**The systemic influence of chronic smoking on skin structure and mechanical function**

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**Abbreviations:**

AFM, atomic force microscopy; AGE, advanced glycation end-products; COPD, chronic obstructive pulmonary disease; DEJ, dermal-epidermal junction; ECM, extracellular matrix; ISZ, *in situ* zymography; MMP, matrix metalloproteinase; SAM, scanning acoustic microscopy; UV, ultraviolet.

**Running title:** Smoking impacts skin structure and mechanical function

**Abstract**

One of the major functions of human skin is to provide protection from the environment. Whilst we cannot entirely avoid, for example, sun-exposure, it is likely that exposure to other environmental factors could impact cutaneous function. A number of studies have identified smoking as one such factor that leads to both facial wrinkle formation and a decline in skin function. In addition to the direct physical effects of tobacco smoke on skin, its inhalation has additional profound systemic effects for the smoker. The adverse effects on the respiratory and cardiovascular systems from smoking are well known. Central to the pathological changes associated with smoking is the elastic fibre, a key component of the extracellular matrices of lungs. In this study we examined the systemic effect of chronic smoking (>40 cigarettes/day; >5 years) on the histology of the cutaneous elastic fibre system, the nanostructure and mechanics of one of its key components, the fibrillin-rich microfibril, and the micromechanical stiffness of the dermis and epidermis. We show that photoprotected skin of chronic smokers exhibits significant remodelling of the elastic fibre network (both elastin and fibrillin-rich microfibrils) as compared to the skin of age- and sex-matched non-smokers. This remodelling is not associated with increased gelatinase activity (as identified by *in situ* zymography). Histological remodelling is accompanied by significant ultrastructural changes to extracted fibrillin-rich microfibrils. Finally, using scanning acoustic microscopy, we demonstrated that chronic smoking significantly increases the stiffness of both the dermis and the epidermis. Taken together, these data suggest an unappreciated systemic effect of chronic inhalation of tobacco smoke on the cutaneous elastic fibre network. Such changes may in part underlie the skin wrinkling and loss of skin elasticity associated with smoking.

**Keywords:** Elastic fibres, Elastin, Fibrillin-rich microfibrils, skin, tobacco smoke exposure

**Introduction**

One of the major functions of human skin is to provide protection from the environment, a major component of which is sun exposure. Chronic ultraviolet radiation (UVR) in sunlight is known to result in profound changes to both the collagenous and elastic dermal matrices resulting in a specific clinical phenotype termed photoageing [1]. Whilst we cannot entirely avoid sun exposure, exposure to other environmental factors, including those where personal choice plays a role, may influence cutaneous function. A number of studies have identified smoking as one such factor [2-5]; these epidemiological studies identify smoking as an independent risk factor for the occurrence of facial wrinkles.

Clinically, facial wrinkles of smokers differ from those of non-smokers; they are narrower but deeper, with sharply contoured periorbital and perioral lines [6]. The physical movement of pursing the lips and squinting (due to smoke irritation of the eyes when inhaling) is likely to enhance wrinkle formation at these anatomical sites. However, as well as direct physical effects, inhalation of tobacco smoke has systemic effects on the smoker. The adverse effects on the respiratory and cardiovascular systems from smoking are well known [7]; each of these systems requires tissue to expand and recoil many millions of times over an individual’s lifespan, mediated by the elastic fibre [8], a key component of extracellular matrices in dynamic tissues such as lungs [9], blood vessels [10] and skin [11].

Elastic fibres are a compound biomaterial, having a central core of amorphous, hydrophobic cross-linked elastin surrounded by a mantle of fibrillin-rich microfibrils (FRMs) [12]. These microfibrils are thought to provide a range of biological functions which include: directing elastogenesis [13]; structural reinforcement of the elastic fibre and force transduction [14,15]; cell signalling via RGD sequences [16]; and allowing TGFβ sequestration [17,18] (for a review on elastogenesis see [19]). Extracted FRMs have a characteristic ultrastructural appearance in electron and atomic force microscopy (AFM) with an average bead-to-bead distance (or periodicity) of 56 nm [20,21]. By using a molecular combing technique it is also possible to characterise the ability of isolated microfibrils to withstand applied surface tension forces [14,21].

A major function of elastic fibres is to provide the host organ with the ability to extend and recoil many times over the lifetime of the individual [22]. Hence, alterations to either the distribution of elastic fibres or to their capacity for recoil will impact significantly upon the tissue in which they reside. Previous studies have identified remodelling of the elastic fibre system in the pathophysiology of emphysema [23], chronic obstructive pulmonary disease [24,25] and aortic aneurysm [26]. Similarly, in human skin, the histological distribution of dermal elastic fibres has been studied [27-29]. These histological morphometric studies identified that the dermal elastic fibres of smokers were fragmented and appeared to be significantly increased in number and that the observed alterations were confined to the reticular and deeper dermis.

We were able to confirm the altered distribution of elastic fibre components in photoprotected skin from smokers. This was done by quantitative immunohistochemistry for both elastin and FRMs and the use of *in situ* zymography (ISZ) to assess whether the remodelling is driven via the induction and increased activity of elastic fibre-degrading matrix metalloproteinases (MMPs). We further characterised the biological impact of changes to the micro-architecture and molecular ultrastructure of the cutaneous elastic fibre network using two independent, though complementary methods. The methods were: scanning acoustic microscopy (SAM) which facilitates investigation of mechanical properties of tissues using ultra-high frequency sound vibrations (100 MHz – 1 GHz) where the reflected acoustic wave speed is related to tissue stiffness and density [30]; and molecular combing, which facilitates quantification of extensibility from extracted, tissue-derived microfibrillar assemblies, observed by AFM [14].

**MATERIALS AND METHODS**

**Skin biopsy procurement**

Studies conformed to The Declaration of Helsinki Principle. The study was approved by the ethics committee of Attikon General University Hospital, and informed consent was obtained from all participants. We recruited 36 volunteers to this study, of these, 18 had never smoked (9 M, 9 F; age range, 25 – 55 years) whilst the remaining 18 (9 M, 9 F; age range, 25 – 63 years) had an accumulated exposure to cigarette smoke of at least 10 pack years (1 pack year is defined as 20 manufactured cigarettes [one pack] smoked per day for one year). Except for their smoking status, all volunteers were in good general health as assessed by a clinician (ETN). All volunteers provided a single 6mm punch biopsy from their photoprotected buttock following 1% lignocaine local anaesthesia. Samples were bisected, one half embedded in optimal cutting temperature compound and the remainder snap frozen in liquid nitrogen. Samples were stored at -80°C prior to analyses. Biopsies were cryosectioned at 7 µm in a single run, using the same blade and the same cryostat settings (Bright OTF cryostat; Cambridge, U.K.).

**Immunohistochemistry**

To investigate the distribution of elastic fibre components in skin of smokers and non-smokers, we performed immunohistochemistry for elastin and FRM as previously described [31,32]. In brief, frozen sections (7 µm) were fixed in 4% paraformaldehyde and hydrated in Tris-buffered saline (TBS; 100 mM Tris, 150 mM NaCl; pH 7.4). Sections were pre-treated with 0.5% Triton X-100 and endogenous peroxidase activity abolished by incubation with 0.6% hydrogen peroxide in methanol. Non-specific binding was blocked by incubation with 3% bovine serum albumin plus 3% normal horse serum. Primary antibodies were applied overnight at 4°C. These were either: mouse anti-elastin (clone BA4; Sigma-Aldrich; Watford, U.K.) diluted 1:1000 or mouse anti-human FRM (clone 11C1.3; ThermoFisher Scientific, Altrincham, U.K.) diluted 1:100. Negative controls were performed by omission of primary antibody. Sections were washed in TBS, prior to incubation with the appropriate biotinylated antibody for 30 minutes. Antibody staining was visualized using a well-characterised immunoperoxidase reaction (VectaStain® *Elite* ABC system; Vector Laboratories, Peterborough, U.K.) utilising Vector SG® as the chromogen. Sections were counterstained with nuclear fast red (Vector Laboratories), dehydrated and permanently mounted. Following randomisation and blinding, bright field images were captured using a BX53 microscope (Olympus Industrial; Southend-on-Sea, U.K.) and image analysis was performed using ImageJ software [33].

***In situ* zymography**

To investigate whether smoking initiated tissue remodelling via induction of MMPs we assessed *in situ* gelatinase activity (MMPs-2 and -9) on a sub-sample of volunteer tissue (non-smokers, n = 10, 5 M, 5 F; age range 26 – 55 years; smokers, n = 10, 5M, 5 F; age range 32 - 63 years). Unfixed frozen sections (7 µm) were incubated with DQTM-gelatin as per the manufacturer’s instructions (ThermoFisher Scientific). As a control, cryosections were also pre-incubated with the MMP inhibitor 1-10-phenanthroline monohydrate (10mM) for 1 hour before continuing with the *in situ* zymography protocol [34]. Following incubation, gelatinase activity was visualised by fluorescence microscopy and intensity analysed using ImageJ software.

**Scanning acoustic microscopy**

To assess whether smoking induced changes in the mechanical properties of skin, we performed scanning acoustic microscopy on a sub-sample of volunteer tissue (non-smokers, n = 4, 3M, 1 F; age range 26 – 45 years; smokers, n = 4, 3M, 1 F; age range 36 - 45 years). Frozen sections (5 µm) were imaged using a SAM 2000 instrument (KSI Gmbh, Heborn, Germany). Images were taken at a frequency of 770 MHz. Experiments were performed at room temperature using distilled water as the coupling solution. For quantitative analysis, three images were recorded for each subject and subsequently analysed as previously described using the *V(f)* method [30].

**Fibrillin-rich microfibril isolation**

A sub-sample of volunteers were used for examination of FRM characteristics (non- smokers, n = 3, age range 26 – 31 years; smokers, n = 3, age range 25-30 years). These assemblies were extracted from skin biopsies using 0.5 mg/mL bacterial collagenase type IA (suspended in 0.4 M NaCl, 0.05 M Tris–HCl, 0.01 M CaCl2 at pH 7.4, and supplemented with protease inhibitors: 2 mM phenylmethanesulfonyl fluoride and 5 mM N-Ethylmaleimide). Skin biopsies were incubated in 2 ml collagenase buffer with agitation for 4 hr at room temperature followed by further digestion overnight at 4°C. The digested tissue was centrifuged to remove any cell debris and the supernatant fractionated by gel filtration using a Sepharose CL-2B column (ÄKTA prime plus system; GE Healthcare, Little Chalfont, U.K.) that was equilibrated in high salt buffer (0.4 M NaCl, 0.05 M Tris–HCl at pH 7.4). FRMs were eluted from the column in the excluded volume (V0) peak.

**Atomic force microscopy, molecular combing and data processing**

FRM ultrastructure was characterized by AFM. Using the Multimode 8 AFM (Bruker AFM Probes, Camarillo, California USA) fitted with ScanAsyst-Air cantilevers, randomly selected 10 x 10 µm locations were scanned at a rate of 1.97 Hz. The morphologic metrics assessed were the number of beads per FRM and inter-bead periodicity. Periodicity was determined by measuring the distance between individual beads. For each experimental group, microfibril periodicity (n = 3000) and bead number per microfibril (n = 150 microfibrils) were determined from AFM images by ImageJ, WSxM scanning probe microscopy software and by routines written in Microsoft Visual Basic 6.0 (MJS). Samples were also subject to molecular combing whereby surface tension forces are employed to cause viscous drag on any partially adsorbed FRM (n = 2000). Inter-bead periodicity is a widely used, reliable and quantitative marker for analysis of FRM ultrastructure [35-37].

**Statistics**

Statistical analysis was performed using GraphPad Prism 8.1.2 (GraphPad Software, Inc. La Jolla, California, and U.S.A). Results were considered significant if P < 0.05 (95% confidence level).

**Results**

**Photoprotected skin of chronic smokers exhibits significant remodelling of the elastic fibre network**

Immunohistochemical analyses of the major dermal elastic fibre components elastin and FRMs were performed on photoprotected buttock skin biopsies from smokers and non-smokers. In the skin of non-smokers, elastin ~~(Figure 1a)~~ and FRMs ~~(Figure 1d)~~ were arranged in distinctive candelabra-like arrays, connecting oxytalan fibres of the dermal-epidermal junction (DEJ) to elaunin fibres of the superficial papillary dermis. In contrast, immunohistochemical staining of elastin ~~(Figure 1b)~~ and FRMs ~~(Figure 1e)~~ in the skin of smokers revealed significant loss of elastic fibre architecture (Figure 1A). This remodelling of the elastic fibre network was accompanied by a significant increased deposition of both elastin (mean ± standard deviation [SD]; non-smokers = 6.4 ± 1.9%; smokers = 8.7 ± 2.2%; p < 0.01; figure 1B & D) and FRMs (non-smokers = 19.3±6.3%; smokers = 23.4±6.0%; p < 0.05; figure 1C & E) in the photoprotected skin of smokers, as compared to age- and sex-matched non-smoker controls.

**Altered elastic fibre function in smokers is not mediated via changes in gelatinase activity.**

To assess whether changes in elastic fibre deposition were mediated via the expression and activity of MMPs-2 and -9 (gelatinases), enzymes known to remodel elastic fibres, we performed immunohistochemistry (pro- and active enzyme isoforms) and ISZ (activity). We observed no alterations in the distribution by immunohistochemistry of MMPs-2 and -9 (data not shown). However, as antibodies identify both the pro- and active forms of these enzymes, we also performed ISZ for MMPs-2 & -9 utilising a gelatin substrate to assess enzyme activity (Figure 2). Here we show these enzymes are primarily localised to the epidermis (Figure 2A & B) and there is no significant difference in their activity (integrated fluorescence intensity; mean ± SD; non-smokers: 50.7 ± 11.1 a.u.; smokers: 46.4 ± 11.2 a.u.; p = 0.402; Figure 2C). As a control, pre-incubation with the MMP inhibitor 1-10-phenanthroline monohydrate blocks gelatinase activity (Figure 2D). Further analysis revealed that, irrespective of smoking status, the epidermal basal keratinocytes were devoid of gelatinase expression (Figure 2E). Within the dermis, gelatinase activity was low and there was no significant difference in activity between smokers and non-smokers (mean ± SD; non-smokers: 4.7 ± 2.5 a.u.; smokers: 5.5 ± 2.5 a.u.; p = 0.50; Figure 2C).

**Smokers’ skin exhibits increased tissue stiffness**

Next, we assessed if alterations to the architecture and abundance of the elastic fibres resulted in changes to the biomechanical properties of the skin of smokers. Tissue stiffness was assessed by SAM, where increasing acoustic wave speed (λ) indicates increasing stiffness. Epidermal and dermal stiffness was determined by performing SAM on cryosections of skin biopsies mounted on glass microscope slides; skin was imaged from the external section edge, through the epidermis and into the dermis (Figure 3A). Using this method we observed that the skin of smokers had a significantly higher acoustic wave speed than non-smokers in both the epidermis (non-smokers: 1579.1 ± 9.4 ms-1; smokers: 1614.7 ± 15.0 ms-1; p < 0.01) and the dermis (non-smokers: 1565.4 ± 10.7 ms-1; smokers: 1611.2 ± 11.3 ms-1; p < 0.001; Figure 3B).

**Extracted fibrillin-rich microfibrils from the skin of smokers are shorter and less able to resist strain than those from the skin of non-smokers.**

To assess whether there were any ultrastructural changes to the FRMs, assemblies from experimental groups were isolated by size exclusion chromatography following bacterial collagenase digestion, as previously described [36,37]. Using AFM, images of FRMs from each of the experimental groups were captured and the number of beads per microfibrillar assembly enumerated.

Microfibrillar assemblies extracted from the skin of smokers were significantly shorter than those extracted from age- and sex-matched non-smokers (mean ± SD; non-smokers: 26.4 ± 21.0; smokers: 21.6 ± 15.8; p < 0.05; Figure 4A-C). Smoking also influenced the inter-bead periodicity of the FRM; characteristically, FRMs have a bead-to-bead periodicity of approximately 56 nm [38]. The mean periodicity for FRMs extracted from non-smokers was in agreement with this (mean ± SD; 55.5 ± 13.4 nm); however, for smokers, periodicity was significantly increased (60.2 ± 14.9 nm; p < 0.001; Figure 4D).

To assess whether these observed changes impact upon the biomechanics of the FRMs, extracted FRMs were subjected to molecular combing to assess whether they were more or less able to resist tensile forces. Following molecular combing, FRM periodicity was significantly increased in the smoking group (mean ± SD; 65.1 ± 19.6 nm) compared with the non-smoking group (57.1 ± 17.6 nm; p<0.001, Mann Whitney U-test; Figure 4E & F). Furthermore, significantly more periodicity measurements (99/2000) were extended beyond 100 nm in the FRMs extracted from smokers as compared with non-smokers (30/2000; χ2 test, p<0.001).

**Discussion**

Smoking is regarded as an independent risk factor for skin ageing [2-5], the clinical presentation of which includes the premature appearance of coarse and fine wrinkles [39]. Here, we confirm the findings of previous studies that identify a significant remodelling of the elastic fibre network occurs in the photoprotected skin of smokers [27-29] and provide further evidence for alterations in both the macromolecular organisation and tensile strength of FRMs, fundamental components of elastic fibres in diverse tissue systems.

Investigators have sought to assess whether smoking has a systemic effect on elastic fibres, with opposing results; Allen and co-workers [40], and more recently Knuutinen and colleagues [41], failed to identify significant changes in the distribution of elastic fibres in photoprotected skin of smokers using histological methodologies. However, studies employing the immunohistological identification of elastin have found significant increases in both the number and total area attributable to elastin-positive fibres in the reticular and deeper dermis of photoprotected skin of smokers [27,29]. In addition to confirming these findings for elastin, we also used immunohistochemical methods to identify a similar increase in FRM abundance and loss of FRM architecture in the photoprotected skin of smokers. One potential mechanism by which elastic fibres may undergo remodelling is through the local activity of MMPs-2 and -9 [42]; therefore, we performed ISZ which has the advantage of localizing active enzyme activity to the tissue under investigation. In agreement with other studies, we showed that the activity of MMPs-2 and -9 was largely restricted to the epidermis [43,44] and that regardless of smoking status, the level of MMP activity was invariant. Our study suggests that MMPs-2 and -9 may not be the main mechanistic driver for the observed alterations in elastic fibres; however, we cannot rule out that small changes to enzyme activity over many years could result in the observed alteration to the cutaneous architecture, abundance and distribution of papillary dermal elastic fibres. The role of MMPs in the pathogenesis of wrinkle formation in the skin of smokers may be more important for the collagenous rather than the elastic fibre network. It has previously been shown that smoking decreases the synthesis of type I and III collagens in skin *in vivo* [41] and alters the balance of dermal extracellular matrix (ECM) turnover [41,45]. However, there are conflicting opinions on precisely which MMPs are responsible for the remodelling of collagen with both MMPs-1 [45] and -8 [41] suggested as likely candidates. Increased activation of circulating neutrophils [46] and neutrophil elastase [47] in response to cigarette smoking have also been noted and may provide a further mechanism by which elastin and FRMs are degraded [48,49].

To further assess whether smoking compromises elastic fibre function, FRM assemblies were extracted from skin biopsies, thus allowing analysis of macromolecular structure. Perhaps unsurprisingly, given the histological description of fragmented fibres in smokers’ skin, we found a significant reduction in the length of FRM assemblies and a significantly increased inter-bead periodicity, as compared to the skin of non-smokers. Extracted FRMs were also put under extension stress via molecular combing. Previous studies have identified the ability of FRMs to reversibly extend up to a periodicity of ~100 nm, after which they are unable to reform [14]. In FRM populations extracted from the skin of non-smokers the proportion of beads with periodicity ≥ 100 nm is between 1-2%; however, when we subjected the FRMs extracted from the skin of smokers to this mechanical force, this proportion significantly increased to ~5%. Hence, FRMs from the skin of smokers appear to be less able to withstand mechanical forces.

Loss of elastic fibre architecture and function ~~in key structural components of the ECM~~ occur not only as the result of tobacco smoke exposure but also in the rare genetic condition *cutis laxa*. Individuals with *cutis laxa* have mutations in key structural components of the elastic fibres (including elastin and fibrillin) causing both excess inelastic skin and pulmonary disease [50-52]. Perhaps indicative of shared biology across systems, it has also been identified that the distribution of elastic fibres in skin correlates well with that in lung [53]. It is therefore tempting to postulate that these weakened ~~elastic fibre structures~~ elastin and FRMs may also impact on the physiological behaviour of the lung and potentially the cardiovascular system in individuals who smoke. Collectively, there is mounting evidence that affirms the biological plausibility underlying the association of pulmonary disease phenotypes with alterations in skin biology in tobacco-exposed individuals. Chronic obstructive pulmonary disease (COPD) is a condition where cigarette smoking is an important risk factor. However, only a subgroup of smokers develops COPD and it is unclear why these individuals are more susceptible to the detrimental effects of cigarette smoking [54]. In individuals with smoking-related COPD there is an association between facial wrinkling and airflow obstruction, suggesting that lung and skin share a common susceptibility to the deleterious effects of tobacco smoke exposure [55]. Individuals with smoking-related COPD have increased elastosis in their photoprotected skin and the degradation of dermal elastin is associated with increased emphysema severity and carotid pulse wave velocity, indicating that elastin breakdown is a systemic condition [56]. More recently, it has been shown that biomechanical loss of skin elasticity is associated with pulmonary emphysema, biomarkers of inflammation, and MMP activity in the skin of smokers with COPD [57]. Functional measurement of elasticity using non-invasive methods is an accessible and objective determinant of the biomechanical properties of skin ECM that has been validated in several studies [58-60], although it’s utility as an indirect measure of elastin degradation in COPD has only recently emerged [57].

Elastic fibres make up only a small proportion of the dermal ECM; therefore, to assess the effects of smoking on skin as a whole we performed SAM. The main advantages of this method, as compared to nanoindentation, for example, is that micromechanical data can potentially be obtained at a higher spatial resolution (~1 μm for 1 GHz lens). Using this method, we identified that the skin of smokers is significantly stiffer than that of non-smokers. It has previously been demonstrated that the skin of smokers has increased activity of lysyl oxidase enzymes [61,62]. Lysyl oxidases primarily drive the catalysis of cross-links in both fibrillar collagens and elastin in the dermis [63]; however, they are also present within the epidermis of both human and mouse skin [62,64-66], where it has been suggested that they play a role in maintaining epidermal homeostasis and normal keratinocyte differentiation [67,68]. While the function of these enzymes in the epidermis remains to be fully elucidated, we suggest that epidermal stiffness could, at least in part, be a consequence of increased lysyl oxidase activity. Furthermore, a recent study employing skin autofluorescence measurements revealed an independent association between the accumulation of advanced glycation end products (AGEs) in the skin and parameters of lung function in subjects with COPD [69]. Thus, the increase in overall skin stiffness described herein may be attributable to an increased level of protein cross-linking within the skin and may be further indicative of an association between changes in the skin and other smoking-related conditions such as idiopathic pulmonary fibrosis [70] and arterial stiffening [71].

This study characterises the effect of cigarette smoking on the dermal elastic fibre system yet the mechanism via which it asserts these effects remains elusive. However, it is clear that dermal homeostasis is disrupted in the photoprotected skin of smokers due to the systemic effects of tobacco smoke. Tobacco smoke is a complex mixture of many thousands of chemical components known to have toxicological, carcinogenic and mutagenic properties [72,73]. Mainstream (smoker-inhaled) smoke is divided into a particulate solid phase (tar) and the gas phase (toxic gases, volatile organic compounds) containing both stable and unstable free radicals and reactive oxygen species (ROS) that have the potential for biological oxidative damage [73,74]. Cigarette smoking enables these toxic free radicals to be distributed systemically via the bloodstream. ROS in the gas-phase promotes the destruction of endogenous antioxidants and impairs the vital role of cellular antioxidant defences [75]. Furthermore, several studies show that antioxidant vitamins are lower in smokers resulting in systemic oxidative stress [76,77]. Thus, the highly vascularised dermis may be particularly sensitive to oxidative stress, leading to a cascade of downstream consequences that ultimately result in ~~ECM~~ elastin and FRM degradation and remodelling [78].

Premature facial ageing, altered skin texture, and skin wrinkling may help to influence a smoker’s decision to quit and are important deterrent factors for the uptake of tobacco products [79]. It has been shown in several studies that smoking cessation has a rejuvenating effect on the skin both in reducing perceived age [80] and improving skin colour [81]. Increased public health awareness of the positive effects of stopping smoking and maintaining abstinence on both facial appearance and respiratory health [82] should be encouraged.

In conclusion, we identified a significant remodelling of the cutaneous elastic fibre system in smokers. This remodelling, which appears to be independent of MMP digestion of elastic fibres, is apparent at both the histological and ultrastructural levels and results in skin which is significantly stiffer in smokers than in non-smokers. These changes may not only impact upon the appearance of skin – via the formation of features associated with premature ageing – but may also influence the function of other elastic fibre-rich tissues, such as those found in the respiratory and cardiovascular systems. Characterisation of the linked pathology between degradation and remodelling of the ECM in skin and other organs as a consequence of tobacco smoke exposure warrants further investigation.

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**Statement of author contributions**

AKL, MJS, RA, BD, REBW and CEMG designed the research. ET-N, AC and AS provided skin biopsies. AKL, HM and XZ performed the experiments and acquired data. AKL, REBW and CEMG wrote the manuscript.

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**References**

1. Kligman AM. Early destructive effect of sunlight on human skin. *JAMA* 1969; **210**: 2377-2380.

2. Kadunce DP, Burr R, Gress R*, et al.* Cigarette smoking: risk factor for premature facial wrinkling. *Ann Intern Med* 1991; **114**: 840-844.

3. Rexbye H, Petersen I, Johansens M*, et al.* Influence of environmental factors on facial ageing. *Age Ageing* 2006; **35**: 110-115.

4. Martires KJ, Fu P, Polster AM*, et al.* Factors that affect skin aging: a cohort-based survey on twins. *Arch Dermatol* 2009; **145**: 1375-1379.

5. Green AC, Hughes MC, McBride P*, et al.* Factors associated with premature skin aging (photoaging) before the age of 55: a population-based study. *Dermatology* 2011; **222**: 74-80.

6. Daniell HW. Smoker's wrinkles. A study in the epidemiology of "crow's feet". *Ann Intern Med* 1971; **75**: 873-880.

7. Kenfield SA, Wei EK, Rosner BA*, et al.* Burden of smoking on cause-specific mortality: application to the Nurses' Health Study. *Tob Control* 2010; **19**: 248-254.

8. Sherratt MJ. Tissue elasticity and the ageing elastic fibre. *Age (Dordr)* 2009; **31**: 305-325.

9. Miller J. The arrangement of the elastic fibres in the bronchi and lung. *J Anat Physiol* 1906; **40**: 162-170.

10. Ayer JP HG, Philpott DE. Aortic elastic tissue; isolation with use of formic acid and discussion of some of its properties. *AMA Arch Pathol* 1958; **65**: 519-544.

11. Dick JC. Observations on the elastic tissue of the skin with a note on the reticular layer at the junction of the dermis and epidermis. *J Anat* 1947; **81**: 201-211.

12. Wagenseil JE, Mecham RP. New insights into elastic fiber assembly. *Birth Defects Res C Embryo Today* 2007; **81**: 229-240.

13. Trask TM, Trask BC, Ritty TM*, et al.* Interaction of tropoelastin with the amino-terminal domains of fibrillin-1 and fibrillin-2 suggests a role for the fibrillins in elastic fiber assembly. *J Biol Chem* 2000; **275**: 24400-24406.

14. Sherratt MJ, Baldock C, Haston JL*, et al.* Fibrillin microfibrils are stiff reinforcing fibres in compliant tissues. *J Mol Biol* 2003; **332**: 183-193.

15. Ashworth JL, Kielty CM, McLeod D. Fibrillin and the eye. *Br J Ophthalmol* 2000; **84**: 1312-1317.

16. Bax DV, Mahalingam Y, Cain S*, et al.* Cell adhesion to fibrillin-1: identification of an Arg-Gly-Asp-dependent synergy region and a heparin-binding site that regulates focal adhesion formation. *J Cell Sci* 2007; **120**: 1383-1392.

17. Olivieri J, Smaldone S, Ramirez F. Fibrillin assemblies: extracellular determinants of tissue formation and fibrosis. *Fibrogenesis Tissue Repair* 2010; **3**: 24.

18. Massam-Wu T, Chiu M, Choudhury R*, et al.* Assembly of fibrillin microfibrils governs extracellular deposition of latent TGF beta. *J Cell Sci* 2010; **123**: 3006-3018.

19. Mithieux SM, Weiss AS. Elastin. *Advances in Protein Chemistry* 2005; **70**: 437-461.

20. Kielty CM, Cummings C, Whittaker SP*, et al.* Isolation and ultrastructural analysis of microfibrillar structures from foetal bovine elastic tissues. Relative abundance and supramolecular architecture of type VI collagen assemblies and fibrillin. *J Cell Sci* 1991; **99 ( Pt 4)**: 797-807.

21. Sherratt MJ. Tissue elasticity and the ageing elastic fibre. *AGE* 2009: 1-21.

22. Gosline J, Lillie M, Carrington E*, et al.* Elastic proteins: biological roles and mechanical properties. *Philos Trans R Soc Lond B Biol Sci* 2002; **357**: 121-132.

23. Janoff A, Sloan B, Weinbaum G*, et al.* Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis* 1977; **115**: 461-478.

24. Deslee G, Woods JC, Moore CM*, et al.* Elastin expression in very severe human COPD. *Eur Respir J* 2009; **34**: 324-331.

25. Maclay JD, McAllister DA, Rabinovich R*, et al.* Systemic elastin degradation in chronic obstructive pulmonary disease. *Thorax* 2012; **67**: 606-612.

26. Campa JS, Greenhalgh RM, Powell JT. Elastin degradation in abdominal aortic aneurysms. *Atherosclerosis* 1987; **65**: 13-21.

27. Frances C, Boisnic S, Hartmann DJ*, et al.* Changes in the elastic tissue of the non-sun-exposed skin of cigarette smokers. *Br J Dermatol* 1991; **125**: 43-47.

28. Boyd AS, Stasko T, King LE*, et al.* Cigarette smoking-associated elastotic changes in the skin. *Journal of the American Academy of Dermatology* 1999; **41**: 23-26.

29. Just M, Ribera M, Monsó E*, et al.* Effect of smoking on skin elastic fibres: morphometric and immunohistochemical analysis. *British Journal of Dermatology* 2007; **156**: 85–91.

30. Akhtar R, Schwarzer N, Sherratt MJ*, et al.* Nanoindentation of histological specimens: Mapping the elastic properties of soft tissues. *Journal of Materials Research* 2009; **24**: 638-646.

31. Watson REB, Craven NM, Kang SW*, et al.* A short-term screening protocol, using fibrillin-1 as a reporter molecule, for photoaging repair agents. *Journal of Investigative Dermatology* 2001; **116**: 672-678.

32. Watson REB, Long SP, Bowden JJ*, et al.* Repair of photoaged dermal matrix by topical application of a cosmetic 'antiageing' product. *British Journal of Dermatology* 2008; **158**: 472-477.

33. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. *Biophotonics International* 2004; **11**: 36-42.

34. Garcia-Alloza M, Prada C, Lattarulo C*, et al.* Matrix metalloproteinase inhibition reduces oxidative stress associated with cerebral amyloid angiopathy in vivo in transgenic mice. *J Neurochem* 2009; **109**: 1636-1647.

35. Eckersley A, Mellody KT, Pilkington S*, et al.* Structural and compositional diversity of fibrillin microfibrils in human tissues. *J Biol Chem* 2018; **293**: 5117-5133.

36. Hibbert SA, Watson REB, Gibbs NK*, et al.* A potential role for endogenous proteins as sacrificial sunscreens and antioxidants in human tissues. *Redox biology* 2015; **5**: 101-113.

37. Sherratt MJ, Bayley CP, Reilly SM*, et al.* Low-dose ultraviolet radiation selectively degrades chromophore-rich extracellular matrix components. *J Pathol* 2010; **222**: 32-40.

38. Kielty CM, Sherratt MJ, Marson A*, et al.* Fibrillin microfibrils. *Adv Protein Chem* 2005; **70**: 405-436.

39. Tsoureli-Nikita E, Watson REB, Griffiths CEM. Photoageing: the darker side of the sun. *Photochemical & Photobiological Sciences* 2006; **5**: 160-164.

40. Allen HB, Johnson BL, Diamond SM. Smoker's wrinkles? *JAMA* 1973; **225**: 1067-1069.

41. Knuutinen A, Kokkonen N, Risteli J*, et al.* Smoking affects collagen synthesis and extracellular matrix turnover in human skin. *Br J Dermatol* 2002; **146**: 588-594.

42. Ashworth JL, Murphy G, Rock MJ*, et al.* Fibrillin degradation by matrix metalloproteinases : implications for connective tissue remodelling. *Biochemistry Journal* 1999; **340**: 171-181.

43. Inomata S, Matsunaga Y, Amano S*, et al.* Possible Involvement of Gelatinases in Basement Membrane Damage and Wrinkle Formation in Chronically Ultraviolet B-exposed Hairless Mouse. *J Investig Dermatol* 2003; **120**: 128-134.

44. Quan T, Qin Z, Xia W*, et al.* Matrix-degrading metalloproteinases in photoaging. *J Investig Dermatol Symp Proc* 2009; **14**: 20-24.

45. Lahmann C, Bergemann J, Harrison G*, et al.* Matrix metalloproteinase-1 and skin ageing in smokers. *Lancet* 2001; **357**: 935-936.

46. Hoonhorst SJ, Timens W, Koenderman L*, et al.* Increased activation of blood neutrophils after cigarette smoking in young individuals susceptible to COPD. *Respir Res* 2014; **15**: 121.

47. Weitz JI, Crowley KA, Landman SL*, et al.* Increased neutrophil elastase activity in cigarette smokers. *Ann Intern Med* 1987; **107**: 680-682.

48. Takeuchi H, Gomi T, Shishido M*, et al.* Neutrophil elastase contributes to extracellular matrix damage induced by chronic low-dose UV irradiation in a hairless mouse photoaging model. *J Dermatol Sci* 2010; **60**: 151-158.

49. Kielty CM, Woolley DE, Whittaker SP*, et al.* Catabolism of intact fibrillin microfibrils by neutrophil elastase, chymotrypsin and trypsin. *FEBS Lett* 1994; **351**: 85-89.

50. Kozel BA, Su CT, Danback JR*, et al.* Biomechanical properties of the skin in cutis laxa. *J Invest Dermatol* 2014; **134**: 2836-2838.

51. Urban Z, Davis EC. Cutis laxa: intersection of elastic fiber biogenesis, TGFbeta signaling, the secretory pathway and metabolism. *Matrix Biol* 2014; **33**: 16-22.

52. Lebwohl MG, Schwartz E, Jacobs L*, et al.* Abnormalities of fibrillin in acquired cutis laxa. *J Am Acad Dermatol* 1994; **30**: 950-954.

53. Just M, Monsó E, Ribera M*, et al.* Relationships between lung function, smoking and morphology of dermal elastic fibres. *Experimental Dermatology* 2005; **14**: 744–751.

54. Eisner MD, Anthonisen N, Coultas D*, et al.* An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010; **182**: 693-718.

55. Patel BD, Loo WJ, Tasker AD*, et al.* Smoking related COPD and facial wrinkling: is there a common susceptibility? *Thorax* 2006; **61**: 568-571.

56. Maclay JD, McAllister DA, Rabinovich R*, et al.* Systemic elastin degradation in chronic obstructive pulmonary disease. *Thorax* 2012; **67**: 606-612.

57. O'Brien ME, Chandra D, Wilson RC*, et al.* Loss of skin elasticity is associated with pulmonary emphysema, biomarkers of inflammation, and matrix metalloproteinase activity in smokers. *Respir Res* 2019; **20**: 128.

58. Langton AK, Graham HK, McConnell JC*, et al.* Organization of the dermal matrix impacts the biomechanical properties of skin. *Br J Dermatol* 2017; **177**: 818-827.

59. Langton AK, Graham HK, Griffiths CEM*, et al.* Ageing significantly impacts the biomechanical function and structural composition of skin. *Exp Dermatol* 2019; **28**: 981-984.

60. Langton AK, Alessi S, Hann M*, et al.* Aging in Skin of Color: Disruption to Elastic Fiber Organization Is Detrimental to Skin's Biomechanical Function. *J Invest Dermatol* 2019; **139**: 779-788.

61. Langton AK, Griffiths CE, Sherratt MJ*, et al.* Cross-linking of structural proteins in ageing skin: an in situ assay for the detection of amine oxidase activity. *Biogerontology* 2012.

62. Langton AK, Tsoureli-Nikita E, Griffiths CEM*, et al.* Lysyl oxidase activity in human skin is increased by chronic ultraviolet radiation exposure and smoking. *Br J Dermatol* 2017; **176**: 1376-1378.

63. Cenizo V, Andre V, Reymermier C*, et al.* LOXL as a target to increase the elastin content in adult skin: a dill extract induces the LOXL gene expression. *Exp Dermatol* 2006; **15**: 574-581.

64. Hayashi K, Fong KS, Mercier F*, et al.* Comparative immunocytochemical localization of lysyl oxidase (LOX) and the lysyl oxidase-like (LOXL) proteins: changes in the expression of LOXL during development and growth of mouse tissues. *J Mol Histol* 2004; **35**: 845-855.

65. Kobayashi H, Ishii M, Chanoki M*, et al.* Immunohistochemical localization of lysyl oxidase in normal human skin. *Br J Dermatol* 1994; **131**: 325-330.

66. Noblesse E, Cenizo V, Bouez C*, et al.* Lysyl oxidase-like and lysyl oxidase are present in the dermis and epidermis of a skin equivalent and in human skin and are associated to elastic fibers. *J Invest Dermatol* 2004; **122**: 621-630.

67. Le Provost GS, Debret R, Cenizo V*, et al.* Lysyl oxidase silencing impairs keratinocyte differentiation in a reconstructed-epidermis model. *Exp Dermatol* 2010; **19**: 1080-1087.

68. Bouez C, Reynaud C, Noblesse E*, et al.* The lysyl oxidase LOX is absent in basal and squamous cell carcinomas and its knockdown induces an invading phenotype in a skin equivalent model. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2006; **12**: 1463-1469.

69. Hoonhorst SJ, Lo Tam Loi AT, Hartman JE*, et al.* Advanced glycation end products in the skin are enhanced in COPD. *Metabolism* 2014; **63**: 1149-1156.

70. Antoniou KM, Walsh SL, Hansell DM*, et al.* Smoking-related emphysema is associated with idiopathic pulmonary fibrosis and rheumatoid lung. *Respirology* 2013; **18**: 1191-1196.

71. Doonan RJ, Hausvater A, Scallan C*, et al.* The effect of smoking on arterial stiffness. *Hypertens Res* 2010; **33**: 398-410.

72. Talhout R, Schulz T, Florek E*, et al.* Hazardous compounds in tobacco smoke. *Int J Environ Res Public Health* 2011; **8**: 613-628.

73. Valavanidis A, Vlachogianni T, Fiotakis K. Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. *Int J Environ Res Public Health* 2009; **6**: 445-462.

74. Huang MF, Lin WL, Ma YC. A study of reactive oxygen species in mainstream of cigarette. *Indoor Air* 2005; **15**: 135-140.

75. Cross CE, Traber M, Eiserich J*, et al.* Micronutrient antioxidants and smoking. *Br Med Bull* 1999; **55**: 691-704.

76. Panda K, Chattopadhyay R, Chattopadhyay DJ*, et al.* Vitamin C prevents cigarette smoke-induced oxidative damage in vivo. *Free Radic Biol Med* 2000; **29**: 115-124.

77. Traber MG, van der Vliet A, Reznick AZ*, et al.* Tobacco-related diseases. Is there a role for antioxidant micronutrient supplementation? *Clin Chest Med* 2000; **21**: 173-187, x.

78. Rinnerthaler M, Bischof J, Streubel MK*, et al.* Oxidative stress in aging human skin. *Biomolecules* 2015; **5**: 545-589.

79. Demierre MF, Brooks D, Koh HK*, et al.* Public knowledge, awareness, and perceptions of the association between skin aging and smoking. *J Am Acad Dermatol* 1999; **41**: 27-30.

80. Serri R, Romano MC, Sparavigna A. "Quitting smoking rejuvenates the skin": results of a pilot project on smoking cessation conducted in Milan, Italy. *Skinmed* 2010; **8**: 23-29.

81. Cho YH, Jeong DW, Seo SH*, et al.* Changes in skin color after smoking cessation. *Korean J Fam Med* 2012; **33**: 105-109.

82. Willemse BW, Postma DS, Timens W*, et al.* The impact of smoking cessation on respiratory symptoms, lung function, airway hyperresponsiveness and inflammation. *Eur Respir J* 2004; **23**: 464-476.

**Figure legends**

**Figure 1:**

**Smokers’ skin contains significantly more dermal elastic fibres than non-smokers.**

Photoprotected skin of smokers contains more elastin-positive fibres and fibrillin-rich microfibrils than photoprotected skin of non-smokers. Immunolocalisations were performed on fresh frozen skin sections using monoclonal antibodies to elastin (BA4) and fibrillin-rich microfibrils (11C1.3). Sections were visualised by immunoperoxidase staining. Discreet elastic fibre-positive assemblies are seen in photoprotected skin of non-smokers whereas the skin of smokers contains fragmented elastin fibres & fibrillin-rich microfibrils (A). Scale bar, 50μm.

Plots reveal a significant increase in both elastin (B & D) and fibrillin-rich microfibrils (C & E) in smokers as compared to non-smokers. \* p < 0.05; \*\* p < 0.01; scale bar = 50 µm.

**Figure 2:**

**Altered deposition does not appear to be mediated via the actions of MMPs-2 & -9.**

*In situ* zymography was performed to assess whether the observed re-organisation of elastic fibre components was driven by MMPs-2 & -9 (A & B). MMP activity is predominantly epidermal and is equivalent in non-smokers and smokers (C). Pre-incubation with the MMP inhibitor 1-10-phenanthroline monohydrate blocks gelatinase activity (D). The epidermal basal layer is devoid of MMP activity (D). Scale bar = 100 µm.

**Figure 3:**

**Smoking results in significant skin stiffening.**

Scanning acoustic microscopy (SAM) was performed on skin samples from smokers and non-smokers. (A) Typical SAM image, showing clearly the acoustic difference between the cellular epidermis and the extracellular matrix-rich dermis. The DEJ is marked by the white line. Data was extracted from scans of 200μm in length extending from the external surface of the skin and into the dermis at a position perpendicular to the DEJ (see red box as an example of the position scanned). (B) Box & whisker plots reveal that both the epidermis and dermis are significantly stiffer in individuals who smoke. Scale bar = 50 µm.

**Figure 4:**

**Fibrillin-rich microfibril ultrastructure is affected by smoking.**

Fibrillin-rich microfibrils extracted from the skin of smokers are shorter and have increased bead-to-bead periodicity (A-D). Molecular combing identifies weakened fibrillin-rich microfibrils in individuals who smoke (E & F).