Preconception origins of asthma, allergies and lung function: The influence of previous generations on the respiratory health of our children.

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Running headline: Preconception origins of asthma and COPD

ABSTRACT

Emerging research suggests that exposures occurring years before conception are important determinants of the health of future offspring and subsequent generations. Environmental exposures of both the father and mother, or exposure to disease processes such as obesity or infections, may influence germline cells – and thereby cause a cascade of health outcomes in multiple subsequent generations. There is now increasing evidence that respiratory health is influenced by parental exposures that occur long before conception. The strongest evidence relates adolescent tobacco smoking and overweight in future fathers to increased asthma and lower lung function in their offspring, supported by evidence on parental preconception occupational exposures and air pollution. While this literature is still sparse, the epidemiological analyses reveal strong effects that are consistent across studies with different designs and methodologies. The results are strengthened by mechanistic research from animal models and (scarce) human studies that have identified molecular mechanisms that can explain the epidemiological findings, suggesting transfer of epigenetic signals through germline cells, with susceptiblity windows in utero (both male and female line) and prepuberty (male line).

The concept that our lifestyles and behaviours may influence the health of our future children represents a new paradigm. This raises concerns for future health in decades to come with respect to harmful exposures, but may open for radical rethinking of preventive strategies that may improve health in multiple generations, reverse the imprint of our parents and forefathers, and underpin strategies that can break the vicious circle of propagation of health inequalities across generations.

KEYWORDS Asthma; lung function; overweight; non-genetic heredity; epigenetics.

INTRODUCTION

Asthma and allergies have reached epidemic proportions, impairing the health of over 300 million persons [1]. In a setting with sufficient resources for efficient medical treatment, the mortality of asthma itself is not high. However, asthma and allergies have high morbidity, in particular because they usually start in childhood and thus may impair health over decades. Further, it has been shown that asthma may lead to twenty times higher risk for chronic obstructive pulmonary disease (COPD) in relatively young adulthood [2]. COPD also leads to high morbidity and mortality and is the third most common cause of death globally [1].

Asthma and allergies appear to follow in the footsteps of economic growth, new forms of production, consumption and urbanisation [1]. However, effective preventive measures cannot currently be taken as the causes are not well understood. This is in contrast to COPD, cardio-vascular diseases and diabetes, where important risk factors are well known. For instance, tobacco smoking and other pollutants from occupation, outdoor air pollution and indoor burning of biomass fuel are important causes of COPD. Nevertheless, the factors underlying the vast differences in susceptibility to risk factors such as smoking, and the wide variations in lung function, are not well understood. Impaired lung function is a key feature of COPD, and also a strong predictor of overall morbidity and mortality [3]. Thus, despite decades of research, there are still wide gaps in our knowledge about the causes of asthma in particular, but also about the underlying risk factors for respiratory health and chronic disease in general. A new scientific approach is urgently needed.

The traditional paradigm of dosage – "the dosage is the poison", i.e. that the dose of exposure determines health effects – is being challenged by a paradigm of timing – "the timing is the poison". For instance, an exposure of little importance in adulthood could have devastating effects if occurring *in utero*, in a more susceptible time window. The majority of research and intervention programs are focused on dosage and (relatively) short-term consequences of exposure to risk factors such as smoking and obesity. In recent decades the long-term consequences of exposures to the pregnant mother for the future health of the child she is bearing, has also become firmly established. The early life origins of a range of non-communicable diseases is documented [4-7], including for respiratory health and disease [8-11]. Maternal smoking in pregnancy is an early life exposure that has received extensive attention and that is decreasing in many societies after successful intervention programs. This appreciation of the early life origins of health and disease has contributed to the knowledge basis for "First 1000 days" programs [12] and other health care programs that aim to improve mother and child health across the globe.

Today, another paradigm of timing is gaining scientific acceptance, the paradigm that phenotype may be determined by factors operating *before conception* – not only immediately before conception in preparation for conception and pregnancy, but years before conception [13, 14]. Substantial evidence from animal studies has identified that environmental impacts can be transferred across generations, driven by epigenetic mechanisms that alter gene expression rather than the sequence of nucleotides in the DNA double helix itself [15]. Several epigenetic layers controlling gene transcription are known, including DNA methylation (DNAm), histone modifications that regulate chromatin accessibility, as well as various types of non-coding RNA (ncRNA). Experimental research in animal and cell models has described the molecular basis for the "cleaning" processes that maintain epigenomic stability, and how epigenetic signals can evade these processes and be transferred across generations, first to offspring born from gametes (oocytes and spermatozoa) directly affected by environmental exposure (intergenerational effect), and thereafter to further generations in the absence of continued exposure (transgenerational effects) [16, 17]. Corresponding mechanistic research is extremely limited in humans given the difficulties in studying an outbred population with uncontrolled environmental exposure over long time frames (decades), but rapidly emerging research is already giving quite amazing results. The preconception origins of respiratory health and diseases, and the potential mechanisms that may contribute to transfer across generations are the focus of this review.

Why is preconceptional origins of disease difficult to study in humans?

The key challenge to the study of preconceptional origins of disease in humans is that the human life cycle spans decades. Unlike in experimental animal models, such as rodents, fish and invertebrates, not only are humans exposed to complex environments that vary over time, also the time between generations spans decades. To investigate how parental factors years before conception may influence offspring health and disease, sufficiently accurate data on exposures, outcomes and key covariates for two or more generations is required, ideally with proactively collected biosamples (including gametes) to enable mechanistic studies. In studies with clinical offspring data and biomaterial from offspring, information about parental exposures from their early life and until conception is rarely available. The availability of cohorts with relevant data is greatly increased by using data reported by a family member - offspring data reported by the parent ("does your child born in xxxx have asthma?" [18]), and parent data reported by the offspring ("did your mother smoke when you were a child?" [19]). There are important studies validating data reported by family members [18-21]. None-the-less, the number of studies with prospectively collected data over two or more generations is extremely limited and those with prospectively collected biosamples to investigate underlying mechanisms even fewer.

Statistical analysis of multi-generation data represents another challenge. Experimental studies, in which the parental exposure is controlled by the investigator, are not feasible – as for most research on, for instance, consequences of smoking and obesity, and follow-up of offspring in cohorts subjected to controlled interventions is rarely feasible. Therefore, it is important to use the most forefront methods available to be able to conclude on cause and effect based on observational data [22].

Human observational studies are of crucial importance, precisely because they study real-life scenarios. However, while epigenetic inheritance is considered relatively established in non-human mammals, observations consistent with preconceptional origins of disease in human multi-generation studies are often questioned due to methodological challenges. In order to take the step from showing an association to interpreting this as a causal association, studies are now applying statistical methods for analysing multiple exposures, multiple mediators and multiple outcomes [23-25]. This greatly increases the knowledge that can be obtained from real-life data.

In order to approach causal inference based on observational data confounding needs to be appropriately accounted for. Human observational studies have the major challenge that an exposure of interest, such as smoking, can be related to a variety of other lifestyle and environmental factors, which could possibly be the true causes of the estimated associations. Even though accounting for as much information as possible, "confounding" by unmeasured and/or unknown variables can rarely be excluded. New studies are using approaches to simulate the presence of unmeasured confounding variables [26, 27], in order to enhance valid causal interpretation.

HUMAN STUDIES (table 1)

A new perspective on smoking, occupation and environmental exposures

Tobacco smoking

Smoking has been a main risk factor for respiratory and other chronic diseases over several decades. Finally, smoking is decreasing in the Western world – with one important exception: The group starting smoking before age 15 years is increasing, in boys and girls, and in practically all regions in Europe [28]. Smoking prevention programs have evidently not been successful in this important age group. The dominating emphasis in research on the impact of smoking on health outcomes has been on effects of personal (including *in utero* as a result of maternal smoking in pregnancy) and second-hand smoke exposure on subsequent diseases in adulthood and older age [10,29]. As part of a focus

on early life origins of health and disease, the *in utero* susceptibility period is being targeted in mother- and child healthcare programs across the world.

Now it is time for a new focus for research on health effects of smoking: the study of effects on future offspring. Tobacco smoking represents a mixed chemical exposure containing irritants, carcinogens, and toxins. It is likely that mutagenic substances may influence germ cells as well as somatic cells. This aspect has been overshadowed by the focus on cancer, but with increasing understanding of transfer of epimutations across generations in animal models [14,15,30], the potential influence of an exposure such as smoking on the germline cells and the offspring born to these cells, is gaining new interest. In this paper we will focus on the role of preconception smoking exposure on respiratory health and disease.

For many years, it was believed observed effects of father's smoking on offspring respiratory health represented passive smoking, second-hand smoke to the child. Father's smoking was found to be a relatively weak risk factor for asthma, that usually would reach statistical significance only in large studies and in meta-analyses [31]. However, when asking the research question "What if the father smoked *only before conception* of the child?", Svanes and Koplin found in a landmark paper, a stronger effect on offspring asthma of father's only smoking preconception, than the effect estimates usually reported for father's smoking at any time during childhood [32]. The estimate was even stronger than that found for maternal smoking in pregnancy in the same analysis and in other studies. Exploring different aspects of father's smoking, such as 1) age of starting smoking, 2) time of quitting before conception of the offspring, 3) number of years smoked before conception and, 4) number of cigarettes/cigars/pipes smoked daily, the study highlighted that *the age when father started smoking* was the essential aspect of father's smoking that influenced offspring health. In comparison to age of smoking debut, other aspects showed limited or non-detectable associations with offspring asthma - duration of smoking ≥10 years prior to conception appeared to be of some importance, while intensity of smoking or time of quitting before conception showed no association with offspring asthma [32].

This study was based on analyses of questionnaire data from the RHINE study of Northern Europe, in which study participants reported about themselves and their offspring and family members. In the (mainly) European ECRHS study, based on interview and clinical data, the same conclusion was reached in an analysis using more advanced statistical approaches [33]. A similar conclusion was reached in an analysis of the TAHS study from Australia, based on exposure data obtained by following the study participants since childhood, and offspring outcome data reported by the parents [34]. A study based on Health Survey of England compared asthma in biological and non-biological offspring as associated with parental smoking habits, and reached a similar conclusion [35]. The studies' findings, that father's early onset smoking is associated with asthma [32,33], are supported by a study of offspring lung function as outcome [36]. This study is based on data from two linked cohorts, the RHINESSA study with offspring data and the ECRHS study with parental data, including lung function measurements in both generations. In order to disentangle only preconception from also postnatal smoke exposure, analyses of the Health Survey of England [35] and the RHINE study [32] did address only preconception smoking with large statistical power; both studies found this to be a consistent risk factor for offspring asthma, as strong as maternal smoking in pregnancy. Thus, in several studies, from different countries and using different study designs, father's smoking onset before age 15 years has been confirmed as an important risk factor for offspring respiratory health.

The impact of father's smoking on offspring health cannot possibly be studied in experimental studies in humans, and important conclusions on cause and effect need to be based on observational studies. Varying statistical approaches are used in the studies cited above, and Accordini et al. has applied methods aiming to ascertain causal inference based on observational data [33,36]. Using simulation techniques, unknown confounding, such as genetic confounding or confounding by factors related to early onset smoking like low social class, was found to only play a minor role for the associations of father's early onset smoking with asthma or low lung function in their offspring.

Regarding mother's smoking and offspring lung health, a role of mother's preconception smoking o offspring asthma or lung function is not identified, as investigated for only preconception smoking [32] and for early onset smoking [33-34, 36]. For comparison, the well-known effects of maternal smoking in pregnancy is replicated in these studies. The authors speculate that this could be due to sex differences in germline cell development between males and females. While the primordial germ cells in females undergo maybe 4-5 cell divisions on their way to mature oocytes, the primordial germ cells in males undergo ten times as many cell divisions in their development to mature sperm; this could render the male germ cells more susceptible to the surrounding environment in the prepubertal developmental window. However, smoking may not serve as the best model to investigate effects of prepuberty exposure in women, since prepuberty is earlier in girls than in boys, and girls would rarely start smoking in the prepuberty age. One could also question whether the effect of mother's smoking in pregnancy could overshadow a very minor effect of preconception smoking exposure –but, to be noted, the associations with father's early onset smoking are strong and consistent.

On the other hand, mother's exposure to smoking while *in utero* -grandmaternal smoking when pregnant with the mother –has been discovered in several analyses to be related to lung health in her offspring, independent of later active smoking in the mother. In most studies, associations have been found for mother's exposure *in utero* - maternal grandmother smoking [33,37-40], but some studies find effects of father's exposure *in utero* - smoking in the paternal grandmother [32,36,41]. Various studies reach consistent conclusions, in spite of different methodology and data sources - registry data as well as cohort data, and from different geographical settings. The findings are biologically plausible, as smoking in pregnancy would affect not only the somatic cells of the foetus but also the primordial germline cells of the foetus, present from very early in pregnancy. Intrauterine life is known as another susceptibility window in germ cell development, in both females and males, with several stages of extensive epigenetic reprogramming.

Overall, there is quite solid substantiation that paternal smoking starting in early adolescence may contribute to cause low lung function and asthma in future offspring. Similar results cannot be found for maternal exposure to smoking in prepuberty, while effects of exposure *in utero* – grandmaternal smoking - is consistently related to offspring asthma in several. Thus, the evidence from human epidemiological studies suggests preconception smoking exposure might be particularly important in two susceptibility windows, future parents' exposure *in utero* and future father's exposure in prepuberty. This is the best evidence from human studies until now of a preconception risk factor for respiratory health and disease.

Occupation and air pollution

Air pollution and several occupational exposures reflect mixed chemical exposures that are relevant for respiratory health and disease, and for a range of chronic diseases. Previous literature involve exposure during pregnancy or during the lifetime, but there are also a few recent studies exploring preconception exposure as related to respiratory health outcomes in future offspring.

It is challenging to obtain information on parental exposure to air pollution from their early life to young adulthood and conception. Kuiper et al. used geocoding of parental residential addresses to define parental exposure to five air pollutants, NO_2, $PM_{2.5}$, PM_{10}, black carbon and ozone [42]. The study concluded that parental air pollution exposure before age 18 years was associated with higher risk for asthma and allergy outcomes in their offspring.

Regarding preconception occupational exposures, young adulthood rather than prepuberty is the relevant time window of exposure. Father's welding before conception was found to be associated with higher asthma risk in offspring in the large RHINE cohort (24,168 parent-offspring pairs); welding 10 or more years before conception was associated with a doubled asthma risk, while father's welding starting after birth of the child showed no influence on asthma in the child [32]. Welding

gives a mixed chemical and physical exposure, and was quite common for the parental generation in the Göteborg and Bergen study centres. In a study of parental exposure to four groups of asthmogens defined by an asthma-specific job exposure matrix (JEM), Pape et al. in an analysis of data from RHINESSA/ECRHS/RHINE in general found little evidence that offspring asthma was related to such parental exposure. There was one exception, maternal exposure to "allergens and reactive chemicals" before or after pregnancy was associated with offspring asthma [43]. In the same cohorts, Tjalvin et al. investigated the specific job exposure category "indoor cleaning (cleaning products, detergents, disinfectants)", encompassing exposure in 21 occupations such as cleaners, nurses, midwives, cooks, hairdressers, etc. [44]. The study included only the maternal line (3318 mother-offspring pairs), in which such jobs were frequent. The authors found that childhood asthma and wheeze were more common in offspring of mothers with occupational exposure to indoor cleaning agents starting before conception, or around conception and pregnancy, while no association was found if mother started in such jobs after the child was born.

In summary, these results on preconception exposure to occupation and air pollution support the studies of tobacco smoking, overall identifying a role for airborne pollutants on future offspring's respiratory health. A preconception susceptibility window in childhood/adolescence was identified with regard to air pollution. The young adult time window is more relevant for occupational exposure than the prepuberty time window. It is urgent to research more extensively the potential impact of occupational exposures on offspring health; today documentation with regard to potential germ cell effects is not required when new substances are introduced in working life.

A new perspective on overweight and metabolic disadvantage

Overweight

In a one-generation perspective, there is solid evidence that overweight plays a role in asthma and in a range of other chronic diseases. A disadvantageous impact from mother's obesity and metabolic disadvantage during pregnancy is also well-known. Recent research describes mechanisms by which overweight and a metabolic disadvantageous environment could also influence germline cells and thereby the next generation [45-48].

Three recent studies show intriguing evidence that overweight in future fathers before conception – not immediately before conception, but in the future father's childhood/puberty – is a risk factor for asthma [49, 50] and lower lung function [51]. One mainly North European two-generation study [49] was based on data for 6347 adult offspring investigated in the RHINESSA study, with parental exposure data from the RHINE and ECRHS studies. A second study [51] based on the same cohorts included 929 adult offspring with clinical data on measured lung function in the offspring as well as a parent. A third study [50] was based on the Tasmanian Longitudinal Health Study in Australia, in which 1822 participants of a cohort that has been followed from age 7 years until the sixties provided data about asthma in 4208 offspring. In this cohort, the parental exposure was defined by BMI trajectories derived from measurements of height and weight from age 7 to 15 years, while offspring asthma was reported by the parents. In the RHINESSA/RHINE/ECRHS cohorts, the parents filled in a validated tool consisting of nine sex-specific body silhouettes – this was used to define overweight in childhood (age 8 years), adolescence (voice break/menarche), and young adult ages. Statistical analyses included counterfactual-based group level (multinomial) logistic regression mediation analyses and adjusted multinomial regressions; the lung function analyses also included simulation analyses to address unknown confounding.

The effects were independent, direct effects, and not mediated by offspring's own overweight – this supports a mechanism in terms of germ cell influence, while genetic inheritance seems less likely. The finding of the simulation analyses, that confounding played a minor role, speaks in contra of genetic confounding [49, 51]. The two RHINESSA analyses compared the role of parental overweight in different time windows, and no effect was found if the father only became overweight after voice

break [49, 51]. The study with the largest number of parent-offspring pairs further identified that father's overweight with onset by voice break but after age 8 years, was the main risk factor [49]. This supports the interpretation that a disadvantageous metabolic environment may be particularly harmful during the developmental phase when the sperm precursor cells undergo extensive epigenetic reprogramming on the way towards mature sperm. The findings that only paternal and not maternal childhood/prepuberty overweight was related to poorer offspring lung health [49-51], are consistent with findings on paternal prepuberty smoking exposure, and agrees with an interpretation in terms of sex-specific germ cell influence of metabolic disadvantage. The lung function analysis [51] mainly identified an effect on lower lung function in sons, not daughters; such sex-specificity is considered typical for some mechanisms for transmission of epigenetic traits across generations. An intriguing finding in this analysis was that father's prepubertal onset overweight also led to lower adult height in sons [51]. This general effect on growth supports a broad role of metabolic disadvantage on developing sperm – a general rather that disease/organ-specific effect would be expected.

Thus, the literature finds that father's overweight in childhood/adolescence appears to be an important risk factor -probably a causal factor- for asthma and low lung function. No such effect could be identified for mother's overweight in childhood/adolescence. The consistency of the results in the studies is amazing given the different study designs, different assessments of parental early onset overweight (through height and weight measured multiple times *versus* a body silhouette tool), different outcomes (personal *versus* parental reporting of asthma, measured lung function), and different analytical approaches, including very advanced approaches aimed at establishing causal relationships based on observational data. These epidemiological findings raise several hypotheses about mechanisms that will be discussed below.

Other hormonal-metabolic factors

The literature is sparse with regard to a potential role of other preconception hormonal-metabolic factors in determining respiratory health. Two studies address maternal use of oral contraceptive pills before conception as related to offspring childhood asthma and allergies. These reach slightly different conclusions: An analysis of 980 children-mother pairs in the Tokyo-Children's Health, Illness and Development Study (T-CHILD) found more wheeze, asthma, and rhinitis in offspring at age 5 years [52] related to maternal oral contraceptive use. In the Norwegian Mother and Child Cohort Study (MoBa), use of oral contraceptives the year before pregnancy was analysed in up to 60,225 mother-offspring pairs; no association between use of combined pills (oestrogen and progestin) and asthma or wheezing in offspring before 3 years old could be identified, but progestin-only pills were weakly related to wheeze in offspring at 6–8 months [53].

A new perspective on infections

The relationship between infections and non-communicable diseases is investigated and discussed from a range of perspectives, and the research in this area has increased exponentially with the current focus on the microbiome as well as specific infections. Regarding lung health and disease, the role of infections has in particular been discussed with regard to the Th1/Th2-type cytokine paradigm. The hypothesis that the influence of infectious agents or microbial diversity on immunological maturation may play a role for the increasing prevalence of allergies [54] is well established. However, recent studies in animals are opening for a new perspective – there are experimental results suggesting that infections may induce immunological changes that can be transmitted across generations [55-58].

The potential health effects in offspring from parental infections before conception in humans is a field with very scarce literature. Two human studies have investigated effects of parents' infections

on offspring respiratory health outcomes. In a Norwegian cohort of 171 parents born 1945-1972 and their 264 offspring born 1969-2003, seropositivity to *Toxocara* was associated with offspring asthma and allergies, following a sex-specific pattern. This was consistent also when adjusting for seropositivity to *Toxocara* in the offspring, pet keeping and other potential confounding factors [59]. In an analysis based on Norwegian registry data including 2965 young adult offspring from 1790 parents with a history of tuberculosis, parental tuberculosis in childhood (*versus* adulthood) was found to be associated with the use of asthma medication in future offspring, more pronounced in the maternal line [60].

Thus, the perspective that the profound immunological responses from some infections can influence immunity across generations so far have very limited supporting evidence, but enough to justify a major scientific effort to better understand the preconception origins of our immune characteristics.

EPIGENETIC MECHANISMS AND ANIMAL STUDIES

Epigenetic mechanisms in sperm

For decades, it was assumed that a father's contribution to the health of his offspring was exclusively anchored in his DNA sequence or rooted in his social behaviour. More recently it has been proposed that intergenerational transport of paternal information involves epigenetic changes in sperm cells. The differentiation of spermatogonia to spermatocytes, including change in RNA types [61, 62], takes place within the testes (Figure 1). The immature spermatocytes then mature stepwise in the epididymis [63], a highly segmented organ with specific microenvironments critical for sperm maturation [64]. During puberty, primordial germ cells develop into spermatogonia, which develop into mature spermatozoa. Therefore, environmental stresses during prepuberty can have long-lasting impacts on the epigenetic make-up of mature sperm cells.

The epigenetic equipment and regulation of male gametes differs in many ways from those of somatic cells. During spermatogenesis, the development of spermatozoa from spermatogonial stem cells, the whole genome of the round haploid spermatids is initially packaged by histones, just as it is in somatic cells [65]. These are replaced in late-stage spermatids in a complex multistep process, involving histone modifications and intermediate small basic proteins by protamines, which eventually results in a tightly packed and silenced genome until fertilization (Figure 2). Still, around 1-2% and ≈10 % of the histones are estimated to escape protamination in mature sperm in mice and humans, respectively, and may influence gene expression in early embryonic development. As for DNA methylation, this is generally erased during embryonic germ cell development in the primordial germ cells once they migrate to the genital ridge [66-68]. Methylation is then re-established as male and female gametes differentiate with sex-specific methylation at imprinted genes and other loci (65]. After fertilisation, almost all epigenetic paternal marks are deleted first and quickly, followed by a slower elimination of maternal marks [69-71]. Nevertheless, certain regions, such as imprinted genes or some transposons, survive this erasure in both sperm and egg genomes. Whether environmentally induced changes in parental DNA methylation marks can also escape global demethylation, is a matter of current debate. In murine models there is limited evidence for this and observations are restricted to epigenetic mosaics (reviewed in [72]) for retrotransposon activity.

Sperm cells carry a cargo of several classes small non-coding RNAs (sncRNAs) that can act as vectors of epigenetic information by regulation of gene expression in the early embryo. Different from DNA methylation, sncRNAs are not erased upon fertilization and are therefore more tractable. These undergo major proportional and pattern changes during the biogenesis and maturation of spermatozoa [73]. The most well-known classes are microRNAs (miRNA), tRNA-derived small RNAs (tsRNAs) and Piwi-interacting RNAs. MicroRNAs regulate gene expression by incomplete binding to messenger RNAs (mRNA), such that one microRNA can regulate hundreds of mRNA targets and one

target can be addressed by many microRNAs, resulting in complex regulatory networks. If transferred to the embryo they could directly influence the expression of genes involved in developmental processes or act indirectly by addressing genes controlling DNA methylation or histone modifying enzymes. tsRNAs are small fragments of RNAs of defined lengths that are generated by cleavage at specific sites from the tRNA trefoil (reviewed in [74]) at least five of which are present in human sperm [75]. They exert pleiotropic epigenetic functions by regulating histone expression [76], inhibiting mRNAs similar to microRNAs [77], and posttranslational gene regulation [78]. The amount of tsRNAs gradually increases during maturation of sperm during their way from the caput to the cauda epididymis, such that mature sperm contain a high proportion of tsRNAs [62, 79]. Vice versa, Piwi-interacting RNAs are abundant in the testis during early spematogenesis and decline with increasing differentiation and maturation [80]. Their main task is thought to be to silence transposons, i.e. mobile DNA elements that can change their position in the genome to maintain genomic stability. Importantly, in a number of animal models, experimental evidence for their role in transmission of information between generations has been provided [81]. For example, Gapp et al. demonstrated that traumatic stress in early life altered mouse sperm miRNA expression and resulted in behavioural and metabolic responses in the progeny. The phenotype of the progeny could be recapitulated by injection of sperm miRNAs into fertilised oocytes in the absence of exposure of the parental generation to traumatic stress [82].

Regarding the evidence from animal models that paternal exposures can influence the sperm epigenome and thereby the offspring phenotype, the currently most plausible transmission route is via sncRNAs in sperm [57]. However, even in highly standardised animal models, individual specific sncRNAs and their pattern vary. Reasons for this may be different techniques in the analysis of sncRNAs, differences in diet composition in nutritional studies, but also distinct microbial colonisation in different animal facilities. Further, it must be clarified to what extent, and in what proportion, the embryo and the placental unit are each controlled by paternal factors. Finally, a study using mice on a low -protein diet that were either devoid of seminal fluid or of sperm, elegantly demonstrated that the seminal fluid can influence vascular function until the F2 generation [83]. In addition, the seminal microbiome may play a role. Therefore, in future studies it will be important to address seminal fluid as well as spermatozoa.

Tobacco smoking and chemical exposures

In humans and animals, smoking has been associated with increased oxidative stress in sperm cells [84]. Artificially induced oxidative stress in bovine spermatozoa induced oxidative stress in the spermatozoa, lowered the physiological DNA demethylation of the paternal genome in the early zygote and led to reduced blastocyst formation. Thus, paternal smoking could potentially affect the expression of crucial developmental genes by distorting the epigenetic reprogramming of the zygote [85]. Sensitivity to oxidative stress of sperm cells by smoking was also shown in a murine model, where exposure to “active” smoking resulted in DNA methylation changes [86]. Mice with a deletion of nrf2, a gene centrally involved in the counter-regulation of oxidative stress, showed the same sperm DNA methylation changes in the absence of cigarette smoke as observed in smoked mice. In contrast to highly hyper- or hypomethylated genomic regions that recovered from smoking-induced changes within 28 days, intermediate DNA methylation did not recover up to 171 days after smoking cessation. Since murine sperm development and maturation in testis and epididymis take only 30 days, the results suggest that smoking prior to conception induces permanent epigenetic changes. These may be routed in testicular spermatogonia from which persistently altered sperm develop.

Changes in spermatozoal DNA methylation can also be induced indirectly via the maternal line as shown in a rat model where dams were exposed to subcutaneous nicotine [87]. Sperm from male F1 offspring had differentially methylated regions that were more abundant in gene bodies than in promoter regions and were also located near genes that control nicotine response and lung

development. An earlier study in the same animal model demonstrated that maternal nicotine exposure resulted in poorer lung function with increased airway resistance and decreased compliance in the F1 to F3 generations [88, 89]. Epigenetic reprogramming of spermatozoa may thus represent a mechanistic link by which lung development is transgenerationally affected.

One study found that intraperitoneal nicotine resulted in hypermethylation of a dopamine transporter in sperm, which was also hypermethylated in the brains of the offspring [90, 91]. Although the mechanisms remained unclear, the similarity of epigenetic changes in fathers and offspring was striking. Many animal models focus on sperm DNA methylation to reflect the epigenetic machinery. This study found that nicotine induced DNA hypermethylation in the CpG island shore region of *mmu-miR-15b* in spermatozoa and in F1 mouse brains which targeted WNT4, thus, connecting two different epigenetic mechanisms [90].

To date, only one murine study has investigated sperm miRNAs after inhalation smoke exposure in the key puberty window [92], demonstrated to be important in human epidemiological studies [33, 36, 93, 94]. While the counts and morphological integrity of the spermatozoa, as well as the proliferation of spermatogonia of smoke-exposed fathers remained intact, 13 miRNAs were upregulated and 32 downregulated and were predicted to target mRNAs associated with lung and early embryo development. Offspring of pubertal smoke-exposed mothers had lower body weights, but offspring from pubertal smoke-exposed fathers tended to higher body weight gain. These findings corroborate with existing epidemiological data, on father's prepuberty smoking as related to offspring obesity [95,96].

Beyond smoking, exposures to wildfires appears to be increasing. Male mice exposed to smoke generated from dried Douglas fir tree needles for forty days, showed 2117 differentially methylated regions in sperm that mapped to genes involved in developmental processes [97]. Potential effects on offspring were not addressed. The pollutant dioxin operates by activating the aryl hydrocarbon receptor (AHR) which is activated from smoking and a range of pollutant, and also plays an important in immune development and differentiation. One murine study found that dioxin influenced cellular immunity across three generations, also altering response to influenza A virus [98], thus, linking exposure to a pollutant to an immunological response in the next generations.

In summary, animal models have provided some insight into the mechanisms underlying inter- and transgenerational effects of tobacco smoke by showing remodelling in the sperm epigenetic landscape. However, a true cause-effect relationship for triggering respiratory disease is still lacking. This would require targeted manipulation of transmitted paternal epigenetic information in the early zygote, like that demonstrated for other exposures in animal models [81]. Further, in the majority of studies to date, subcutaneous or oral routes were used to administer cigarette compounds, which does not correspond to human smoking.

Diet induced obesity

Intergenerational studies

Paternal pre-puberty overweight was found in human studies to increase the risk for asthma and low lung function in offspring [49-51]. One could postulate that this could be explained by parental obesity affecting epigenetic markers in sperm cells that influence early embryogenesis and ultimately the phenotype of the next generation.

Several animal models of high-fat diet induced obesity have been used to study a potential connection between altered sperm cell DNA methylation marks and molecular events in offspring. For example, a high number of differentially methylated regions (DMRs) was seen in the sperm cells of obese male rats, and these DMRs were predicted *in silico* to affect genes controlling embryonic growth such as Wnt, Hedgehog, TGF-beta, and Notch [99], which are also central to lung development. These signalling pathways were also dysregulated in resorbed, i.e. non-surviving,

embryos sired by obese fathers, suggesting dramatic effects on the embryo of deregulated transferred sperm cell DNA if methylation patterns are not properly re-set in fertilisation. This indicates that DNA methylation may be a prominent vector of epigenetic information in sperm cells. Others did not see changes in sperm methylation in obese males, but an enrichment of histone 3 in sperm of obese male mice near genes and at more distance to gene bodies of H3K4me1 - a marker for active transcription [100].

Imprinted genes such as the insulin-like growth factor-2 (Igf2)-H19 locus are expressed in offspring in a parent-of-origin allele specific manner, i.e. depending on whether they are inherited from the mother or the father, and these can escape demethylation in the early embryo. The gene igf2 is paternally imprinted and controls embryonic development, while H19, - a precursor of various microRNAs -, is maternally imprinted. In mice, a paternal high-fat diet led to hypomethylation of the Igf2/H19 locus in sperm cells [101]. Hypomethylation of Igf2/H19 was also seen in the liver of F1 offspring, suggesting that a paternal high-fat diet may alter the epigenome of sperm cells and pass it on to the offspring. Similarly, paternally expressed genes (Pegs) are highly expressed in the placenta to control the transport of nutrients to the embryo and foetus. Three of nine Pegs quantified by qPCR were downregulated in murine placentas of female mice mated to obese males, one of which was hypermethylated at CpG sites in its promoter region [102]. The same gene, Peg9, was also a hypermethylated in sperm cells, suggesting transmission of paternal exposures to maternofoetal tissues. Different from the study above [101], no changes were seen for igf2r and H19.

Intergenerational studies in mice also found altered miRNAs in sperm cells of fathers with high-fat diet induced obesity [103]. When mated to lean females, the F1 blastomeres had a highly sex-specific transcriptional response with only upregulated genes in male F1 and predominantly downregulated genes in female F1. Of note, close to three quarters of F1 female blastocyst genes were predicted targets of the identified paternal sperm miRNAs. Vice versa, two downregulated paternal microRNAs were predicted to target 18 transcripts in male F1, thus explaining their upregulation. Although formal proof that paternal miRNAs indeed regulate gene expressions in the pre-implantation embryo was lacking, the results are concordant with the hypothesis of paternally mediated programming. Beyond miRNAs, specific tsRNAs [62,104-108] and long-noncoding (lnc) RNAs have been implicated in diet-induced paternal programming, where paternal and male offspring sperm lncRNAs were highly correlated and predicted to control pathways involved in growth [104].

To substantiate that environmental modifications of snclRNAs in sperm can act as epigenetic transmitters of disease risk to the next generation, sperm-derived snclRNAs have been injected into zygotes that were then implanted into foster dams. In a seminal paper, Chen et al. demonstrated that injection of tsRNA fractions from sperm of obese male mice into normal zygotes recapitulate metabolic traits in the F1 offspring that are unrelated to DNA methylation at CpG-enriched regions [105].

A major advantage of animal models is the possibility to test interventions. Several studies have shown that for example exercise in obese male mice can normalize or at least modify sperm sncRNAs and also improve metabolic characteristics in offspring [109-111]. There are not, to our knowledge, similar experimental studies investigating offspring respiratory health outcomes related to paternal obesity/overweight.

Transgenerational studies

Few studies have investigated how paternal obesity is transmitted transgenerationally[112-115]. Although none of these animal studies looked at offspring lung health, they provide mechanistic insight into the processes that may operate in humans [107,112,113,115,116]. Raad et al. fed mice over five generations, from F0 to F4, with a high-fat high-sugar diet. The mice showed progressively higher body weight per generation up to the fourth generation and systemic inflammation from the

third generation onwards, suggesting that environmental experiences can be passed down even to great-grandchildren at least in mice. RNA sequencing of sperm from the exposed F0 and F4 generations and control animals showed that the number of deregulated small RNAs decreased significantly from the F0 to the F4 generation. Nevertheless, most of the deregulated F4 small RNAs were already present in the F0 generation. Injection of total sperm RNA from diet exposed F0 and F4 into zygotes yielded heavier male offspring than injection of RNA from controls, showing that sperm RNA was causally linked to the offspring phenotype [117]. Others set male mice on a "chronic" (≈7 months) versus a shorter (≈2 months) high-fat diet. Sons of chronically exposed fathers had changes in various classes of sncRNAs in their sperm cells, most prominently for two microRNAs, which was not seen in the F2 generation. In contrast, grandsons of fathers receiving the diet for a shorter period had several microRNAs changed in their sperm [112]. Thus, the numbers of differently expressed sperm sncRNA varied significantly in each generation and for each type of ancestral exposure, suggesting considerably plasticity of the sperm epigenomic response to environmental challenges.

Overall, there is evidence from animal studies that high-fat diet induces molecular changes that can be transferred through sperm to offspring, and also some evidence of transgenerational transfer. These studies do not directly address lung health outcomes in the offspring, but mechanisms that are likely to or known to influence airways/lung pathology, such as adverse metabolic conditions. This mechanistic literature thus gives support to the notion that paternal preconception overweight could impact offspring phenotype, through molecular processes transferred, among other, through sperm.

Infections

A study of preconception helminth infection in female mice, cleared before mating, revealed persistent alterations in immunological characteristics in the pups of exposed mothers. Immunological changes from the infection in the mother was partly transmitted to offspring through breastmilk [55]. Encouragingly, in this study an infection *enhanced* immune competence in the next generation. Another study found effects on the microbiome as well as immune characteristics in the pups of mothers infected with a helminth that was cleared before conception [56]. Regarding the male line, a study found that infection with *Toxoplasma gondii* infection in male mice caused small RNA profile changes in their sperm and led to subsequent altered behaviour in the offspring [57]. Another study found that sepsis in mice induced alterations in sperm methylome and dampened immune responses in male offspring [58].

Thus, animal studies give a complicated picture suggesting that an infection may influence offspring immunity by transfer of immune cells in breastmilk, by impact on microbiome and through changes to epigenetic signals that are transferred across generations through sperm. This scarce literature supports the even more limited human studies, to advocate future research on transfer of immune competence across generations and the potential relevance to better understand the preconception origins of asthma and allergies.

EVIDENCE FOR EPIGENETIC MECHANISMS IN HUMAN STUDIES

As noted above, the identification of potential trans- and intergenerational epigenetic inheritance in multi-generational human studies is difficult. None-the-less, observational studies have suggested that, as for other mammalian species, transgenerational effects may exist that cannot easily be attributed to cultural and/or genetic inheritance [118, 119]. There is much more limited mechanistic evidence in human studies, not least due to the difficulty of sampling the appropriate tissue at key time points. However, two major sources of evidence suggest that mechanisms similar to those described above in animal models may also operate in humans. Firstly, a number of studies demonstrate that epigenetic profiles of human spermatozoa vary according to environmental exposures [15]; environmental toxicant exposures such as phthalates, alcohol, flame retardants and

chemotherapy treatments, as well as exercise, are all correlated with sperm DNA methylation changes. Changes in miRNA expression in sperm have been seen after exposures such as early life stress [120].

Of relevance to offspring respiratory health, both paternal obesity and tobacco smoke exposure have been shown to be associated with specific sperm DNA methylation patterns [91,121-123] and sperm miRNA content [122,124,125]. Sperm of men exposed to cigarette smoke have also been shown to have an altered ratio of histones to protamines [126].

There are limited studies of epigenetic profiles of offspring in relation to parental pre-conceptional exposure. Studies of the Dutch famine of 1944-45 observed that offspring born during the famine were smaller compared to those born the year before the famine, and that they had increased risk of metabolic and cardiovascular disease in adulthood [127]. In offspring, lower DNA methylation of the imprinted IGF2 gene was observed in famine exposed individuals compared with their unexposed, same-sex siblings [128]. A genome-wide analysis showed that prenatal malnutrition-associated differentially methylated CpG sites preferentially occurred at regulatory regions and map to genes enriched for differential expression during early development [129]. These associations were specific for periconceptional exposure, reinforcing that very early mammalian development is a crucial period for establishing and maintaining epigenetic marks, even though this may not involve epigenetic inheritance.

More recently, in the RHINESSA cohort, association between paternal pre-conceptional cigarette smoke exposure and offspring DNA methylation in blood has been observed. In a pilot study several differentially methylated regions were identified to be associated with paternal smoking, annotated to genes involved in innate and adaptive immunity, fatty acid synthesis, development and function of neuronal systems and cellular processes [130]. More recently, in a much larger analysis of the RHINESSA cohort, Kitaba et al. demonstrated that father's preconception smoking, particularly in puberty, was associated with offspring DNA methylation and that these changes were different to those induced by both maternal smoking in pregnancy and personal lifetime smoking [131]. This study suggests that paternal preconception smoking induces epigenetic changes in offspring, which provides a plausible mechanistic explanation for the association of paternal smoking with offspring respiratory health.

CONCLUSIONS

Starting parenting already in childhood?

The literature discussed above suggests that it is highly likely that respiratory health and disease in part originate long before conception. The literature on the role of the preconception for health and disease is very sparse, compared to the vast literature on the early life origins of health and disease with focus on the *in utero* and perinatal exposure period. However, the epidemiological observations on preconception origins of asthma and lung function are from quite strong analyses, the results are consistent across studies with different designs and methodologies, and the findings are supported by mechanistic research. Molecular research in animal models and (scarce) human studies provides evidence of biological mechanisms that can contribute to explain the epidemiological findings, mainly pointing to transfer of epigenetic signals through germline cells with vulnerability windows in utero (both male and female line) and prepuberty (male line). Seen together, there is reasonable evidence, in particular regarding airborne pollutants, that the preconception environment and exposures of parents to be are important for respiratory health in the next generation(s).

The paradigm of timing appears to be gaining an extended meaning: male prepuberty stands out as a potentially very important period with regard to the health of future offspring. In the literature presented above that explores several potential susceptibility windows, exposure starting before age 15 years or between childhood and voice break in boys show astonishingly strong associations with

outcomes in future offspring, comparable to exposures *in utero*. Regarding the prepuberty susceptibility window, the pattern in males appears to be quite different from that of females. Given the very different development of the germ cells into mature oocytes and sperm, this supports an interpretation in terms of transfer of environmental influences through germ cells that is sex-specific and dependent on the developmental stage of the gametes.

If the environmental influences are transferred across generations through alterations to the epigenetic make-up of the germ cells, it seems plausible that the effects on health and disease are broad and not specific, e.g. for respiratory outcomes. This is beyond the scope of this review, but appears to be true. Adult height represents a general aspect of growth, and was identified to be one consequence of father's prepuberty overweight [51]. There are further human and animal studies demonstrating associations of various ancestral exposures with phenotypic and molecular outcomes [132-135].

An exposome approach, including the preconception exposome [136], is very likely needed in further research, to try to capture the complexity of multiple environmental factors, lifestyles and behaviours that may impact the germline cells during their development and other components relevant for transfer of environmental effects across generations.

Perspective

The understanding of a preconception origins of health and disease - that our lifestyles and behaviours may influence the health of our future children – represents a new paradigm in research on respiratory health and disease, and a new perspective on pathophysiology and health in general. This opens for a new paradigm in intervention, "killing two (at least) birds with one stone". This could open for highly efficient preventive programs, for example, an intervention aimed at promoting healthier choices in children approaching puberty may improve lifelong health in the individuals themselves as well as in their future offspring – and possibly further generations. Thereby more health can be gained from less resources, a sustainable approach to public health.

Inequalities in health are known to be propagated across generations. There are several possible mechanisms in addition to genetic inheritance, including transfer of behaviours and maybe even microbiome across generations. Epigenetic inheritance of environmentally induced changes to the germ line cells adds a different perspective that opens for identifying potentially vulnerable groups and develop new strategies to counteract a vicious circle of transferring vulnerability and health inequalities across generations.

Table 1. Epidemiological studies of preconception exposures related to asthma, allergies and lung function.

Reference	Study, country	Major findings
Smoking, occupation and environmental exposures		
Svanes et al. Int J Epidemiol 2017 [32]	RHINE, Northern Europe	Father smoking starting <15 years, father's welding preconception, and paternal grandmother's smoking; all strongly associated with offspring asthma
Accordini et al. Int J Epidemiol 2018 [33]	ECRHS, Europe (Australia)	Father smoking starting <15 years, and maternal grandmother's smoking in pregnancy, appears to cause offspring asthma
Liu et al. Eur Respir J 2022 [34]	TAHS, Australia	Father smoking starting <15 years associated with offspring asthma
Potts et al. Eur Respir J 2019 (abstract) [35]	Health Survey of England	Father smoking starting <15 years associated with offspring asthma in biological offspring, not in non-biological offspring
Accordini et al. Eur Respir J 2021 [36]	RHINESSA (offspring), RHINE/ECRHS (parents	Father smoking starting <15 years, and paternal grandmother's smoking in pregnancy, appears to cause lower lung function in offspring
Li et al. Chest 2005 [37]	CHS, USA	Maternal grandmother's smoking in pregnancy associated with offspring asthma
Lodge et al. Clin Exp Allergy 2018 [38]	NSC, Sweden	Maternal grandmother's smoking in pregnancy associated with offspring asthma
Bråbäck et al. Pediatr Allergy Immunol 2018 [39]	Swedish national health registry-based cohort	Maternal grandmother's smoking in pregnancy associated with offspring asthma
Magnus et al. Thorax 2015 [40]	MoBa, Norway	Maternal grandmother's smoking in pregnancy associated with offspring asthma
Miller et al. Chest 2014 [41]	ALSPAC, UK	Paternal grandmother's smoking in pregnancy associated with offspring asthma
Kuiper et al. Int J Environ Res Public Health 2020 [42]	RHINESSA (offspring), RHINE/ECRHS (parents)	Parental air pollution <18 years associated with offspring asthma and allergy outcomes
Pape et al. Int Epidemiol 2020 [43]	RHINE/ECRHS (parents), RHINESSA (offspring)	Parental preconception job exposure to asthmogens not clearly associated with offspring asthma
Tjalvin et al. J Allergy Clin Immunol 2021 [44]	RHINE/ECRHS (parents), RHINESSA (offspring)	Mother's cleaning/disinfectant exposure preconception associated with offspring asthma
Overweight and metabolic disadvantage		
Johannessen et al. J Allergy Clin Immunol 2020 [49]	RHINE/ECRHS (parents), RHINESSA (offspring)	Father's overweight onset before voice break appears to cause offspring asthma
Lønnebotn et al. Nutrients 2022 [51]	RHINE/ECRHS (parents), RHINESSA (offspring)	Father's overweight onset before voice break appears to cause lower lung function in offspring

Bowatte et al. J Allergy Clin Immunol 2022 [50]	TAHS, Australia	Father's higher BMI trajectory at ages 7-15 years associated with offspring asthma
Yamamoto-Hanada et al. Allergol Int 2016 [52]	T-CHILD, Japan	Mother's oral contraceptive use associated with offspring asthma and allergies
Hancock et al. Pediatr Allergy Immunol 2011 [53]	MoBa, Norway	No association of mothers combined contraceptive pills with offspring asthma, weak association of progestin only pills with infant wheeze
Infections		
Jögi et al. Clin Exp Allergy 2018 [59]	Bergen ECRHS and RHINESSA, Norway	Parental helminth *Toxocara* seropositivity associated with more offspring allergies
López-Cervantes et al. Int J Tuberc Lung Dis 2022 [60]	Tuberculosis registry (parents), prescription registry (offspring), Norway	Parental childhood-onset tuberculosis associated with offspring asthma

CHS: Children's Health study in southern California; ALSPAC: Avon Longitudinal Study of Parents and Children; MoBa: Norwegian Mother and Child Cohort Study; RHINE: Respiratory Health In Northern Europe; ECRHS: European Community Respiratory Health Survey; NSC: Nationwide Swedish Cohort; RHINESSA: Respiratory Health In Northern Europe Spain and Australia; T-CHILD: Tokyo-Children's Health, Illness and Development Study

FIGURE LEGENDS

Figure 1. The differentiation of spermatogonia to mature sperm, and proportional changes in classes of small non-coding RNAs during the differentiation [61, 62].

Figure 2. Alterations in epigenetic characteristics from mature sperm with tightly packed and silenced genome, to demethylated paternal DNA in the zygote after fertilisation.

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Conflict of interest

The three authors declare no conflict of interest related to the topic of the review

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