Title: **Sex Specific effects of *in utero* and adult tobacco smoke exposure**

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Running Head: Timing of Tobacco Exposure Alters the Affected Sex

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Abstract

Tobacco smoke has harmful effects on a multi-organ level. Exposure to smoke, whether *in utero* or environmental, significantly increases susceptibility. This susceptibility has been identified to be divergent between males and females. However, there remains a distinct lack of thorough research into the relationship between sex and exposure to tobacco. Females tend to generate a more significant response than males during adulthood exposure. The intrauterine environment is meticulously controlled, and exposure to tobacco presents a significant factor that contributes to poor health outcomes and susceptibility later in life. Analysis of these effects in relation to the sex of the offspring is yet to be holistically reviewed and summarised. In this review, we will delineate the time-dependent relationship between tobacco smoke exposure and sex-specific disease susceptibility. We further outline possible biological mechanisms that may contribute to the identified pattern.

Sex-Specific effects of *in utero* and adult tobacco smoke exposure

Background

The intrauterine environment is highly regulated to enable healthy fetal development. Alterations to the molecular and chemical milieu predispose the fetus to chronic diseases later in life. The 'developmental origins of disease' hypothesis describes that changes to prenatal conditions induces adaptions which alter anatomical structure and physiology to promote growth and success (27). These adaptations, although aiding intrauterine survival, may not offer protection perinatally (27). A recent review details the effects of maternal exposure to heavy metals, stress, tobacco smoke, and alcohol on the health outcomes of offspring (17), identifying that tobacco smoking negatively impacts multiple organ systems (17).

Tobacco smoking is a leading risk factor for non-communicable diseases (NCDs). Although on the decline, smoking rates remain high worldwide. Smoke exposure remains the leading preventable cause of chronic diseases such as chronic obstructive pulmonary disease (COPD), cancer, and congenital heart disease (CHD) (3, 22, 38). Approximately 50% of women who smoke continue after discovering they are pregnant (48). As such, maternal tobacco smoke (MTS) is the leading preventable cause of abnormal pregnancy outcomes (48). MTS increases the likelihood of the newborn to suffer from various NCDs in childhood and adulthood (6, 45, 54, 62). The biological mechanism driving this is yet to be elucidated however hormones, epigenetics, oxidative stress, and DNA mutations have all been postulated.

Active smoking has an overall adverse health impact, and sex-specific effects are recognised. Adult tobacco smoking has greater consequences in females, suffering higher morbidity, and greater severity of tobacco-related disease (44). A meta-analysis found smoking intensity increased the magnitude and likelihood of stroke in women compared to men (53). Females had increased risk and severity of COPD and colorectal cancer with similar smoking levels (3, 22). Despite a clear pattern in adulthood, sex-specific prenatal effects of tobacco smoke exposure are rarely investigated appropriately or thoroughly.

This review aims to highlight the importance of fetal sex in response to tobacco smoke exposure *in utero* and compare these patterns to adult smoke exposure.

Sex Differences in Development

Sexual dimorphism exists *in utero*, with the male and female fetus' undergoing distinct physiological and developmental processes. This is apparent in lung development. Lung maturation is advanced in female fetuses, with mouth movement and surfactant production starting at 18 weeks, whereas males reach this milestone after 20-23 weeks (54, 56). As a result, the female fetus can generate a robust response to various exposures (56). The phenomenon of dysanapsis describes differences in fetal lung development between the sexes. Male lungs have larger but fewer airways, while females have more abundant yet smaller airways, enabling higher airflow rates (32). Increased flow-rate enables greater adaptability in females to cope with adverse environments, sustaining stable growth. This phenomenon persists until 18 years of age, when male lung function overtakes females (32). The cause of delayed lung growth in males is

unknown. It is a leading factor predisposing pre-pubertal males to asthma and lower respiratory tract infections (LRTI) (55), from which young females appear to be protected.

Sex differences in fetal brain, heart, and overall growth also exist. Genes located on the male-specific Y-chromosome drive sexual dimorphism in brain development (65) The *SRY* gene, primarily known for testicular maturation (65), controls a cascade of androgens regulating male brain development *in utero* (14). As evidence of dimorphism, boys are liable to the creation of white matter by androgen stimulation; on the other hand, estrogen in females is inhibitory (14). Fetal heart rate patterns also differ, highlighting a divergence in male and female development. Males demonstrate increased heart-rate variability (16), implying a lack of stability in growth compared to females.

Here, we present inherent differences between the sexes in the absence of exogenous influences. However, the presence of this sexual dimorphism may leave either sex more susceptible to the effects of in-utero exposure to external toxicants.

Sex-Specific susceptibility due to Tobacco Smoke

Modern medicine strives to design patient-specific 'personalised medicine.' The ideology is based on the heterogeneity of symptoms, pathology, and response to treatment. This concept explains why interventions are effective for some individuals, yet ineffective for others. As such, precise and distinct mechanisms regulate physiological processes in everyone. Although sex is a fundamental characteristic, it is widely overlooked or under-investigated as a determining factor in disease and is considered a co-variate or confounding factor. As such, limited thorough research exists investigating sex-specific effects of MTS and adult tobacco smoke exposure on disease susceptibility.

Here we will detail sex-specific pathological sensitivity due to both *in utero* and adult tobacco exposure.

Lung Function Decline

The process of dysanapsis is well-described and closely linked to sex differences in respiratory outcomes (5, 76, 79). Males have decreased lung function compared to females who consistently have higher maximal expiratory flow at functional residual capacity (V'max FRC) perinatally (76, 83). MTS compounds this sex difference in lung function, as shown by various cohort studies. A study of 6,740 Chinese children reported independent negative correlation between MTS and post-natal smoke exposure on lung function, with a greater response in males overall. Male adjusted odds ratio (OR) for decreased forced vital capacity (FVC) in response to MTS OR6.46 (95% confidence interval (CI) 2.58 - 16.17) compared to the female OR 2.16 (95% CI 0.96 - 4.88) (35). Interestingly, this authors note an apparent protective effect of asthma diagnosis, with non-asthmatic offspring presenting a more pronounced decrease in lung function outcomes (35). A large North American cohort found male MTS-exposed offspring consistently experience worse lung function outcomes (FEV_1, FEV_1/ FVC, $FEF_{65-75\%}$) than female counterparts, when measured from 8 – 12 years (15). Therefore, MTS has a lasting impact on lung development throughout childhood. The study is limited by its sample size of MTS exposed children, most likely caused by underreporting of smoking by mothers, reducing the ability differentiate MTS from postnatal smoke exposure. Nonetheless, a reduction in male offspring lung function from MTS is supported by multiple case-control cohort investigations (19, 31, 47, 52).

Chronic Obstructive Pulmonary Disease

COPD is primarily an adult-onset disease, instigated by long-term exposure to noxious gases causing chronic progressive lung function decline (70). As such, it is challenging to associate MTS to COPD incidence as it is near impossible to differentiate prenatal influence from life-long exposures. Generally, males report increased COPD incidence; however, evidence supports greater female susceptibility (26). A cross-sectional US cohort study, concluded a significantly greater percentage (64%) of adults diagnosed with COPD were female from a cohort of 2,113 patients, possibly due to lifetime environmental tobacco exposure(22). The authors also report no significant relationship between MTS and COPD incidence ($p = 0.051$). Foreman et al. (2011), , find females predominate early-onset COPD due to adult exposure (26). An alternative perspective and interpretation was presented by a recent systematic review of 16 studies, the authors assert MTS impairs physiological development and hence lung function resulting in an increased risk of COPD development in adulthood (67). Therefore, as there is a male-biased effect of MTS on lung function decline, men are predisposed towards COPD development later in life. However, the age window of susceptibility may vary between the sexes (71), requiring further investigation.

Asthma

The pattern of male sex being predominately affected by MTS disappears when investigating asthma. A large Australian cohort reported that when mothers smoke more than a pack a day, female offspring had an almost two-fold increased risk of asthma; OR 1.96 (95% CI 1.25 - 2.08) (1). Males showed no association between MTS and asthma at any age, with asthma risk-reducing upon postnatal smoke exposure (1). A Finnish

study investigated the effect of mothers smoking greater than and less than 10 cigarettes during pregnancy, and identified no significant difference in asthma incidence between the sexes. A higher crude OR for female offspring of smoking mothers, although, males demonstrate higher cumulative incidence of asthma irrespective of maternal smoking level (39).During prepubescence, males dominate asthma incidence. At puberty, this pattern reverses, and females dominate diagnoses with an increase in female cases rather than a decline in male cases, with disease incidence equalising post-menopause (61). This natural pattern confounds many MTS asthma studies, which assess asthma diagnosis at age 14. As such, it is difficult to determine a definitive sex-link between MTS and asthma. Thus, the methodology of future studies requires careful consideration to sufficiently differentiate the temporal incidence of asthma with the effects of MTS. In general, women are more likely to use healthcare compared to men when matching for asthma status, indicating worse disease prognosis (44). Asthmatic females have greater bronchial hyper-responsiveness to cigarette smoke, highlighting another sexual dimorphism (44). In support, a large Swedish cohort, found smoking increases female relative risk of adult-onset asthma with an increased rate ratio 1.3 (95% CI 1.0 – 1.6) compared to men (74). Therefore, the respiratory effects of adult smoke exposure are more pronounced in women.

Multi-Organ Effects

Sexual dimorphism in disease susceptibility due to tobacco smoke exposure extends beyond the respiratory system, having multi-organ disease influences. Childhood cancers such as leukemia, childhood brain tumors (CBT), and lymphomas report higher incidence in boys than girls (6). Adult female smokers have a greater rate of cancer,

despite lower smoking intensity than males (3). Anderson et al. (2010) found a statistically significant increased risk of advanced colorectal neoplasia for mild smoking (10-30 pack years) females OR 4.11 (95% CI 1.88 – 9.01). Males only show increased disease development risk with heavy smoking (≥ 30 pack years) OR 3.10 (95% CI 1.71 – 5.65), which is similar to females at this smoking intensity (3). This contrasts with prenatal exposure closely which is linked to increased risk of disease in males. Tettamanti et al. (2016), identified in a large Swedish cohort, that smoking during pregnancy indicated a trend towards increased childhood brain tumor incidence in male children aged between five and nine years old, with a non-significant OR 1.23 (95% CI 0.91 – 1.67) (73). A recent review supports this trend asserting that nicotine affects the male brain development greater than in females (14). However, in contrast, large cohort studies find either a slight increase or no link between MTS and the development of CBT (28, 78).

Intellectual and social disability is linked to the action of nicotine on fetal brain development (14). Meta-analyses and cohort studies find increased male susceptibility to intellectual disability (36, 37). MTS is asserted to primarily affect brain microstructure in males, causing less-coherent fibers and myelination (13). This male-dominated risk is attributed to genetic differences defined by the Y-chromosome (not present in females), resulting in differential expression of genes in specific brain regions (65).

Smoke exposure increases congenital heart disease (CHD) risk with teratogenic effects during early pregnancy fetal heart development. The incidence of CHD is higher in children of smokers (60.9%) compared to non-smokers (35.8%) (42). A study of 365 neonates found an increased OR 2.75 (95% CI 1.66 – 4.58) of CHD due to peri-conceptual smoking, increasing with smoking intensity (42). Thus, the effects of tobacco

smoke are highly persistent, possibly affecting gametes. A Brazilian longitudinal cohort at follow-up, found female offspring exposed to MTS presented decreased HDL levels (33). Although an indirect indicator, the authors assert females may be more sensitive to cardiovascular disease due to MTS, due to reduced circulating HDL. Another study found that the female offspring of smokers had lower HDL levels than males in a large British cohort (62). These findings are reported in adult females, without pediatric effects of MTS exposure reported, limiting the ability to make conclusions regarding the life-long effects of smoke exposure. Adult female smokers have an overall increased risk of cardiovascular disease, supported by a 50% increased risk of myocardial infarction due to smoking (38, 63). Male mice offspring exposed to prenatal nicotine demonstrated a higher risk of hypertension and increased arterial thickness, with no significant effect observed for females (82). Alexander et al. (2011) assert that precise timing of *in utero* exposures significantly impacts the subsequent cardiovascular outcome (2), accounting for significant variability in the findings of the literature relating to cardiovascular disease.

Sex-Specific susceptibility due to E-Cigarette Exposure

The recent popularity of e-cigarettes or vapes as an alternative to traditional tobacco smoking is important to acknowledge. Due to their perception of being “safer”, e-cigarettes usage in pregnancy equals tobacco cigarette consumption (80). A detailed review of 800 articles Limited studies of the long-term physiological effects of e-cigarette products exist. McGrath-Morrow et al. (2015) identify a modest impairment of lung growth by neonatal e-cigarette exposure but do not investigate any effect of sex (49). An epidemiological study reported males comprise approximately 69% of patients suffering lung injury associated with vaping, indicating a greater pathogenic effect in males (59). A

deleterious multi-organ effect driving increased inflammatory response and cytotoxicity supported by clinical and *in vitro* studies (8, 23). Striking similarities between tobacco and e-cigarettes responses warrants further research of the long-term and sex-specific effects of e-cigarettes. Across all NCDs discussed, the effect of MTS was weakened with age (11), indicating a more substantial impact on pediatric outcomes and less of a life-long impact. We have identified a distinctive male predominate effect of prenatal smoke exposure with higher incidence and susceptibility across multiple organ systems. Some studies report MTS-related effects in females; however, this is confounded by unaccounted post-natal environmental and biological factors. The male fetus appears to be more susceptible to the adverse effects of tobacco smoke, while female fetuses are protected. This protection only lasts for the childhood years, with adult females consistently experiencing more extreme forms of chronic disease from tobacco smoke exposure (3, 12, 76). Our findings are summarised in Table 1.

Table 1: Summary of sexual dimorphism in disease susceptibility due to tobacco smoke exposure prenatally and during adulthood.

Condition	Evidence	Tobacco Smoke Exposure Time	
		Prenatal	**Adult**
Cancer	• Tettamanti et al. 2016 (73) • Botsivali & Kyrtopoulos 2019 (6) • Anderson et al. 2011 (3) • Drake et al. 2015 (21)	Male Male Male	Male Female
Respiratory	• Jaakola & Gissler 2007 (39) • Dotterud et al. 2013 (20) • Hu et al. 2017 (35) • Cunningham, Dockery & Speizer 1994 (15) • Hayabakhsh et al. 2009 (31)	Female Male Male Male Male	

	• Toren & Hermansson 1999 (74) • Kynyk, Mastronarde & McCallister 2011 (44) • Miller et al. 2014 (51) • Moshammer et al. 2006 (52) • Eisner et al. 2005 (22) • Foreman et al. 2011 (26)	 Female Male 	Female Female Female Female
	•		
Neurological Conditions	• Hutchinson et al. 2009 (37) • Cross, Linker & Leslie 2017 (14) • Appelman et al. 2015 (4) • Peters, Huxley & Woodward 2013 (60) • Chang et al. 2016 (13)	Male Male Male	 Female Female
Cardiovascular Diseases	• Power, Atherton & Thomas 2010 (62) • Horta et al. 2011 (33) • Prescott et al. 1998 (63) • Huxley & Woodward 2011 (38) • Xiao et al. 2008 (82)	Female Female Male	 Female Female

Mechanisms of Susceptibility

Cleary, sexual dimorphism exists in disease susceptibility due to tobacco smoke exposure. Importantly, the affected sex differs depending on *in utero* verse adulthood exposure. Here, we outline possible mechanisms that may contribute to sex differences.

Sex Steroid Hormones

Hormones are a critical biological factor responsible for various phenotypic differences between males and females. The primary male hormones are androgens (testosterone), while in females; estrogens (estradiol) predominate, although both function in either sex. Estrogens are pro-inflammatory increasing production of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-12, while downregulating the anti-inflammatory cytokine IL-10 (68). Androgens have the opposite effect, increasing IL-10

levels and suppressing production of IFN-γ (68). Increased estrogen levels correlates with greater production of cytochrome P450 enzymes; central to the metabolism of tobacco into toxic intermediaries (72). As result, there is accelerated production of oxidative smoke metabolites, which contribute to lung injury and inflammation. The prevalence and strength of the estrogen's effects change over time. Mature young females generate a more robust immune response, compared to males and older women, which is attributed to higher blood estrogen levels (58). The immune response in male mice is increased with exogenous estrogen, indicating a similar function of the hormone in both sexes (50, 68). Conversely, androgens act to suppress the innate immune response up-regulating IL-10 production (30). Maternal testosterone levels are increased by 11% higher in smokers, whilst no effect is seen for estrogen (75). Generally, post-menopausal women tend to response similar to males with estrogen levels declining with age. However, this remains an understudied phenomenon. Brand et al. (2011) find tobacco increases circulating levels of both male and female associated hormones in post-menopausal women but do not compare to perimenopause (9). Therefore, a close relationship exists between tobacco smoke exposure and sex hormone levels in circulation.

Epigenetics

Males and females have comparable genomes, except for the male-specific Y-chromosome; therefore, sex-biased gene regulation is likely to occur. Our group has previously shown that sexual dimorphism is maintained *ex vivo* (64), supporting the notion that an internal mechanism other than hormones drive observed sex differences. MTS is well-established to alter the epigenome causing adverse effects in the lung (84). Epigenetics is well-established as a mechanism influencing transcription, showing clear

sexual-dimorphism *in utero* (46, 54). Ladd-Acosta et al. (2016) describe MTS-induced sex-specific methylation patterns, where MTS-exposed female preschoolers had increased methylation, with decreased methylation in males near the *HLA-DPB2* gene locus (46). Other studies identify that genes involved in the immune response and defense against infection are altered in female infants, while genes regulating cancer, developmental and neurological processes are affected in male infants (43). This partially explains why males incur more developmental problems, particularly in childhood, whereas, women experienced more smoking-induced chronic inflammatory diseases in adulthood. Many smoking-related genes are differentially methylated *in utero* with *AHRR* and *CYP1A1;* both central in tobacco smoke metabolism*,* and *GFI1*; which is essential role to developmental processes and histone modification, (41). Of significance, differential methylation caused by smoking shows no differences between males and females in whole blood (86). Upon deeper investigation, the authors identify that males have an increased tendency for DNA methylation alteration, with 42 differentially methylated sites in males compared to 10 sites in females. Females demonstrate alteration to immune-related genes due to adult smoke exposure, such as *CCL13, CCL2,* and *FCN1* (18). Breitling et al. (2011) assert that sexual dimorphism of DNA methylation does not occur due to adulthood tobacco smoke exposure (10). Figure 1 highlights this dynamic relationship between MTS and gene expression *in utero* presents as highly valuable future research pathways to generate a holistic picture between sex, the epigenome, and tobacco smoke.

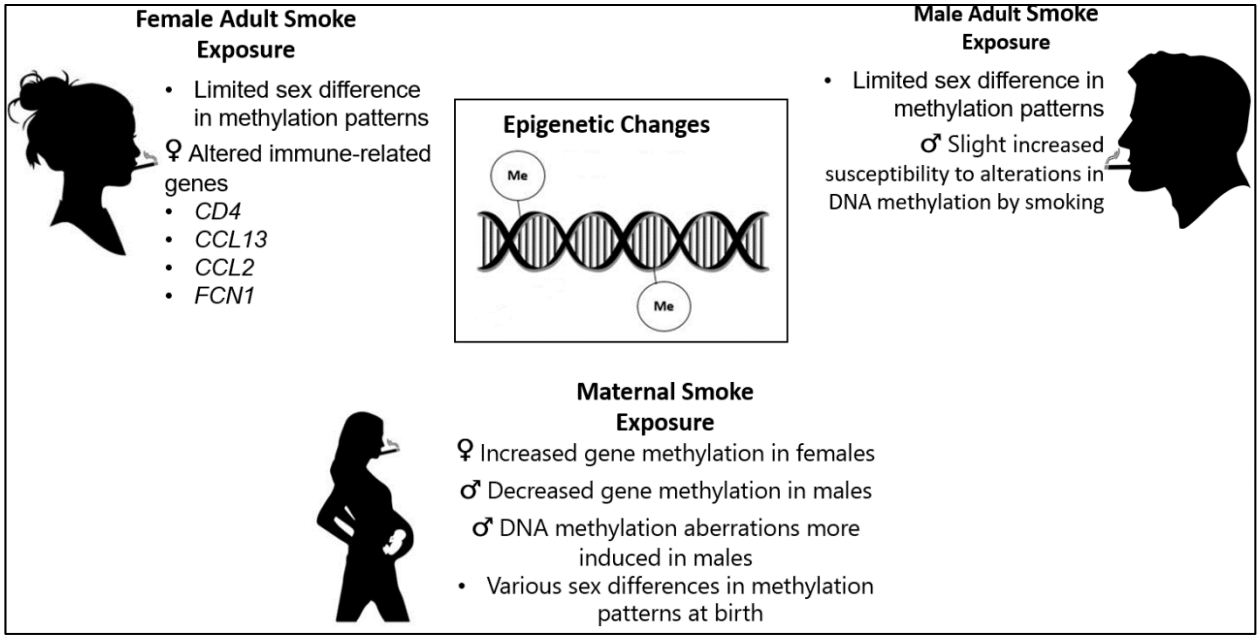


Figure 1: Sexual dimorphism in epigenetic changes between males and females depending on the time of tobacco smoke exposure. ♂ = male specific effect, ♀ = female specific effect.

Telomere Length

Telomeres are complicated nucleotide sequences located at the end of chromosomes, functioning in cell division, chromosome stabilisation, and apoptosis (66). Telomere length (TL) contributes to a variety of diseases. Shorter TL is linked with atherosclerosis and cardiovascular diseases, while long TL is associated with cancers as cellular longevity is increased (25). An association between the sex-specific relationship between telomere length and smoking has been identified, and is summarised in Figure 2. MTS is linked with shorter TL in males (24). However, studies also find females generally have longer TL than males across all ages, confounding these findings (25). An *in vitro* investigation of telomeres in cord blood found only male telomere length was negatively affected by smoke exposure *in utero* (24). The generally longer TL in females may enable more stability and resilience to exposures, subsequently offering protection

from short TL-associated diseases postnatal (29). Therefore, it is pertinent to investigate how smoking directly influences TL and its link to sex-specific disease susceptibility.

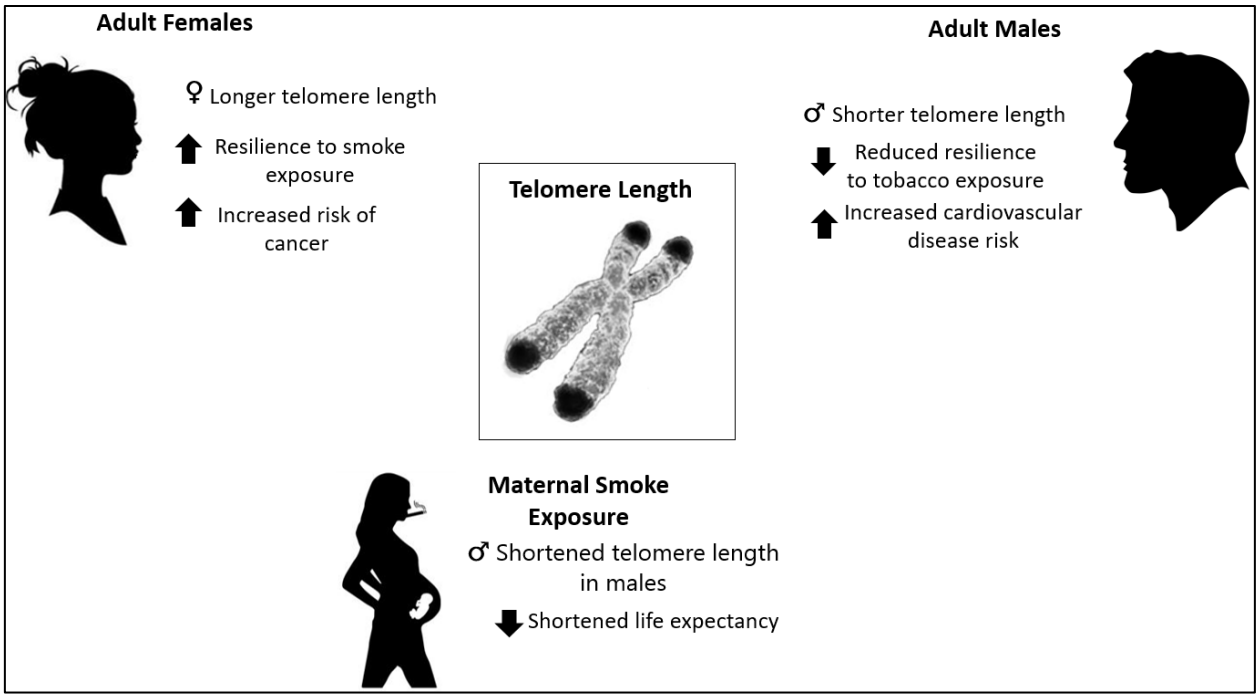


Figure 2: Sexual dimorphism in the effect of tobacco smoke exposure on telomere length depending on the time of exposure between males and females. ♂ = male specific effect, ♀ = female specific effect.

Mitochondria

Mitochondria produce the energy currency of the cell adenosine-tri-phosphate (ATP). But, mitochondrial functions extend beyond ATP energy production from hormone synthesis to ionic regulation and apoptosis (77). The breakdown of mitochondria bioenergetics *in utero* impacts various biological processes, proving detrimental to fetal health. Tobacco smoke stimulates increased mitochondrial DNA copy number to compensate for a decline in cellular respiratory function (34). MTS induces a breakdown in enzymatic activity of placental mitochondria (7), negatively impacting the ability to sustain optimum intrauterine conditions. Toxic products in tobacco smoke accumulate in

the placenta (7), with tobacco smoke metabolized at a higher rate in the fetus. Therefore, the interaction between smoking by-products: CO, nicotine, and thiocyanate contributes to a breakdown in mitochondrial function (7). Our group recently found male mice offspring exposed to smoke *in utero* showed an increase in mitophagy markers (Drp-1 and Opa-1, while no effect was seen in females (57). These proteins are markers of mitochondrial fission and fusion, dysregulation of these markers can correlate with disease severity through altered airway structure and function in asthma and COPD.. Optimised function of mitochondria in females was recognised in peripheral blood mononuclear cells, where significantly greater mitochondrial activity was reported in women (69). It is hypothesised that as mitochondria are maternally inherited, spending more time under selection in females they are optimised for function in females rather than males. Due to a greater functional ability female mitochondria may have more resilience and improved ability to respond to stressors such as environmental smoke, protecting females from reactive oxygen species (ROS) and oxidative stress. Ventura-Clapier et al. (2017) thoroughly detail the tissue and sex specificity of mitochondria (77). Figure 3 illustrates temporal sex differences of mitochondrial response, highlighting that the mitochondria may significantly contribute to the disparity in disease susceptibility between the sexes and warrants more in-depth investigation.

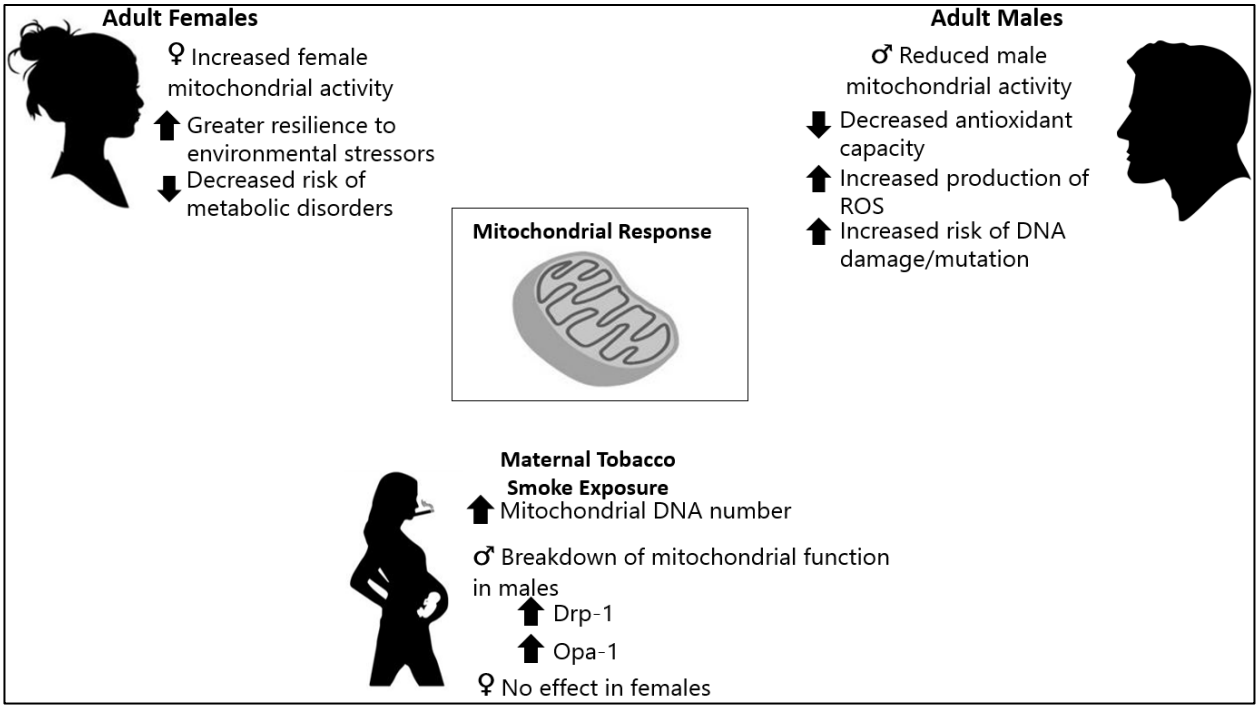
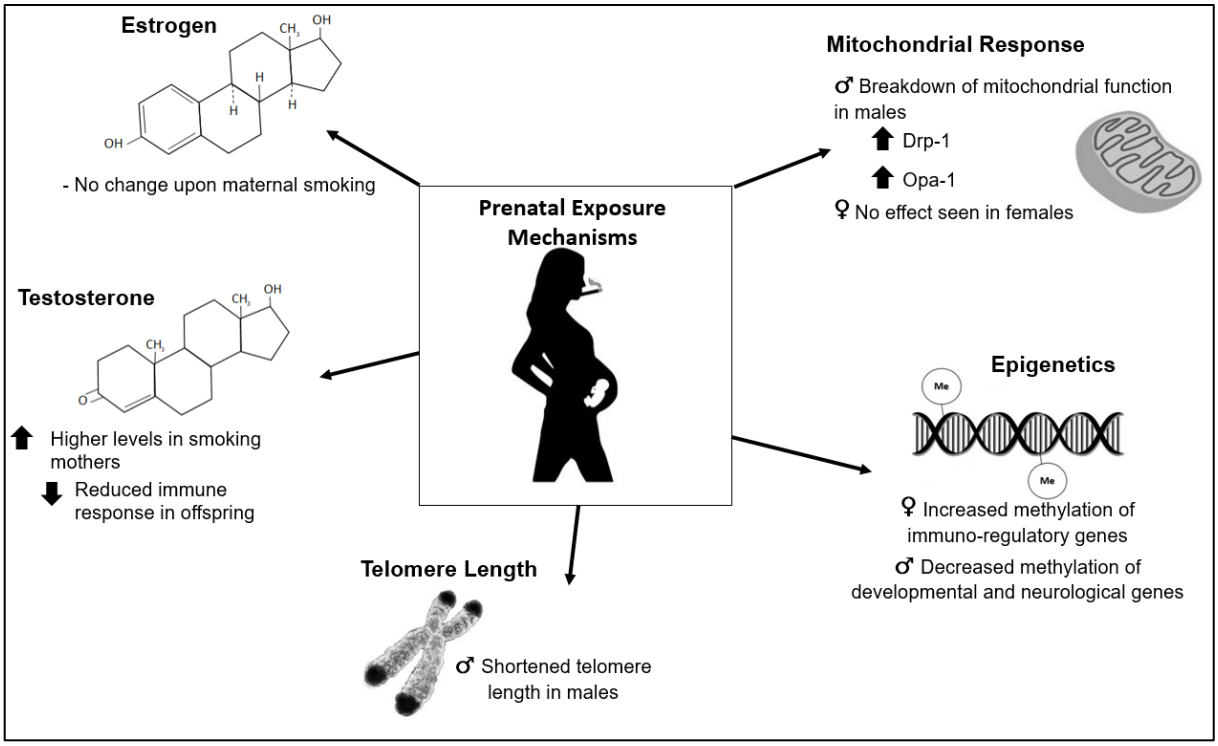


Figure 3: Sexual dimorphism in mitochondrial responses between adult males and females, and sex-specific effects of prenatal tobacco smoke exposure on fetal mitochondria. ♂ = male specific effect, ♀ = female specific effect.

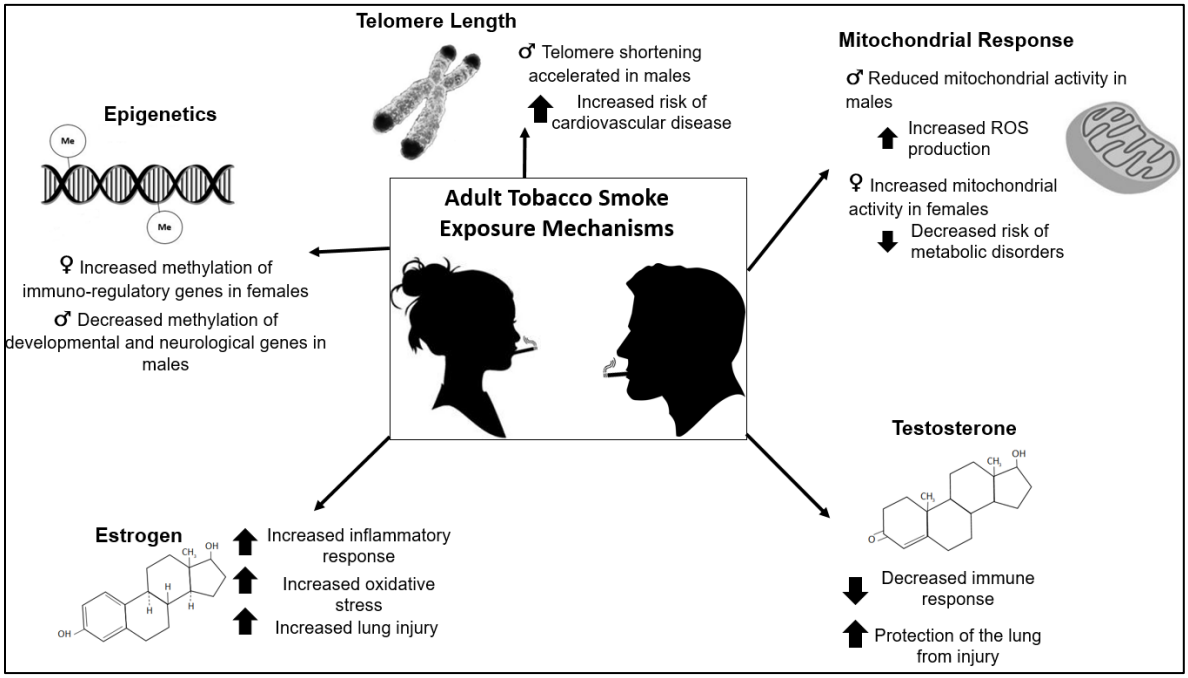
Conclusions

The intrauterine environment is a highly regulated and dynamic milieu of factors. Hence, exposure of the fetus to tobacco smoke threatens outcomes for the offspring. We have reviewed sexual dimorphism in disease susceptibility with the time of tobacco smoke. We found compounding evidence that male offspring exposed to cigarette smoke *in utero* have increased sensitivity to disease development in multiple organ systems, with this trend reversed in adult exposure, where females show greater susceptibility (4). Therefore, a distinct sexual dimorphism exists regarding the effects of tobacco smoke exposure, with the time of exposure having a defining impact, as illustrated in Figure 4 and Figure 5.

We have also discussed possible biological mechanisms driving sexual dimorphism. Sex hormones, epigenetics, telomere length, and the mitochondrial response may contribute either independently or concurrently to the sex disparity. Other possible mechanisms not discussed include, genetic polymorphisms, chromosomal aberrations, and the sex chromosomes. As a distinct temporal switch in sex-susceptibility exists, it is possible different mechanisms function at different life-stages. Future research must focus on the timing and intensity of exposures to develop a holistic understanding. The outcomes of subsequent mechanistic studies can inform clinical responses, enabling patient-specific interventions, and more effective treatments.



348 **Figure 4: Schematic summarising molecular mechanisms contributing to sex differences**
349 **in disease susceptibility of offspring in response to maternal tobacco smoke exposure.**
350 ♂ = male specific effect, ♀ = female specific effect.



351
352 **Figure 5: Schematic summarising molecular mechanisms contributing to sex differences**
353 **in disease susceptibility caused by adulthood tobacco smoke exposure.** ♂ = male specific
354 effect, ♀ = female specific effect.

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Disclosures

The authors have nothing to disclose

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