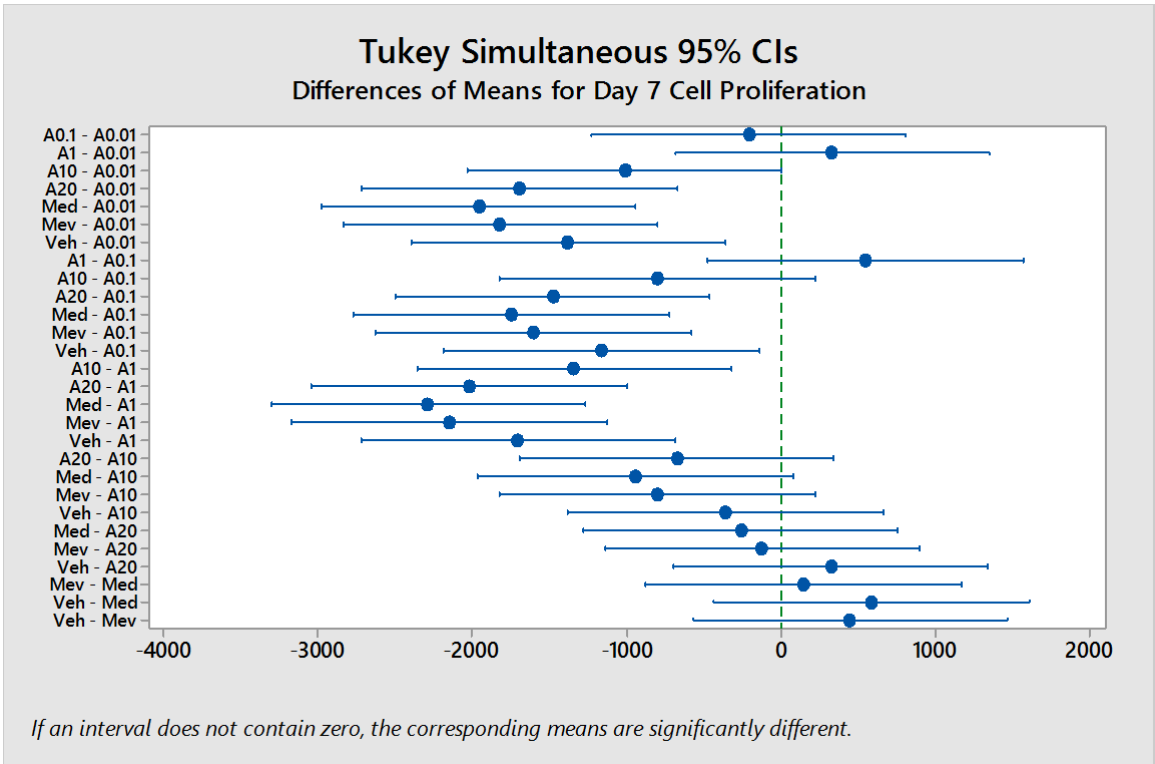


If an interval does not contain zero, the corresponding means are significantly different.

Day 7



The pooled standard deviation was used to calculate the intervals.



If an interval does not contain zero, the corresponding means are significantly different.

Metabolic Activity

AB 15000 (Alamar Blue, 15000 cells/well)

Day 1. A10 and A20 resulted in significantly less metabolic activity compared with Med as control (P=0.010 and P<0.0001, respectively). There was no difference in metabolic activity among other comparisons

Day 3. A1 resulted in significantly more metabolic activity compared with Med as control (P=0.027) and A20 resulted in significantly less metabolic activity compared with Med as control (P=0.003). There was no difference in metabolic activity among other comparisons.

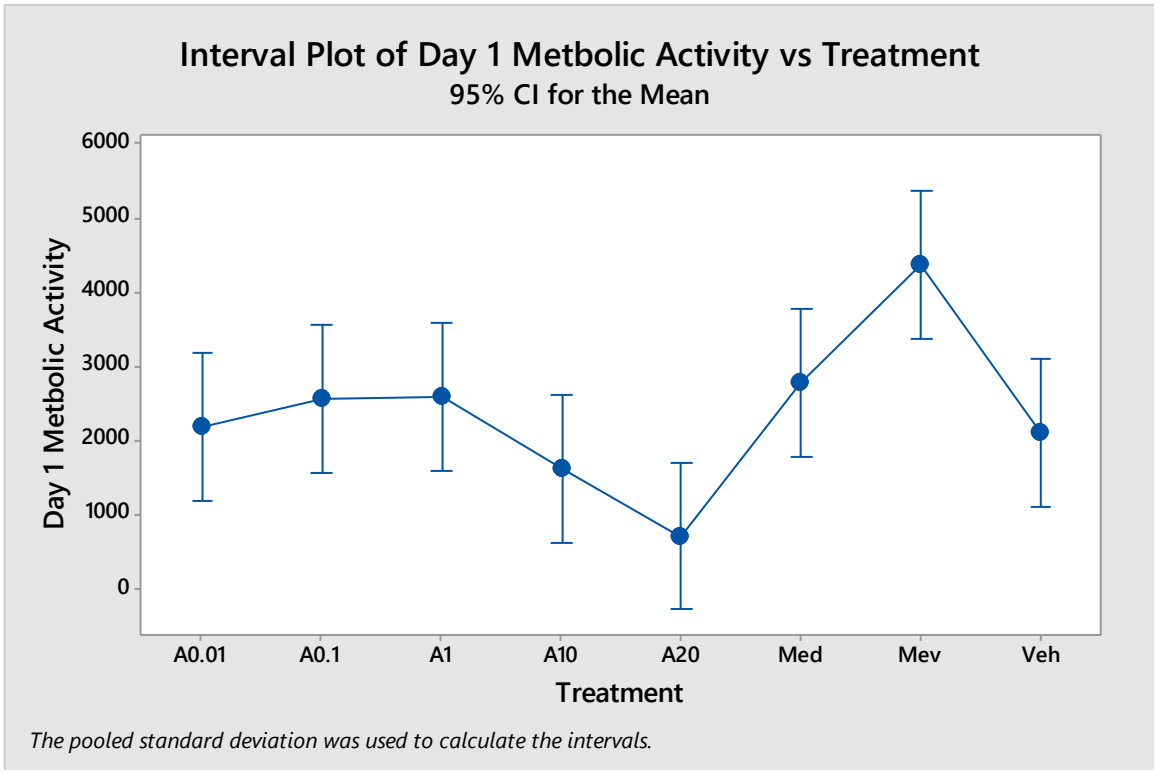
Day 5. A20 resulted in significantly less metabolic activity compared with A0.01 (P=0.012), A0.1 (P=0.003), A1 (P=0.002), Med as control (P=0.001) and Veh as control (P=0.004). There was no difference in metabolic activity among other comparisons.

Day 7. A0.01 resulted in in significantly more metabolic activity in comparison to A10 (P<0.0001), A20 (P<0.0001) and Meval as control (P<0.0001). A0.1 resulted in in significantly more metabolic activity in comparison to A10 (P<0.0001), A20 (P<0.0001), Med as control (P=0.002) and Meval as control (P<0.0001). A1 resulted in significantly more metabolic activity in comparison to A10 (P=0.007), A20 (P<0.0001) and Meval as control (P=0.008). A10 resulted in significantly less metabolic activity in comparison to Veh as control (P=0.007). A20 resulted in significantly less metabolic activity in comparison to Med as control (P=0.001) and Veh as control (P<0.0001). There was no difference in metabolic activity among other comparisons.

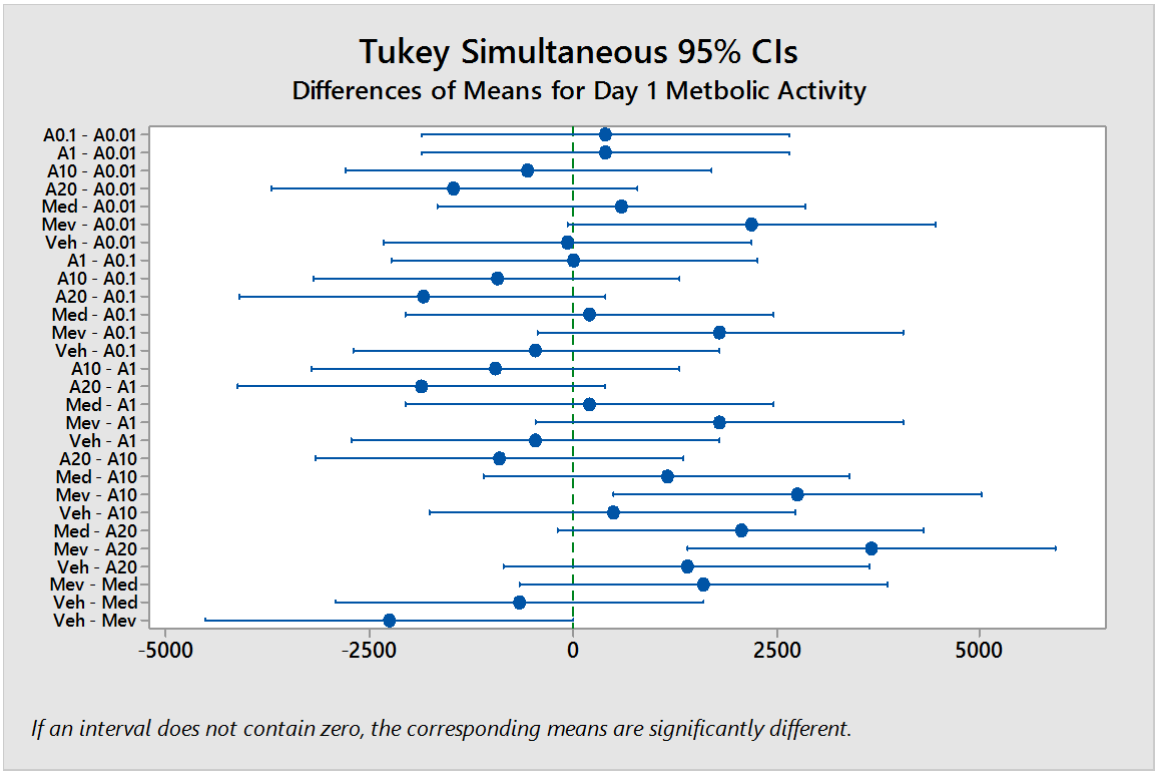
Figures to Demonstrate The Effect of Atorvastatin Concentration on Metabolic Activity Over 4 Time Points in a Densely Seeded Cell Population

AB 15000 (Alamar Blue, 15000 cells/well)

Day 1

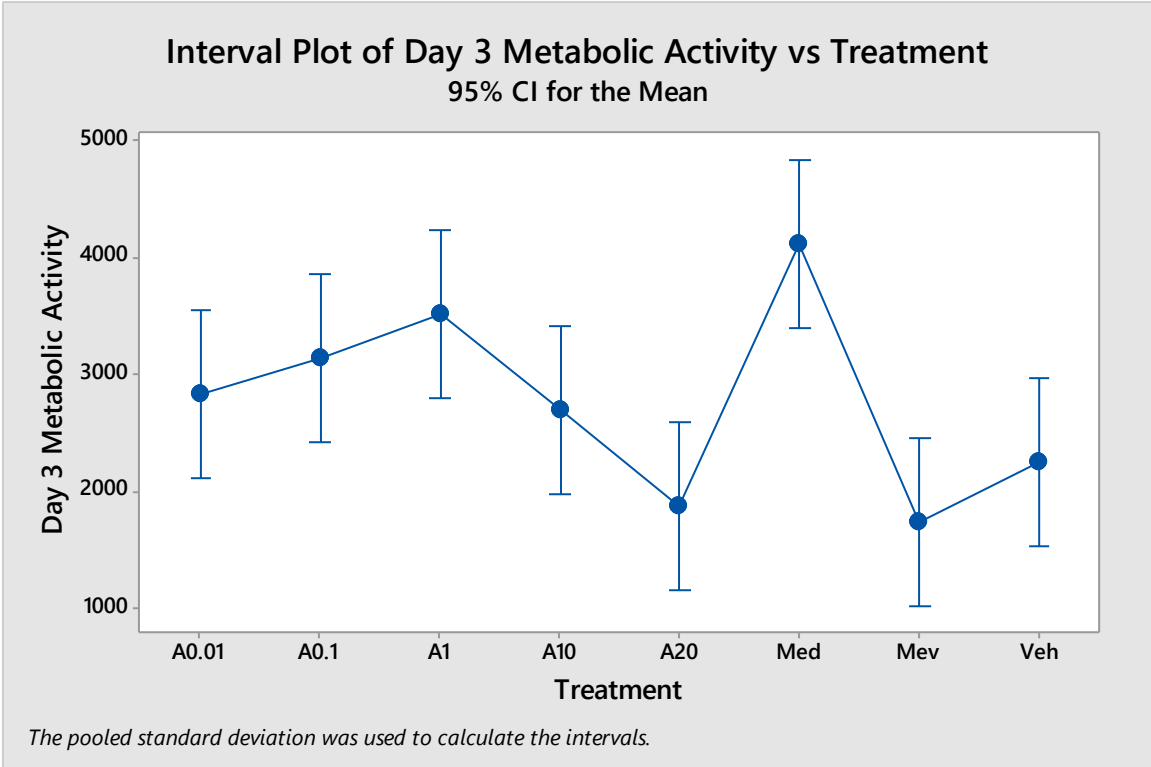


The pooled standard deviation was used to calculate the intervals.

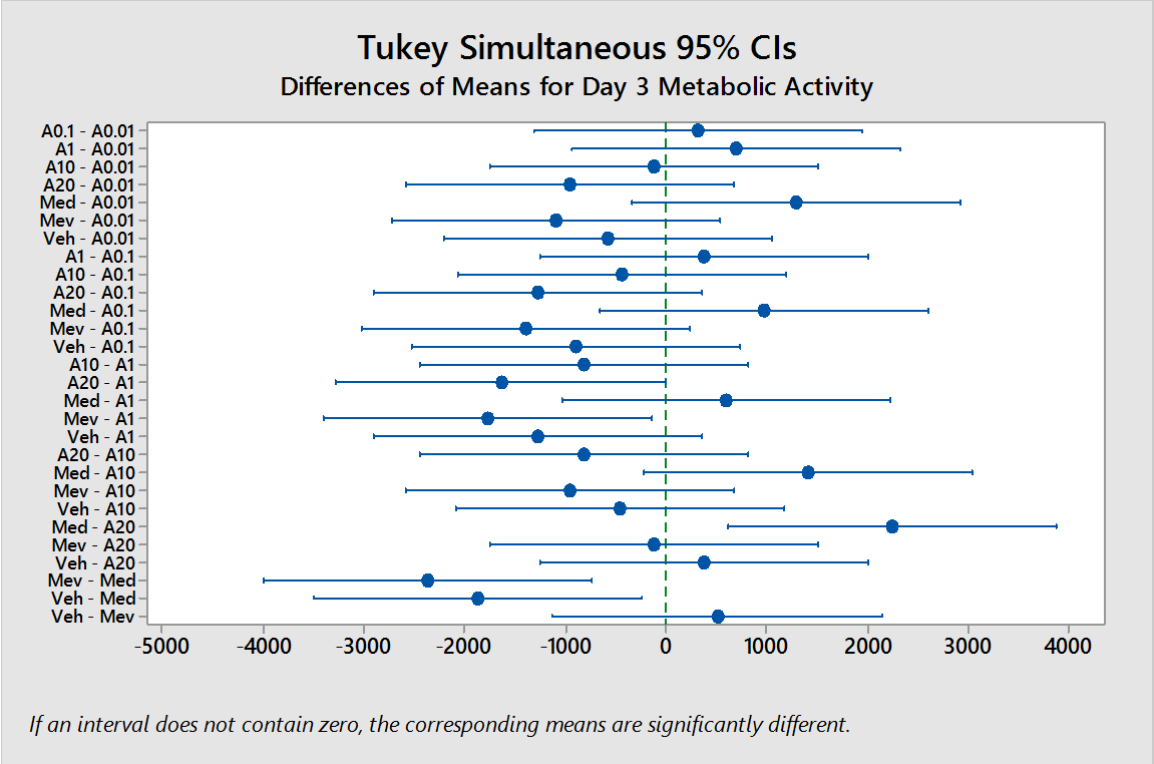


If an interval does not contain zero, the corresponding means are significantly different.

Day 3

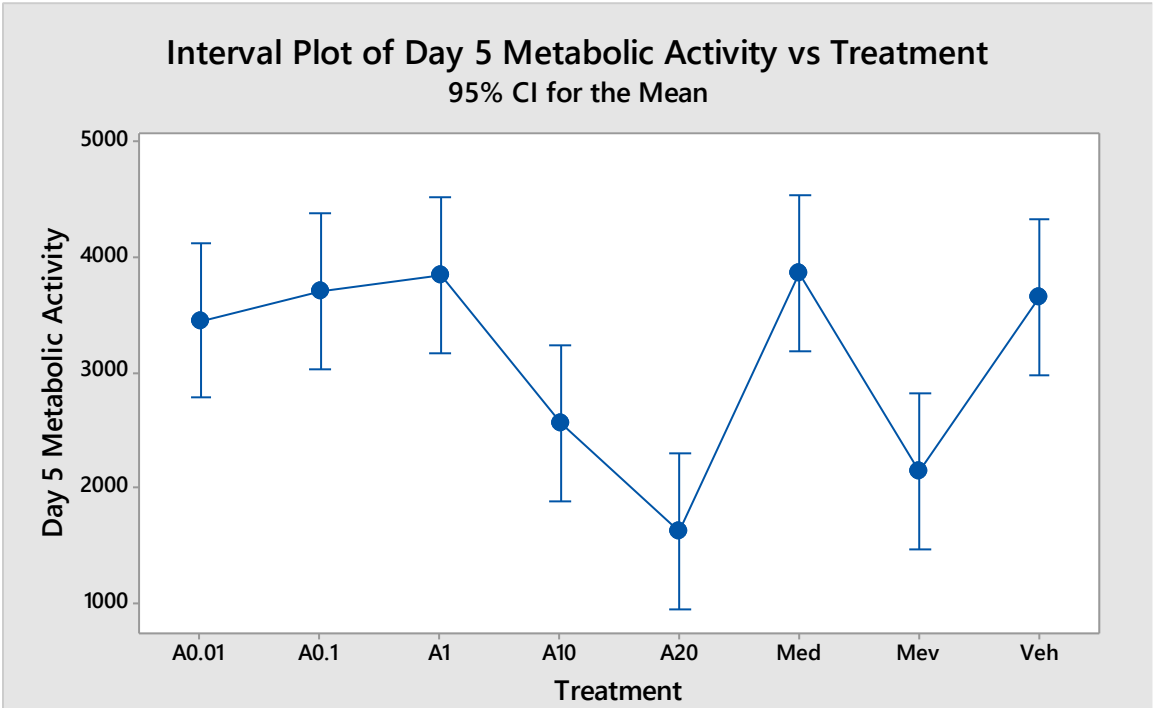


The pooled standard deviation was used to calculate the intervals.

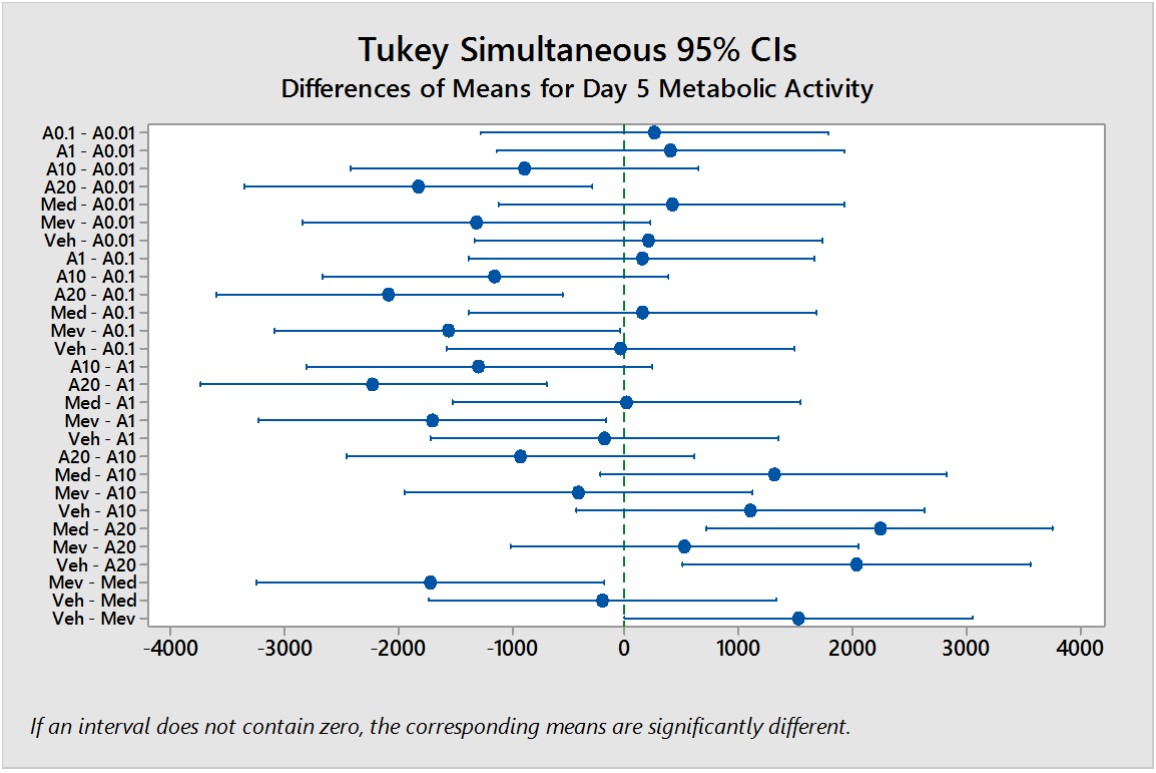


If an interval does not contain zero, the corresponding means are significantly different.

Day 5

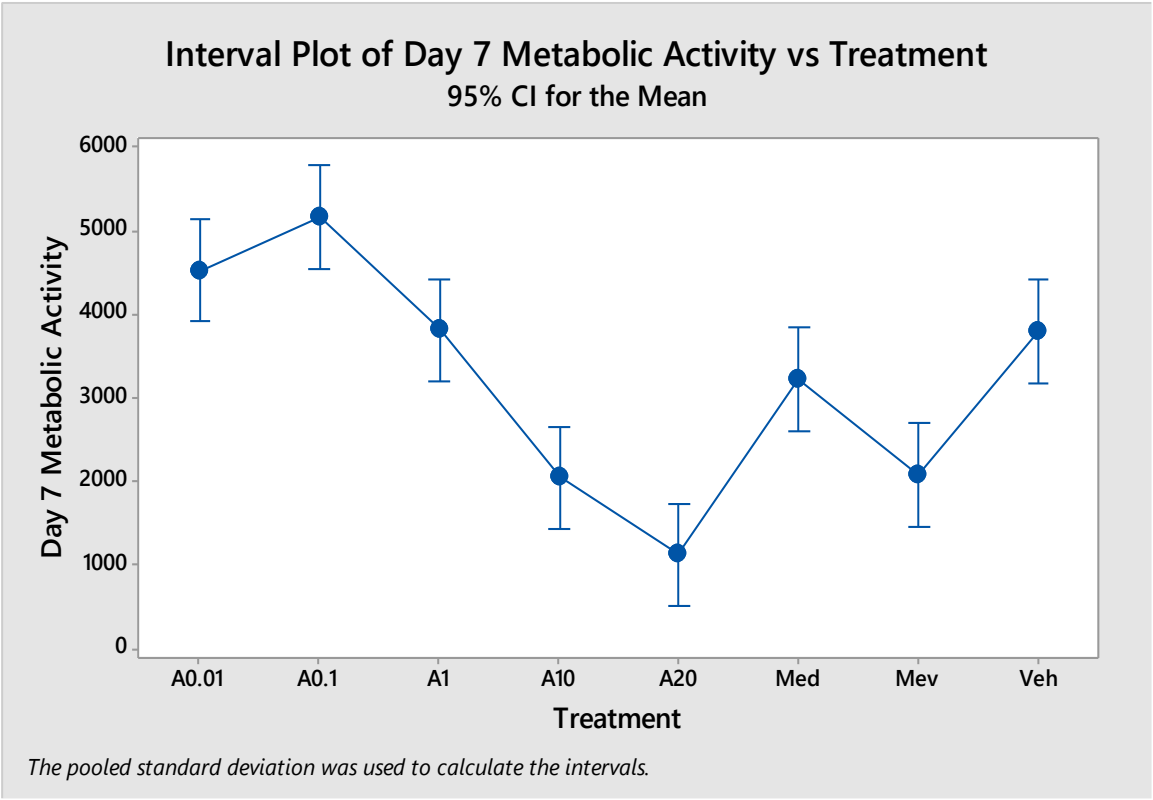


The pooled standard deviation was used to calculate the intervals.

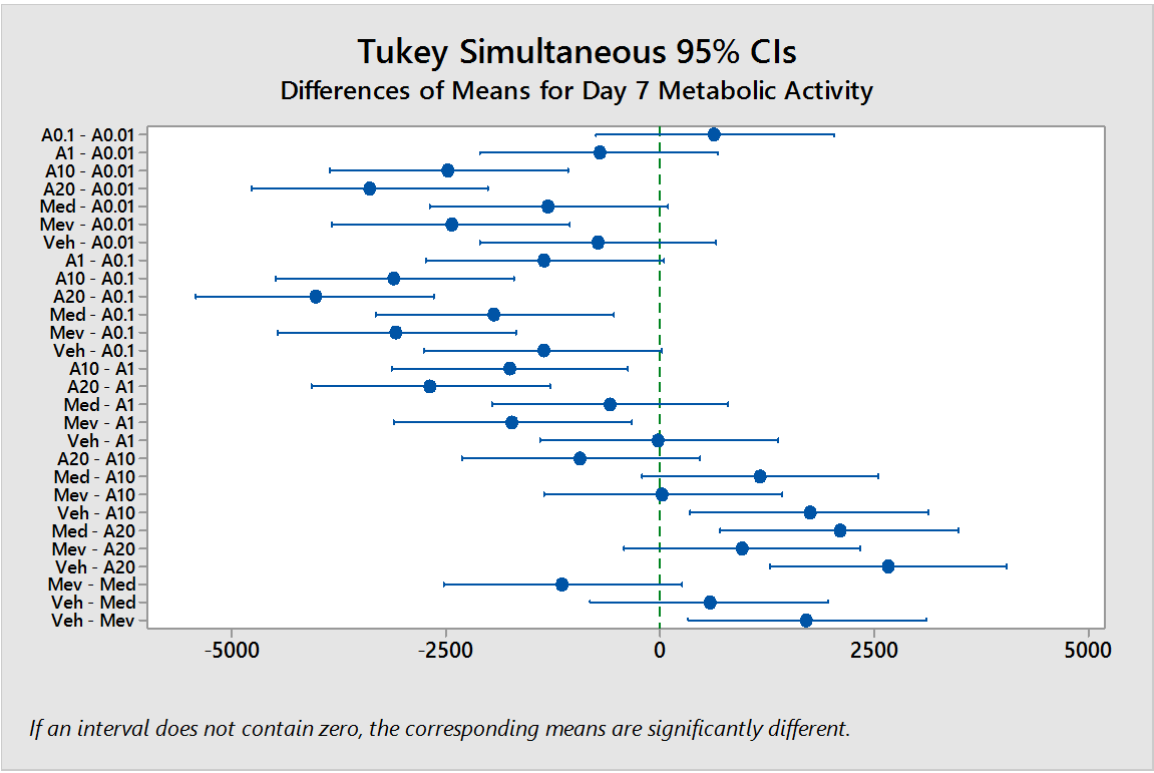


If an interval does not contain zero, the corresponding means are significantly different.

Day 7



The pooled standard deviation was used to calculate the intervals.



If an interval does not contain zero, the corresponding means are significantly different.

AB 2500 (Alamar Blue, 2500 cells/well)

Units on the Y axes of the figures displaying metabolic activity are Absorbance. Numerical values were generated by the plate reader, using the settings described in the methods section.

Day 1. A0.1 resulted in in significantly more metabolic activity in comparison to A1 (P=0.027) and A20 (P=0.006). There was no difference in cell proliferation among other comparisons.

Day 3. There was no difference in metabolic activity among other comparisons.

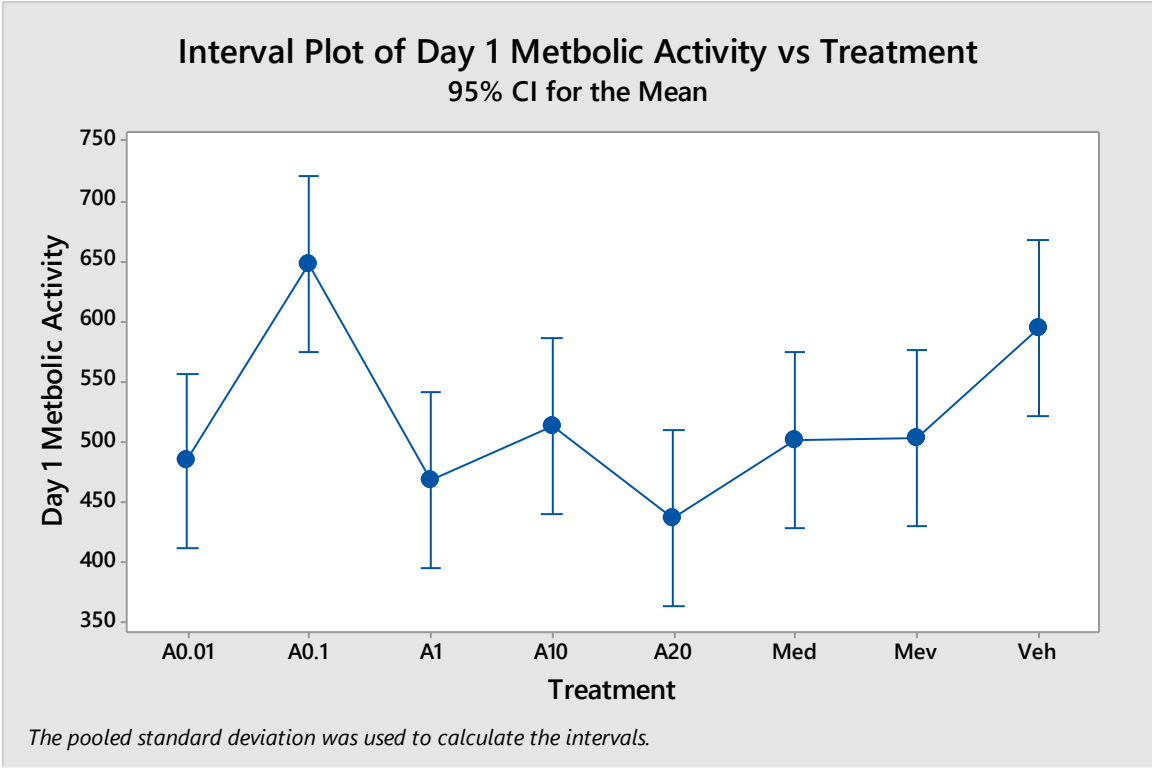
Day 5. A0.01 resulted in in significantly more metabolic activity in comparison to A10 (P=0.003), A20 (P=0.001), Med as control (P=0.008) and Meval as control (P=0.003). A0.1 resulted in in significantly more metabolic activity in comparison to A10 (P=0.021), A20 (P=0.006), Med as control (P=0.002) and Meval as control (P=0.022). A1 resulted in significantly more metabolic activity in comparison to A10 (P=0.007), A20 (P<0.0001) and Meval as control (P=0.008). Veh as control resulted in significantly more metabolic activity in comparison to other treatments. There was no difference in metabolic activity among other comparisons.

Day 7. A0.01 resulted in significantly more metabolic activity in comparison to A20 (P<0.0001) and controls [Veh (P=0.003), Med (P<0.0001), Meval (P<0.0001)]. A0.1 resulted in significantly more metabolic activity in comparison to A20 (P=0.001) and controls [Veh (P=0.017), Med (P<0.0001), Meval (P=0.001)]. A1 resulted in significantly more metabolic activity in comparison to A10 (P=0.004), A20 (P<0.0001) and controls [Veh (P<0.0001), Med (P<0.0001), Meval (P<0.0001)]. There was no difference in metabolic activity among other comparisons.

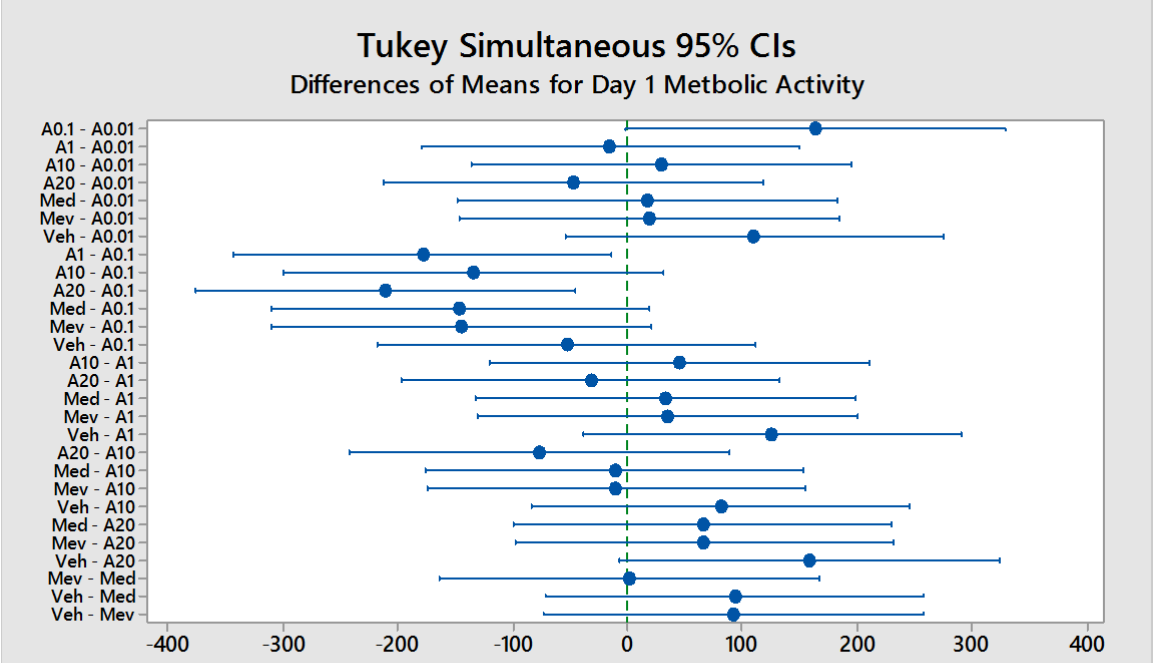
Figures to Demonstrate The Effect of Atorvastatin Concentration on Metabolic Activity Over 4 Time Points in a Sparsely Seeded Cell Population

AB 2500 (Alamar Blue, 2500 cells/well)

Day 1

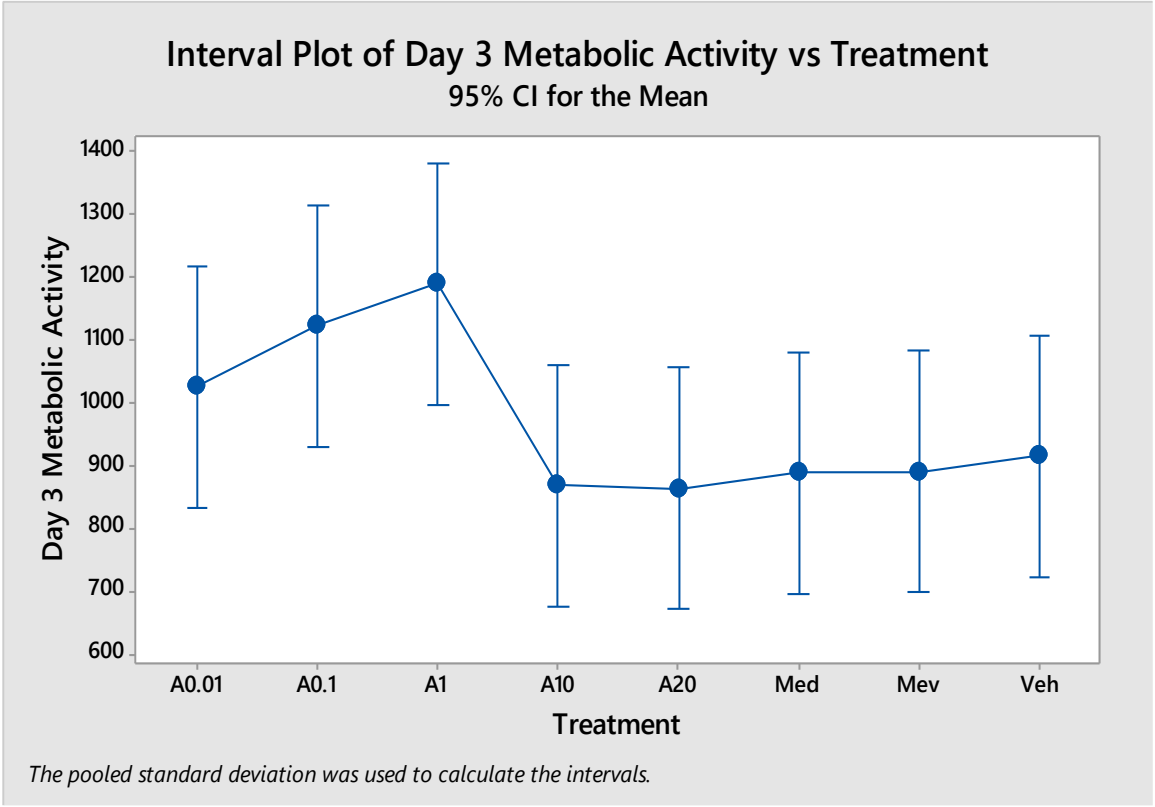


The pooled standard deviation was used to calculate the intervals.

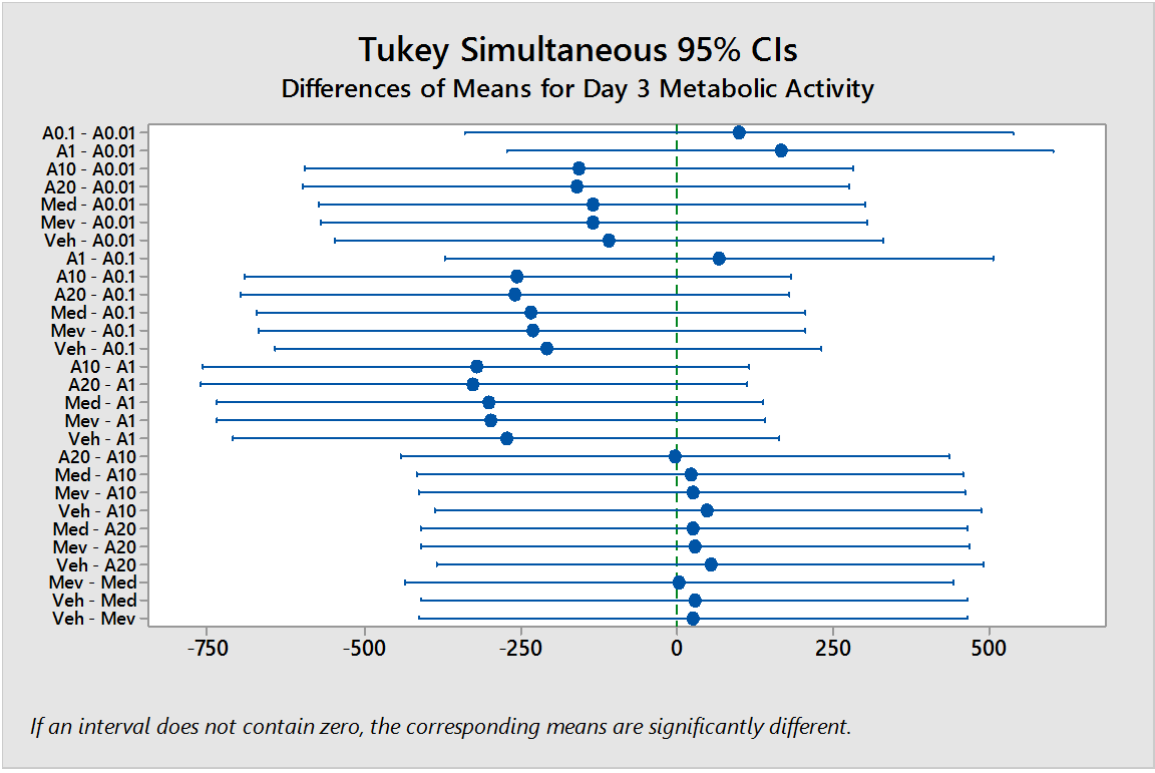


If an interval does not contain zero, the corresponding means are significantly different.

Day 3

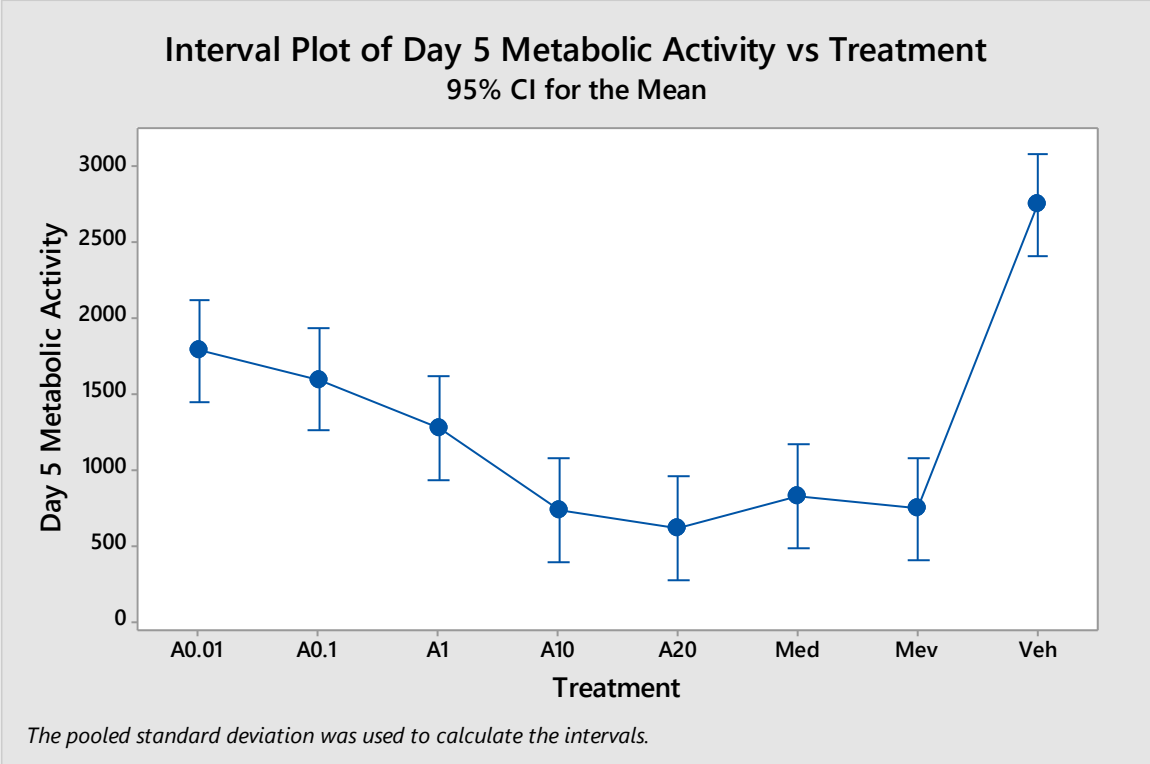


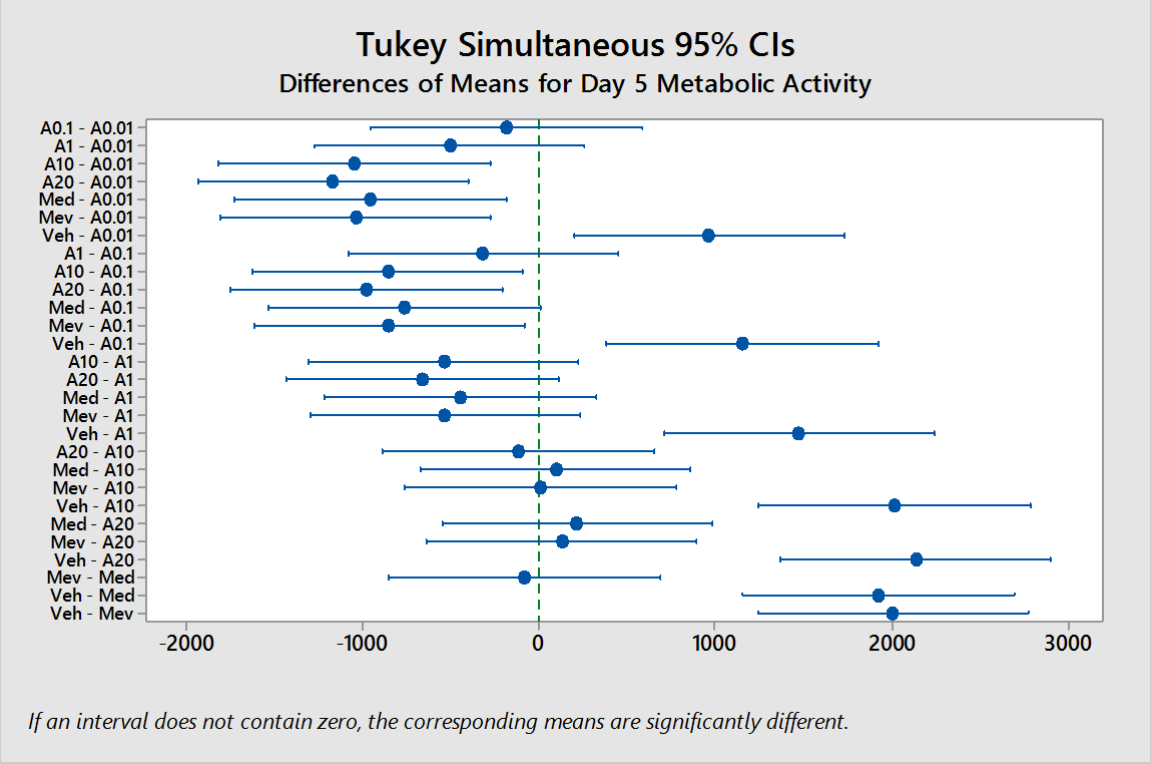
The pooled standard deviation was used to calculate the intervals.



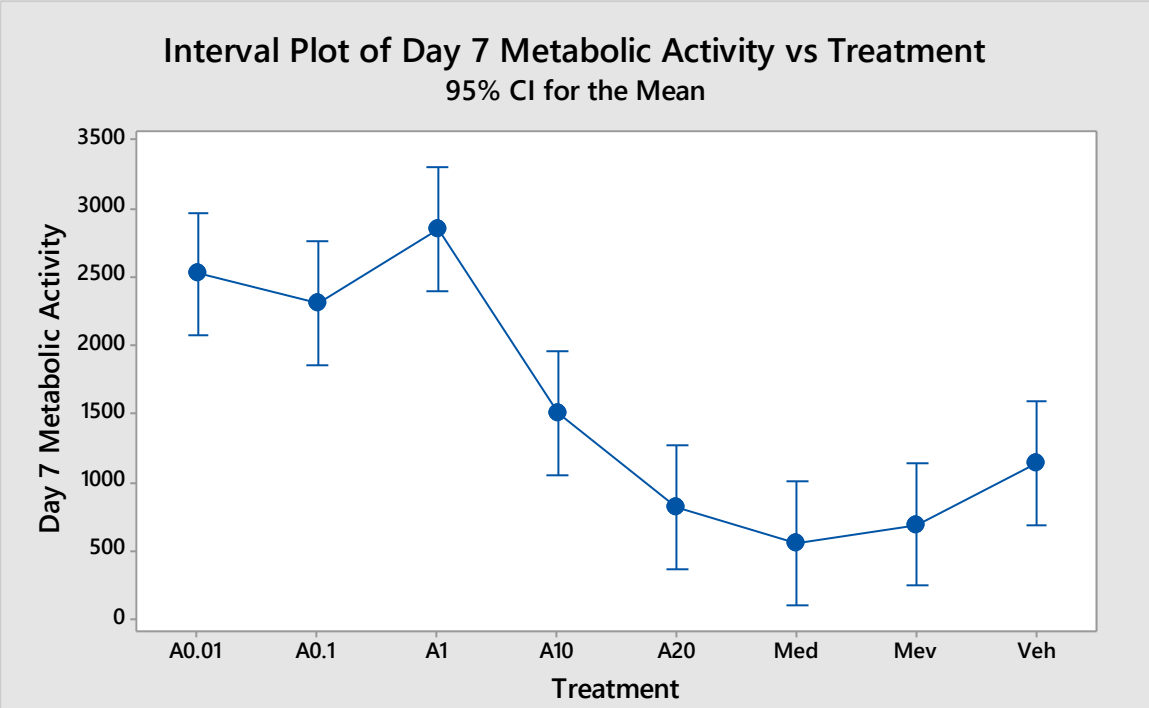
If an interval does not contain zero, the corresponding means are significantly different.

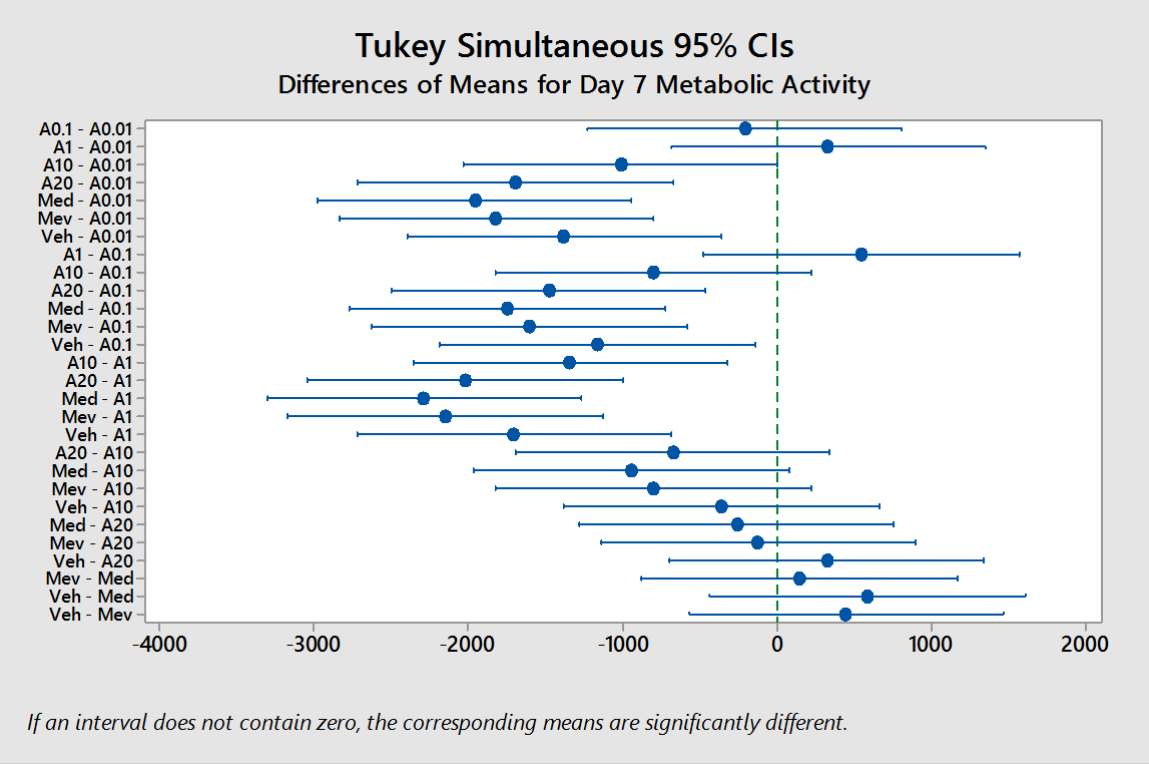
Day 5


Interval Plot of Day 5 Metabolic Activity vs Treatment
95% CI for the Mean
Day 5 Metabolic Activity
3000
2500
2000
1500
1000
500
0
A0.01
A0.1
A1
A10
A20
Med
Mev
Veh
Treatment
The pooled standard deviation was used to calculate the intervals.


Tukey Simultaneous 95% CIs
Differences of Means for Day 5 Metabolic Activity
A0.1 - A0.01
A1 - A0.01
A10 - A0.01
A20 - A0.01
Med - A0.01
Mev - A0.01
Veh - A0.01
A1 - A0.1
A10 - A0.1
A20 - A0.1
Med - A0.1
Mev - A0.1
Veh - A0.1
A10 - A1
A20 - A1
Med - A1
Mev - A1
Veh - A1
A20 - A10
Med - A10
Mev - A10
Veh - A10
Med - A20
Mev - A20
Veh - A20
Mev - Med
Veh - Med
Veh - Mev
-2000
-1000
0
1000
2000
3000
If an interval does not contain zero, the corresponding means are significantly different.

Day 7


Interval Plot of Day 7 Metabolic Activity vs Treatment
95% CI for the Mean
Day 7 Metabolic Activity
3500
3000
2500
2000
1500
1000
500
0
A0.01
A0.1
A1
A10
A20
Med
Mev
Veh
Treatment
The pooled standard deviation was used to calculate the intervals.


Tukey Simultaneous 95% CIs
Differences of Means for Day 7 Metabolic Activity
A0.1 - A0.01
A1 - A0.01
A10 - A0.01
A20 - A0.01
Med - A0.01
Mev - A0.01
Veh - A0.01
A1 - A0.1
A10 - A0.1
A20 - A0.1
Med - A0.1
Mev - A0.1
Veh - A0.1
A10 - A1
A20 - A1
Med - A1
Mev - A1
Veh - A1
A20 - A10
Med - A10
Mev - A10
Veh - A10
Med - A20
Mev - A20
Veh - A20
Mev - Med
Veh - Med
Veh - Mev
-4000
-3000
-2000
-1000
0
1000
2000
If an interval does not contain zero, the corresponding means are significantly different.

The Clinical Study

Methods

Ethical permission for this study was approved by national research ethics service (NRES) Committee South Central - Oxford C (Appendix 1)**.** The study was sponsored by The Royal Liverpool University Hospital. Data was recorded in a prospectively managed database. All patients undergoing major surgery requiring an ileocolonic, colocolonic or colorectal anastomosis at The Royal Liverpool University Hospital colorectal unit, between August 2013 and September 2014 were invited to participate in the study. The study was clearly explained to each patient pre-operatively, and patients were provided with literature to reinforce the details of the study, and with contact details of the investigators. The patients understood that participating in the project would have no bearing whatsoever on decisions surrounding the management of their disease, or on their clinical course. The patients also understood that they would have to make no further personal contribution to the study, other than the pre-operative interview. The patients all understood that a small amount of healthy tissue may be taken from the operative specimen for cell culture, and that they were entitled to withhold consent for tissue sampling should they wish. Those patients who agreed to participate in the study signed a consent form pre-operatively; every patient in the study was consented by the lead investigator. Following consent, pre-operative data was collected using Clinical Research Form A (CRF A, appendix 2). The data included patient demographics, medical comorbidities, current medications, smoking history, alcohol intake, the details of the planned operation, and the underlying disease being treated. Results of pre-operative blood tests, including lipid profiles, were also recorded. Operation notes for each patient were reviewed to confirm details of the

operation. Each patient's post-operative course was analysed by reviewing the notes between 30- and 45-days post-operation. Patients were excluded if they had a defunctioning ileostomy or did not have an anastomosis created.

Anastomotic leak was defined as a clinically suspected leak, with radiological or intra-operative confirmation. Computed tomography (CT) scans were ordered at the discretion of the clinical team responsible for the patient.

All statistical analysis was performed using dedicated statistical software (SPSS v20, IBM Corp, Armonk, NY, USA). All data are presented as median (Interquartile range) unless otherwise stated. Group comparisons were performed using Mann-Whitney U Tests and Fishers Exact Test.

Results

During the study period 124 patients undergoing major surgical resection consented to take part in the study. Seven patients had a defunctioning ileostomy created, two underwent a Hartmann's procedure and a further two patients were excluded due to incomplete documentation. Data from 113 patients was therefore available for analysis. Of the 113 procedures, 45 patients underwent right hemicolectomy, 9 underwent left hemicolectomy, 20 underwent sigmoid colectomy, 6 underwent reversal of Hartmann's procedures, 27 underwent anterior resections and 6 other major colorectal operations. Demographics of the patient cohort are displayed in Table 2, and are representative of the patient cohort served by and inner-city teaching hospital with a high proportion of patients from a deprived area.

38.9% of patients were taking statins pre-operatively. Patients taking statins had significantly more co-morbidities than those patients not taking statins (Table 2). Those patients also had a significantly greater number of recognised risk factors for AL.

The AL rate in our cohort was 5% (6 patients). Factors predictive of leak were demonstrated in Table 4. Statins had no effect on AL rates in our study, neither were they associated with a significant difference in maximum 7 day CRP (128mg/L (83-206) v 142 (85-238), p=0.697). The nature of the operation, and therefore anastomotic site, was not associated with AL in our study (p=0.269). Factors found to be significantly associated with anastomotic leak in our study were impaired renal function, measured by low pre-operative eGFR, and maximum 7 day CRP.

Multivariate regression was not performed due to the low numbers of AL encountered.

Descriptor		**Cohort**
Age		67 (57-75)
Male Sex		62 (54%)
Body Mass index (BMI)		26.9 (23.4-30.6)
Smoking	Never Smoked	41 (36.3%)
	Current Smoker	49 (43.4%)
	Ex-Smoker	23 (20.3%)
ASA Score	1	26 (23%)
	2	64 (56.6%)
	3	21 (18.5%)
	4	2 (1.8%)
Chronic Kidney Disease		3 (2.7%)
Ischaemic Heart Disease		14 (12.4%)
Hypertension		39 (34.5%)
Diabetes		26 (23.2%)
Statin USe		44 (38.9%)
Antiplatelet Use		33 (29.2%)

Table 2: Summary of demographics and comorbidities of the patient cohort

Descriptor		Statins	No Statins	p value
Age		73 (66-76)	61 (49-70)	<0.001*
Male Sex		26 (59.1%)	36 (52.2%)	0.471
Body Mass index (BMI)		27.8 (24-33)	25.8 (23-30)	0.148
Operation for malignancy		34 (77.3%)	36 (52.2%)	0.007*
Pre-operative radiotherapy		3 (6.8%)	7 (10.3%)	0.737
Smoking	Never Smoked	13 (30.2%)	26 (41.9%)	0.01*
	Current Smoker	27 (62.8%)	22 (35.5%)	
	Ex Smoker	3 (7%)	14 (22.9%)	
ASA Score	1	2 (4.7%)	24 (37.5%)	<0.001*
	2	28 (65.1%)	34 (53.1%)	
	3	11 (25.6%)	6 (9.4%)	
	4	2 (4.7%)	0	
Chronic Kidney Disease		1 (2.3%)	2 (2.9%)	1
Ischaemic Heart Disease		11 (25%)	3 (4.3%)	0.002*
Hypertension		27 (61.4%)	12 (17.4%)	<0.001*
Diabetes		17 (38.6%)	9 (13.2%)	0.003*
Antiplatelet Use		24 (54.5%)	9 (13.4%)	<0.001*
Haemoglobin (g/L)		123.5 (112-136)	133 (119.5-142)	0.015*
White cell count (10^9/L)		8 (6-10)	7 (6-9.5)	0.335
Albumin (g/L)		42 (39.3-44)	43 (40.3 – 45)	0.110
eGFR (mL/min/1.73m^2)		70 (54.5-85)	80 (40.24 – 45)	0.001*
Total cholesterol (mmol/L)		4 (3-4)	5 (4-5)	<0.001*
Triglycerides (mmol/L)		1 (1-2)	1 (1-2)	0.584
HDL (mmol/L)		1 (1-2)	1 (1-2)	0.239
LDL (mmol/L)		2 (1-2)	3 (2-3)	<0.001*

Table 3: Summary of demographics and recognised risk factors for anastomotic leak amongst the clinical study population, comparing those patients taking statins, and those patients not taking statins at the time of surgery.

Descriptor		Leak	No Leak	p value
Age		57 (50-75)	67 (58-75)	0.493
Body Mass index (BMI)		26.8 (23.9-29.5)	26.7 (23.4-31)	0.917
Smoking	Never Smoked	3 (50%)	36 (36.4%)	0.856
	Current Smoker	2 (33.3%)	47 (47.5%)	
	Ex-Smoker	1 (16.7%)	16 (16.2%)	
ASA Score	1	1 (16.7%)	25 (24.8%)	1
	2	4 (66.7%)	58 (57.4%)	
	3	1 (16.7%)	16 (15.8%)	
	4	0	2 (2%)	
Chronic Kidney Disease		0	3 (2.8%)	1
Ischaemic Heart Disease		1 (16.7%)	13 (12.1%)	0.557
Hypertension		1 (16.7%)	38 (35.5%)	0.663
Diabetes		1 (16.7%)	25 (23.6%)	1
Pre-operative radiotherapy		2 (33.3%)	8 (7.5%)	0.089
Malignancy		6 (100%)	64(59.8%)	0.081
Antiplatelet Use		2 (33.3%)	31 (29.5%)	1
Statin Use		3 (50%)	41 (45.8%)	0.676
Haemoglobin (g/L)		115 (105-144)	131 (117-141)	0.327
White cell count (10^9/L)		9.5 (6.8-14)	8 (6-10)	0.127
Albumin (g/L)		41.4 (35.8-43.3)	43 (50-45)	0.207
eGFR (mL/min/1.73m^2)		60.5 (35.75-72)	78 (65-89)	0.019*
Total cholesterol (mmol/L)		4 (2.75-4.5)	4 (4-5)	0.265
Triglycerides (mmol/L)		1 (1-1.25)	1 (1-2)	0.256
HDL (mmol/L)		1 (1-2)	1 (1-2)	0.605
LDL (mmol/L)		2 (1-3)	2 (2-3)	0.526
Max 7 Day CRP		258 (217-377)	126 (83-208)	0.003*

Table 4: Summary of demographics and recognised risk factors for anastomotic leak amongst the clinical study population, comparing the patients who were diagnosed with anastomotic leak with those patients who did not experience anastomotic leak.

Discussions and Conclusions

Discussion

The effect of statins on primary cultured human colonic myofibroblasts

The early part of the laboratory work was dedicated to learning and refining a reproducible method for primary cell culture from specimens of healthy human colon, harvested at the time of surgery, then ensuring that adequate numbers of early passage cells could be grown and maintained. Using primary patient derived cells in research is one of the most challenging parts of cell biology and *in vitro* cell culture. It is absolutely, rightly valued as the most relevant research that can be conducted outside of a body. As myofibroblasts are the main secretors of extracellular matrix, they drive the restoration of tissue integrity after injury(118). Myofibroblasts are fundamental to tissue healing from the very earliest stages of the process, therefore it is plausible that any factor that may influence their function and proliferation could promote, or impair, tissue healing.

As described earlier in this thesis, there is early evidence to suggest that patients taking statins may have a reduced incidence of anastomotic leak following colorectal surgery. Although statins are widely used to reduce serum cholesterol levels in patients believed to be at high risk of cardiovascular disease, they are recognised to have pleiotropic effects; effects which influence tissue healing. A number of statins are in widespread use throughout the developed world, and most of these compounds are available for use in *in vitro* work. Atorvastatin was chosen for this study as it remains stable in a stock solution, and can be reliably dissolved in a non-toxic concentration of dimethyl sulfoxide (DMSO) (referred to as vehicle). Unlike

Simvastatin, Atorvastatin does not require extra steps to activate it in cell medium. In accordance with other *in vitro* studies that have investigated the effects of statins on cell function, a range of concentrations was created, to include sub-therapeutic and supra-therapeutic levels, in addition to therapeutic equivalent concentrations. This range is particularly important in the study of statins, as a non-linear, biphasic effect is often observed (as described in the introduction). The effect of the interventions was compared to control conditions: plain medium, medium containing DMSO at the same concentration as that used to make the stock Atorvastatin solution, and medium containing both Atorvastatin and mevalonate, to consider whether any effects observed were due to inhibition of the mevalonate pathway, or were independent of that pathway.

Effect of Atorvastatin on Cellular Metabolism

The influence of Atorvastatin on metabolic activity of primary cultured human colonic myofibroblasts was assessed using Alamar Blue, with wells seeded at low cell density and high cell density. For both densities a non-linear relationship is observed, with the considerable inhibition of metabolic activity being associated with higher concentrations of Atorvastatin. Increased metabolic activity was seen at lower concentrations, although concentrations in the middle of the range promoted the greatest level of metabolic activity, which is consistent with other *in vitro* work described in the introduction. The observation that the lowest level of metabolic activity is seen with the higher concentrations of Atorvastatin is seen across all days, and over both densely seeded and sparsely seeded conditions. The observation that lower doses of statins promote increased metabolic activity compared to control

conditions is particularly interesting; such an effect *in vitro* may ultimately have clinical relevance in the use of statins to promote tissue healing. The effect of the higher concentrations may simply reflect a toxic effect of Atorvastatin at these concentrations.

Effect of Atorvastatin on Cell Proliferation

As previously described, cell proliferation over time was measured using the CyQUANT assay. This data was generated from the same cell populations that were analysed for the Alamar Blue metabolic activity assay. A very similar trend to that observed in metabolic activity is seen in this study, with the lower responses being seen at higher concentrations, again suggesting an inhibitory effect of Atorvastatin at higher concentrations. Although the effect is similar to that seen with the assay of metabolic activity, the effect of variation in concentration of Atorvastatin on cell proliferation is less pronounced than the effect on metabolic activity.

Comparing the effects observed in the Alamar Blue and CyQUANT studies, it is seen that metabolism is more obviously affected than proliferation, with a more pronounced non-linear relationship. This suggests that Atorvastatin is promoting intracellular activity, and not having its effect by promoting cell proliferation. For both Alamar blue and CyQUANT studies, the control groups were considered together, as there was no significant difference between them. In both studies, the outcomes from the control condition of Atorvastatin and mevalonate were the same as Atorvastatin only; mevalonate therefore did not reverse the influence of

Atorvastatin, indicating that the effects are independent of the mevalonate (cholesterol synthesis) pathway.

Direction for Future Studies

Primary derived myofibroblasts were utilised for this study, as they are amongst the first cells to respond to tissue injury, and to promote repair by secreting extracellular matrix. To the author's knowledge this is the first study to investigate the effect of statins on cells that have been cultured from fresh specimens derived from human colon. This study, simply from the success of doing that, has demonstrated that it is a feasible model which has the potential to form the basis of valuable non-animal, primary human cell research. Future research could include investigating other markers of myofibroblast function and ECM secretion, including collagen secretion into the cell medium. The use of multiplex enzyme-linked immunosorbent assay (ELISA) panels would allow simultaneous assays of a range of matrix metalloproteinases (MMP), and would represent a valuable approach to adding more detail into the knowledge obtained. Future research could also investigate the function of other cell types fundamental to tissue healing, such as microvascular endothelial cells, macrophages and the sub types of macrophages, as these sub populations and their predominance and role in inflammation and wound healing are the subject of much research relating to skin wound healing.

In future research, the laboratory research could be linked to the clinical course of the donor patients; it would then be possible to identify whether differences in cell

function and ECM secretion were associated with the risks of complications, specifically anastomotic leak.

Clinical Study Discussion

The beneficial effects of perioperative statin use have been demonstrated in cardiac and vascular surgery, but have only been investigated to a much lesser extent in other specialities. As already described, there is conflicting data regarding the influence of statins upon outcomes in major colorectal surgery, particularly regarding AL.

This study has not demonstrated an overall reduction in AL risk in patients taking statins. An important observation, however, is that patients taking statins had a greater burden of co-morbidities and AL risk factors than those not taking statin therapy, even so, they did not demonstrate the expected higher incidence of AL. That observation has also been shown in other retrospective reviews of major colorectal practice. It is therefore reasonable to suggest that statin therapy might reduce the risk of AL in these high-risk patients; statin use may effectively be a form of pharmacological prehabilitation. That observation is particularly pertinent to the cohort of patients in this study. The Royal Liverpool University Hospital serves a socioeconomically deprived population, with many patients having a variety of comorbidities. Although specific deprivation data was not collected during this study, The English Indices of Deprivation, a report produced by the Department for Communities and Local Government, showed that at the time of data collection for this study, 45% of neighbourhoods within the local authority district of Liverpool

were in the most deprived 10% of neighbourhoods nationally(119). The 2016 Health Survey for England found that 14% of adults were prescribed lipid lowering medication whereas 38.9% of the patients in this study was prescribed a statin. The difference is likely to reflect both a relatively unhealthy population, but also the diligence of the local primary care services in ensuring that appropriate primary and secondary prevention of cardiovascular disease is addressed.

As discussed earlier in this document, there are many proposed pre-operative risk factors, and peri-operative clinical findings believed to be associated with an increased risk or incidence of leak, however very few are robustly demonstrated. In this study, only two of the measured values were significant; a low eGFR, reflecting poor renal function, and a high CRP level 7 days post-operatively. Both of these factors have been shown to be associated with an increased incidence of AL in previous studies, so those observations in this study can be interpreted as validating the model and the method of the study.

In this study, a number of risk factors for AL identified in other studies, were not shown to be associated with AL in this study. Particularly significant risk factors not associated with AL in this study include smoking, diabetes and obesity, as these have frequently been identified as risk factors in other studies. It is likely that the relatively small patient population, and low number of leaks accounts for these observations. It is widely accepted that AL is a multifactorial entity, moreover, leaks will occur in patients with either no, or very few, obvious risk factors, whilst some patients with several risk factors will not experience leak. For those reasons, it is extremely challenging to identify individual risk factors for AL.

Limitations of the Study and Direction of Future Research

This study has the inherent limitations of a retrospective study, carried out in a single centre with a population that may not be representative of the wider society. AL was diagnosed based on radiological or operative confirmation of a clinically suspected AL; although some small leaks may have been undetected, the definition used in this study is conventional in observational studies investigating AL. The small number of patients in the study, and small number of ALs reported and the effect this has on the power of our study is also acknowledged. The decision to exclude patient with a defunctioning stoma may have also influenced the findings of the study, as low anterior resections are recognised as having a higher incidence of AL. This group was excluded as anastomotic deficiency in defunctioned patients is often not recognised until the anastomosis is investigated radiologically or clinically prior to planning a closure of stoma. As time to stoma reversal is variable, and frequently several months, follow-up for these patients may well have fallen outside of the data collection period.

The limitations of this study may be borne in mind when considering future study design. Designing a randomised controlled trial to investigate the relationship between statins and leak risk would be highly compromised, as this would rely on either recruiting patients with no indications for statins, or denying patients with an indication for statins the appropriate medication. It would also be necessary to control for other recognised risk factors for leak, so would require a study population of tens of thousands. Snapshot studies and registries are likely to represent the most realistic way of investigating a relationship between AL and statin use. The European

Society of Coloproctology (ESCP) collaborative group snapshot audit of right hemicolectomy recruited 3208 patients within a 2-month period, and the subsequent left sided resection audit recruited 3676 patients within a 10-week period(120, 121). Such an extensive multicentre approach is a valid way to collect the volume of data required to investigate problems with a complex multifactorial aetiology. The American College of Surgeons' National Surgical Quality Improvement Program (NSQIP) is a validated, outcomes-based program designed to measure and improve quality of surgical care. This is essentially a prospectively maintained rolling audit programme, and collects vast amounts of data from every patient treated in the institutions enrolled into the program, and it is possible that further research into the relationship between statins and leak may be conducted through the ACS NSQIP.

Beyond the consideration of statins, this observation emphasises the potential importance of optimal management of comorbidities in the preoperative phase, in order to mitigate against the risk of complications associated with comorbidities. This study also raises the broader issue of considering the influence of the co-morbidities and pre-operative medication taken by patients undergoing colorectal surgery; this is an under investigated issue. Two recent comprehensive and authoritative reviews of the challenges presented by anastomotic leaks, and strategies to minimise the incidence have been published(122, 123). Both reviews highlight areas for future research, yet neither mentions assessment of the patient's current medications.

In conclusion we have demonstrated that patients taking statins had more comorbidities than those not taking statins, but those patients did not demonstrate the higher anastomotic leak rate that might have been expected. Further studies

should be directed towards identifying whether statins do indeed modify the risk of AL, and indeed other perioperative complications in high-risk patients. As many high-risk patients will already be taking statins, and low-risk patients may be unlikely to derive a benefit from statins, an RCT is unlikely to be an ethically acceptable study design, therefore a prospectively held multi-centre registry would be the most appropriate method of investigation, and would also provide the opportunity to investigate the effect of other medications upon perioperative outcomes.

Conclusions

Anastomotic leak remains one of the most devastating complications of colorectal surgery; efforts to reduce the incidence of this complication are therefore paramount. These laboratory and clinical studies were designed and conducted to further investigate the suggested relationship between statins and anastomotic leak; specifically, that those patients taking statins may have a reduced risk of anastomotic leak. To the author's knowledge, this is the first study to investigate the effects of statins on primary cultured cells, from fresh specimens of human colon. This research experimentally evidenced an effect of Atorvastatin upon the metabolic activity of colonic myofibroblasts. The method of isolating and culturing primary human myofibroblasts was developed and could now be repeated and provided as a reproducible technique, and provides a valuable human cell model for further research.

Anastomotic leak is widely accepted to be a multifactorial entity, therefore reducing the incidence of leak will require multiple risk factors to be addressed. It is certainly not the suggestion of this author that statins, or any other single medication, will

represent a mechanism to prevent AL. However, medications such as statins, that are shown to promote tissue healing may have a role in limiting complications such as leaks, especially for those patients with a high risk of anastomotic leak, due to comorbidities such as underlying cardiovascular disease.

The clinical study that was run in parallel to the laboratory work was based upon prospectively collected data, to investigate both potential effects of statins, and also to allow other risk factors to be considered. Although statins were not shown to reduce the risk of AL, there was no observed increased incidence of AL in high-risk individuals with comorbidities. The number of patients that is required to elucidate the effect of a single risk factor or intervention in an entity as complex as AL is much higher than could be collected in a single centre over 12-18 months. Data from randomised controlled trials is regarded as being the most robust data to direct change in clinical practice. It is unlikely that an RCT would be an appropriate method to investigate the relationship between statins and AL, as a scientifically robust version of that study would require patients who depend on statins being denied that medication, and would therefore not be ethically acceptable. This highlights the importance of the “big-data” approach, and it is therefore likely that studies such as the ESCP snapshot studies, or studies delivered from the ACS NSQIP will represent the best way of investigating relationships such as that between AL and the use of statins.

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Appendices

Appendix 1: Outcome of Research Ethics Committee Review of the Clinical Study.

NHS
Health Research Authority
NRES Committee South Central - Oxford C
Bristol REC Centre
Level 3, Block B
Whitefriars Building
Lewins Mead
Bristol
BS1 2NT

Telephone: 0117 342 1335
Facsimile: 0117 342 0445

26 June 2013

Mr Paul Rooney
Royal Liverpool and Broadgreen University Hospitals Trust
9Z Link, Royal University Hospital
Prescot Street
Liverpool
L7 8XP

Dear Mr Rooney

Study title:	**The effect of statins upon colonic cells, and colonic tissue healing.**
REC reference:	**13/SC/0351**
IRAS project ID:	**120643**

The Proportionate Review Sub-committee of the NRES Committee South Central - Oxford C reviewed the above application on 24 June 2013.

Issues discussed with the researcher

1. The committee was happy with the study design but noted that the PIS might alarm participants who did not have cancer by mentioning cancer.
   Mr C Battersby agreed to modify the PIS.

2. The committee requested clarification on sample storage and use with reference to anonymity and to consent for use in other research.
   Mr Battersby confirmed that samples would be linked-anonymised and for use in this study only. The consent form would be modified to reflect this.

A revised participant information sheet and consent form were forwarded to the committee, who was satisfied with same.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Investigator CV	P S Rooney	
Letter from Sponsor		09 April 2013
Letter from Statistician		
Other: CV for C Battersby		
Other: CV for J Hunt		
Participant Consent Form	1.2	25 June 2013
Participant Information Sheet	1.4	25 June 2013
Protocol	1.3	02 April 2013
REC application	3.5	14 June 2013

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Naazneen Nathoo, nrescommittee.southwest-bristol@nhs.net.

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
information is available at National Research Ethics Service website > After Review

13/SC/0351 **Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

pp. Dr David Scott
Alternate Vice-Chair

Email: nrescommittee.southcentral-oxfordc@nhs.net

Enclosures: *"After ethical review – guidance for researchers" [SL-AR2]*

Copy to: *Heather Rogers, Royal Liverpool and Broadgreen University Hospitals Trust*

NRES Committee South Central - Oxford C

Attendance at PRS Sub-Committee of the REC meeting on 24 June 2013

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Avinash Gupta	Clinical Research Fellow	Yes	
Mrs Susan Lousada	Lay Member	Yes	
Dr David Scott	Pharmacist	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Naazneen Nathoo	REC Coordinator

Appendix 2: Clinical Research Form A

CRF A- Demographic Data and Preoperative Assessment

For patients undergoing elective colorectal resection

Patient Information

Patient Name

D.O.B:

Hospital Number:

Consultant:

ESTIMATED DATE OF OPERATION: ……./……../……..
dd mm yyyyy

DATE OF THIS ASSESSMENT ……./……../……..
dd mm yyyyy

Person completing this form: .....

Patient Characteristics

Gender **Male ☐** **Female ☐**

BMI (or weight and height) **…… kg/m^2** **(.....kg ..... cm)**

Smoker? **Never smoked ☐** **Ex-smoker ☐** **(when stopped? ……)** **Current smoker ☐** **(Pack years……..)** **(20/day for a yr = 1 pack yr)**

Alcohol **Non Drinker☐** **<10 units/wk ☐** **10-30 units/wk ☐** **>30units/wk ☐**

1 unit = 1 bottle of beer, 1 glass of wine or 1 measure of spirits.

Previous Surgery NO ☐ YES ☐

Procedure?.....

Medications:

Taking a Statin NO ☐ YES ☐

If Yes, Dose, name of statin and duration of therapy_____

Taking an Antiplatelet NO ☐ YES ☐

If Yes, name, dose and duration_____

Comorbidities:

Other Medications:

CRF A- Demographic Data and Preoperative Assessment (continued)

On Steroids or Immunosuppressed within past 6 months? **NO ☐** **YES ☐**
Indication and duration ……..

Drug + dose .....

Radiotherapy and chemotherapy history:

Pre-op Radiotherapy? **NO ☐** **YES ☐**
If yes: Details of regime:

Pre-op Chemotherapy? **NO ☐** **YES ☐**
If yes, details of regime:

Previous Pelvic Radiotherapy **NO ☐** **YES ☐**

Appendix 3: Clinical Research Form B

CRF B. Intra-Operative Data

Study Number: _____

Date: _____

Operation: _____

Consultant: _____

Tissue Collected: _____

_____

Appendix 4: Patient Consent Form

The Royal Liverpool and
Broadgreen University Hospitals
NHS Trust

Department of General Surgery

Colonic Tissue and Blood Sample Collection

PATIENT CONSENT FORM

Name of Researcher: _____

I confirm that I have read and understood the patient information sheet dated June 2013 (Version 1.4) for Surplus Colonic Tissue Samples and Analysis. I have had the opportunity to ask questions and have had these answered satisfactorily.

I understand that my participation in this project is entirely voluntary and that I may withdraw at any time without needing to give a reason, and without my medical care or legal rights being affected.

I understand that sections of my medical notes may be looked at by responsible individuals involved in this research or from regulatory authorities where it is relevant to my taking part in this research. I give permission for these individuals to have access to my medical records.

I give permission for the samples of my blood and bowel to be used for this project.

I am happy for the future publication and dissemination of research involving my gifted sample, which may contain genetic information

I agree to take part in the above study.

Who has reviewed this study?
All NHS research is reviewed by an independent group of people called a research ethics committee. This study was reviewed and approved by the Health Research Authority Oxford C committee.

Patient Name	Signature	Date
Name of Researcher	Signature	Date
Name of person taking consent (if different)	Signature	Date

When completed: 1 for patient, 1 in medical notes, 1 (original) for researcher site file

Statins and colonic healing
Patient Information Sheet

Affix patient identification sticker here

Chief Investigator

Mr Paul Rooney
Consultant Surgeon
Registrar
Royal Liverpool University Hospital
Prescot Street
Liverpool
L8 7XP

Principal Investigator

Mr Christopher Battersby
General Surgical and Research
Royal Liverpool University Hospital
Prescot Street
Liverpool
L7 8XP

The Purpose of this Form

This form will help you decide if you want to participate in the research study. As you read this form, the Principal Investigator (the person in charge of this research) or a member of the research team will describe this study to you and answer all of your questions. You should have all your questions answered before you give your permission to be in the study. We encourage you to take time to discuss this with your family and friends.

If you do want to be in the study, you will need to sign this form to give your consent. This is called *informed consent* form because it informs you before you sign to give your consent. You will get your own copy of this signed form for your records.

Why have I been invited to take part?

You are being asked to be in this research study because you need to have surgery to remove part of your bowel. You are being asked to allow the researchers to carry out a small number of extra tests on the bowel tissue that is removed at the time of your operation. The researchers are investigating whether medications that are commonly taken by patients, for other conditions, have any effect upon tissue healing in the bowel. The researchers may also request a sample of blood to analyse cholesterol levels.

The bowel that is removed will undergo all of the standard investigations, and almost all of the extra investigations for the purpose of research will carried out after the standard routine investigations are complete. We will not be taking samples of cancer tissue.

What will happen to me if I take part in the study?

Whether you decide to participate in this study or not, will make no difference to your medical care. Information about you and your disease will be recorded. This information includes height, weight, age, race, smoking and alcohol consumption, the kind of disease you have and medications you are taking. Your planned operation, and post-operative care will be identical whether you participate or not. The only difference will be that a small number of extra investigations will be carried out on the tissue that has already been removed. Researchers who have no involvement in your clinical care will not be given your name, or any details that would enable them to find out who the sample is from.

The principal investigator will review your hospital notes at some point (1-2 months) after your operation to assess your post-operative recovery. This will not require any further input from you or your family.

Do I have to take part in the study?

No. If you decide not to take part, you will receive exactly the same level of care.

Who can take part?

Anyone who is being considered for planned surgery to remove a section of large bowel will be allowed to take part.

What will happen to my sample?

The section of bowel that is removed will be sent to the pathology laboratory, and analysed according to standard practice. The researchers will then carry out the extra tests on remaining tissue. Any remaining tissue will be stored in the histopathology laboratory, in the standard manner. If there is any leftover tissue not used in this project, it will be stored and may be used in other projects in the future without your knowledge. The researchers might ask to take a small sample of normal bowel tissue before it is sent to the laboratory. This will be done in the operating theatre, at the same time as the operation, and will not in any way affect the nature of the operation, the analysis of the sample, or your post-operative care. Any samples that are taken at the time of the operation (fresh tissue) will be disposed of safely at the end of the experiment, and will not be stored in a laboratory for future use.

How will my sample be anonymised?
When your sample is transferred from the operating theatre, it will be labelled with a study number. There will be no way for the scientists to find out who the sample came from.

What are the benefits of donating my sample to this study?
There will be no direct benefit to you for participating in this study. The findings from this study may benefit other patients in the future.

What are the risks of taking part in this study?
Deciding to take part in the study will make no difference to what happens to you during your operation and hospital stay and so there are no risks to taking part in the study.

Will I find out the results of research performed on my sample?
You will not receive the results of research performed on your samples.

How will my personal information be shared?
We are asking for your permission to gather information about you for this study. This information will include personal information such as name and age. It will also contain information about height, weight, your medical history, information about medicines you take, how much you smoke and how much alcohol you drink. When your sample is released to researchers, your name will be removed and replaced by a sample number. There will be no way to connect this sample number with you.

What if I change my mind after I've donated a sample?
If you decide that your tissue may be kept for research, but later change your mind, contact any member of the research team at any time. You will be under the continued care of your surgeon, and can contact them to discuss withdrawing your consent.

Will I be paid for donating my sample?
You will not be paid to donate your sample.

Who is organising this study?
The study is organised and run by scientists and doctors from the University of Liverpool and Royal Liverpool University Hospital.

Who can I ask for advice on whether to take part?
We recommend that you speak to your friends and family before you decide whether or not to take part in this study. You can also ask to speak to a surgeon who is not involved in the study to get independent advice on whether to take part. If you would like to speak to someone independent, please ask your doctor.

For further specific information about this study please contact Mr Christopher Battersby (General Surgical Registrar) on 0151 794 4227 or christopherbattersby@nhs.net

What if I have a complaint about the study?

If you are not happy with the general care and treatment you receive during the study, please speak first to your study doctor who will try to resolve the problem. They can tell you about the hospital's standard complaints procedure in case you wish to take the matter further. You can do this by contacting Royal Liverpool University Hospital Patient Advice and Liason Service (PALS).

You may also report a concern about a study or ask questions about your rights as a research subject by contacting the Research Ethics Committee. When you call with a concern, please give as much information as you can. Include the name of the study leader and details about the problem. This will help officials look into your concern. When reporting a concern, you do not have to give your name.