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A POSSIBLE MECHANISM FOR LEPTIN'S ROLE IN LUNG FUNCTION INDEPENDENT OF BODY MASS INDEX: INVESTIGATING LEPTIN'S GENOTYPE, DNA, METHYLATION, AND SERUM PROTEIN LEVELS

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Dedication

To the parents and children who participated in the Isle of Wight Birth Cohort Study and the research team that collected the data and made this work possible.

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ACKNOWLEDGMENTS

There are many people I would like to thank without whom this academic milestone would not have been possible. First and foremost, I would like to thank my advisor, mentor, and committee chair, Dr. Wilfried Karmaus. Most of what I know about epidemiology I learned from him and I feel fortunate to have worked with someone who truly loves what they do and cares about his students. His perpetual enthusiasm for emerging areas of research have been an example to me to not hesitate simply because something is new and unknown. This is the very spirit of research and it has been valuable to see it in practice. Next, I would like to thank Dr. Hongmei Zhang who is not only a brilliant biostatistician but also a great role model as a woman in science. Her poise, confidence, and kindness have been an inspiration to me. I would like to thank Drs. Jim Burch and Myriam Torres who also served on my committee and contributed their invaluable expertise. It is time consuming and strenuous to serve on someone's committee and I do not take their hard work for granted.

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during the coursework stage of my PhD program. Thank you especially to Errol who calmly and swiftly came to my rescue countless times over IT-related emergencies. Thank you to Dr. Alicia Snider who was the best roommate a girl could ask for, she made a house a home during my time in South Carolina. Thank you, Dr. Meredith Ray, Zoey Phelps-Bergeron, and Kera Bergeron, for welcoming me to Memphis, Tennessee when I knew no one in the city, and for introducing me to a great group of people.

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parents, Nezam Yousefi and Simin Mahmoudi, who were the first people to show me what it is to be brave and to dream big. Thank you for their love, strength, sacrifices, and hard work in leaving Iran and giving me a life full of opportunity. I would not have the life I have today without everything that they did.

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ABSTRACT

Background: Leptin, initially believed to simply be a satiety hormone responsible for obesity, is now recognized as a pleiotropic cytokine that is involved in many biological processes; including the body's host inflammatory response. Clinically, leptin may affect lung function although research in this area is limited. It is also known that the leptin receptor is necessary for the activation of the leptin protein, making it an important protein to consider. Furthermore, single nucleotide polymorphisms (SNPs) and DNA methylations of the leptin and leptin receptor genes (*LEP* and *LEPR* respectively) may provide important insight on the relationship that leptin has with inflammation in the body.

Objectives: This dissertation focused on: 1) the association between leptin and leptin receptor gene polymorphisms and lung function (forced vital capacity, FVC; forced expiratory volume in 1 second, FEV_1; and FEV_1/FVC) at ages 10 and 18. 2a) The association between *LEP* SNPs and *LEP* DNA methylation. 2b) The association between *LEP* DNA methylation and serum leptin levels. 3) The association between leptin and FVC, FEV_1, and FEV_1/FVC controlling for body mass index (BMI).

Methods: The Isle of Wight (IOW) birth cohort, a population-based sample of 1,456 infants born between January 1989 and February 1990, was prospectively assessed at ages 1, 2, 4, 10, and 18 years. FVC, FEV_1, and leptin were collected at 10 and 18-year follow-ups. SNP and DNA methylation data was analyzed from blood that was collected at birth and 18 years follow up respectively. Regarding associations between *LEP* and

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LEPR SNPs and FVC, FEV_1, and FEV_1, FVC, forty-two independent repeated measurement analyses were conducted to test their association. Linear regression analyses were used to test the links between *LEP* SNPs and *LEP* DNA methylation, as well as the association between *LEP* DNA methylation and serum leptin protein levels at age 18. Finally, linear regression analyses were applied to investigate the association between serum leptin levels at ages 10 and 18 and FVC, FEV_1, and FEV_1/FVC at ages 10 and 18.

Results: *LEPR* SNPs rs6669354, rs1137101, and rs3762274 were associated with decreased lung function from ages 10 to 18. Those with the AC genotype of rs6669354 had 0.092 L lower FVC and 0.10 L lower FEV_1 than those with the AA genotype (Adjusted P-value=0.015 for both tests). A similar pattern was observed for SNPs rs1137101 and rs3762274 and the association with decreased FEV_1/FVC (Adjusted P-values 0.04 and 0.02 respectively). *LEP* SNPs rs11763517 and rs4731429 were both found to be related with DNA methylation sites cg00666422 (5'UTR region) and cg24862443 (3'UTR region) and *LEP* SNP rs4731429 was associated with cg00840332 (TSS200 region). The results were replicated in the second-generation (F2) cohort. Regarding leptin in F1, increased methylation of cg00840332 interacting with rs11763517 and rs4731429 was associated with decreased leptin serum levels. Lastly, in boys, an increase in leptin levels from ages 10 to 18 was related to a 0.017 L decreased FVC at age 18 (STD=0.007, P-value=0.018), while in girls increased leptin between ages 10 to 18 was associated with FEV_1 at age 18 decreasing by 0.013 L (STD=0.006, P-value=0.029). These associations were seen even after controlling for BMI.

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Conclusions: *LEPR* SNPs are associated with decreased FVC, FEV_1, and FEV_1/FVC from ages 10 to 18. A possible mechanism for this association can be explained through the activity of leptin. First, *LEP* SNPs are associated with increased *LEP* DNA methylation at the start of the gene and decreased *LEP* DNA methylation at the 3'UTR region of the gene; DNA methylation is linked to circulating serum leptin protein levels. Second, an increase in leptin protein between the ages of 10 and 18 is associated with decreased FVC in boys and decreased FEV_1 in girls at age 18.

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LIST OF SYMBOLS

ρ Spearmen correlation value

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List of Abbreviations

BMI ....body mass index

COPD ....chronic obstructive pulmonary disease

CpG .... cytosine-phosphate-guanine site in the DNA

DNA-M ....DNA methylation

FEV1 ....forced expiratory volume in one second

FEV1/FVC ..... the ratio indicates the proportion of the vital capacity that can be expired in the first second of forced expiration

FVC..forced vital capacity

FDR.. false discovery rate

Ig ....immunoglobulin

IL- ....interleukin-

JAK2 .... Janus Kinas 2

LEP ....gene the codes for the protein leptin

LEPR...gene that codes for the protein leptin receptor

LEPROT ....gene that codes for the protein leptin receptor overlapping transcript

Meth-QTL .... methylation quantitative trait loci

SNP ....single nucleotide polymorphism

STAT3.. signal transducer and activator of transcript 3

TNF..tumor necrosis factor

Th-..T Helper cells

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Treg .... T regulatory cells

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Chapter 1

Introduction and Literature Review

1.1 Background

Since the cloning of the *LEP* [1] and *LEPR* [2] genes and the identification of leptin as a pleiotropic hormone [3-20], leptin has been the extensive focus of studies regarding hormone regulation of hunger [21], energy expenditure [4,22], and immune system homeostasis [5,23-27]. Given that weight gain and reduced physical activity in mice was the most visible response to leptin deficiency and that leptin was found to be expressed and secreted mainly in white adipose tissue [1,3], it was initially believed that leptin's primary role was metabolic. However, it has since been recognized that the adipose tissue is not inert but rather is an endocrine organ that releases pro-inflammatory and anti-inflammatory factors, among them cytokines such as leptin [28]. Leptin is now known to play an important role is the body's host inflammatory response [5], and by extension, in the respiratory system [29-35]. Leptin is also produced in lower concentrations by other tissues, namely peripheral lung tissue (bronchial epithelial cells, alveolar type II pneumocytes, and lung macrophages) [36]. There is also evidence that suggests that leptin plays a key role in lung maturation and development [36,37], that it might be a stimulant of ventilation [29-35,38], and that it is involved in respiratory diseases (i.e., obstructive sleep apnea hypopnea syndrome, chronic obstructive pulmonary disease, lung cancer, and asthma) [37,39-51]. It is becoming increasingly clear that the role of leptin in the body's

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immune response is widespread, complex, and needs to be better understood, especially in humans.

To start understanding the mechanism behind leptin's role in the body's host inflammatory response and, more specifically, in respiratory illnesses, this dissertation focused on the association between leptin and leptin receptor gene polymorphisms and lung function (**FVC**-forced vital capacity; **FEV_1**-forced expiratory volume in 1 second, and their ratio FEV_1/FVC) while also considering the role of the circulating serum leptin protein as an intermediary in the above association. These measurements were considered at two different time points, ages 10 and 18 years of age. FVC, FEV_1, and FEV_1/FVC assessments are measured markers that clinicians rely upon to identify asthmatic patients and are therefore useful in human models. The *LEP* gene codes for the leptin protein and is vital in understanding this relationship. However, knowledge of a potential association between *LEP* and lung function is currently ambiguous as there are few studies that have been conducted in humans and those available yield conflicting results. For example, a single nucleotide polymorphism (SNP) of *LEP* (rs2167270) has been found to be associated with FVC and FEV_1 [52] in one study while another study has found no association between the same SNP and lower respiratory quotient [53].

Given that leptin activates subsequent processes by binding to the leptin receptor (LEPR), the *LEPR* gene also needs to be considered. The leptin receptor is a member of the class I cytokine receptor super-family and is present in many tissues, including the lung epithelium [52]. A complementary step is to investigate a possible link between *LEPR* SNPs and lung function. As with *LEP* genes, *LEPR* and *LEPROT* (leptin receptor overlapping transcript) genes have not been extensively explored in human populations

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and leave room for further inquiry. Hansel et al found 21 *LEPR* SNPS to be associated with FEV_1 decline in patients with chronic obstructive pulmonary disease [54] while van den Borst et al found no association between two *LEPR* SNPs (that were included in the 21 SNPs from Hansel et al) and FEV_1 or FVC [52].

Not only does the association between *LEP* and *LEPR* SNPs with lung function need to be further explored but there needs to also be a better understanding of how the protein product of these genes influences/mediates this relationship. This involves first considering a potential association between SNPs and circulating leptin levels and second, examining a relationship between protein leptin levels and lung function. It is important to note that plasma leptin levels have been observed to be higher in girls than boys during and after puberty [55-58]. Blum et al found that plasma leptin levels increased in girls, and decreased in boys after pubertal assessment of Tanner stage 2 and found a significant gender difference, especially in late puberty and adolescence, even after adjustment for body mass index (BMI) or body fat percent [55]. This is in accordance with the observation that testosterone inhibits the expression of leptin in the body while ovarian sex steroids have the opposite effect [55,59,60]. Therefore, boys and girls should be considered separately when evaluating leptin protein levels. It is also important to look at leptin levels throughout puberty (ages 10 and 18 years in our data) because this is the period when sex hormone levels fluctuate substantially in boys and girl, thereby influencing leptin levels.

When investigating the effect of genetic activities on protein leptin levels additional consideration of DNA methylation can provide a more complete picture. DNA methylation represents a change in the activity of the gene that does not involve a

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mutation (genetic variation or single nucleotide polymorphism, SNP). This change can be inherited as a result of past environmental exposures. In a recent article, Yousefi et al found that that *LEPR* SNPs in interaction with *in utero* smoking exposure were associated with *LEPR* DNA methylation levels and that *LEPR* DNA methylation was in turn associated with serum leptin levels. This two-step analysis revealed that a study of DNA methylation and genetic variants is more powerful than the single analysis of genetic variants [61]. As part of understanding the mediating effect of circulating body leptin levels, this dissertation repeated the above-mentioned analysis with *LEP* SNPs and *LEP* DNA methylation and additionally, taking circulating body leptin levels into account. This investigation provides the needed synthesis of the interplay of SNP, DNA methylation, and environmental exposures on protein levels that is currently missing in the literature (briefly mentioned below and explained in more depth in the literature review).

Depending on the SNP and SNP location, *LEP* genes have been associated with increased or decreased protein leptin levels. Fourati et al found that the following *LEP* SNPs were associated with elevated plasma leptin levels (hyperleptinemia): rs1349419, rs12535708, rs10487506, rs11770725, rs12535747, rs7799039, and rs2167270 [62]. Two other human studies have found an inverse relationship between *LEP* SNPs and plasma and breast tissue leptin levels [63,64]. Regarding *LEPR* SNPs in humans, the *LEPR* SNP (rs1137101) and rs17412175 have been shown to be associated with increased leptin levels in Pacific Islander [65] and English populations [61] respectively, while two other studies observed no association between genetic variations of the *LEPR* gene and serum leptin levels [66,67].

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In a study by Hillemacher et al that looked at 130 men and 34 women 7 days after alcohol cessation, *LEP* DNA methylation in the promoter region of the gene was associated with increasing serum leptin levels in alcohol dependent patients who were going through withdrawal [68]. There is a body of evidence that points to prenatal factors influencing adult risk of developing various chronic diseases [69]. Therefore, it was important for us to investigate environmental factors that can influence prenatal conditions. With regard to leptin, there is specific evidence that leptin plays an important role in prenatal development; *LEP* methylation has been found to be negatively correlated with placental gene expression in male infants [70]. Given the placenta's role in controlling *in utero* growth and development [71] and there is evidence placental genetic and DNA methylation profiles may serve as markers of the intrauterine and extra-uterine environment [72]. In addition, studies have found a positive, significant correlation between leptin levels in cord blood and birth weight [73-75]; large for gestational age babies had higher leptin levels in cord blood compared to appropriate for gestational age babies [75]. To further knowledge on prenatal influences, we considered the *in utero* environmental exposure, maternal smoking. Beyond that, genetic variants also have been found to affect the susceptibility of genes to methylation, a process named allele-specific genotype-dependent DNA methylation, or methylation quantitative trait loci methQTL [76-82]. Inconsistent reports on how protein leptin levels are associated with lung function may now be explained by accumulating evidence that leptin is both a pro-inflammatory and anti-inflammatory cytokine [83]. Leptin contributes to protection from infectious agents but also to a loss of tolerance and autoimmunity. Reduction in the level of circulating leptin (from malnutrition or genetic leptin deficiency) results in impaired Th1 response and

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induction of Treg cells, thus reducing the immuno-competence in mice [84] and humans [85] increasing susceptibility to infection. (Th1 helper cells are involved in response against intracellular parasites). Conversely, a high amount of leptin secreted by adipocytes (from obesity or genetic leptin receptor deficiency) leads to a high frequency and expansion of Th1 cells and increased secretion of pro-inflammatory cytokines, on one side, and a low proportion of proliferation of Treg cells infiltrating adipose tissue, on the other [83]. Leptin is involved with body's innate and adaptive response and has effects on many cells that are ultimately related to IL1, IL6, IL-10, IL-1Ra, nitric oxide, LB4, CTA1, COX-2, CD1 1b, IL-1β, IL-8, TNF-α, INF-γ, IL-12, IL-18 and IgG1 [86,87].

Contradictory findings on the association between serum leptin levels and lung function could therefore be due to the fact that leptin signals different immune pathways depending on its concentration in the body. An example of these contradictory findings are van den Borst et al not finding an association between serum leptin levels and FEV_1 [52] while a large middle-age US sample found a relationship between impaired FEV_1 and increased serum leptin levels [88]. Hickson et al detected an inverse association between serum leptin levels and predicted FEV_1 and predicted FVC % independent of adiposity (total body weight, waist circumference, and BMI) in an African American population women of a normal body weight (the models were also adjusted for age, education, smoking status, pack-years of cigarette smoking, respiratory medication use, and menopausal status in women) [89]. Childhood lung infection and asthma have also been related to increased plasma leptin levels, and may be related to maximally attained lung function [48].

This dissertation investigated 1) an association between *LEP* and *LEPR* SNPs with

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FVC, FEV_1, and FEV_1/FVC in a longitudinal analysis looking at ages 10 and 18 years, 2) the relationship between *LEP* and *LEPR* SNPs and protein leptin levels at age 18, considering intermediary role of *LEP* DNA methylation, and 3) the association between leptin and FVC, FEV_1, and FEV_1/FVC independent of BMI in concurrent and time delay models at ages 10 and 18.

1.2 Overall Document Structure

This dissertation is divided into the following sections:

i. A literature review (Chapter 1, continuation of current chapter) that summarizes previous findings investigating the role of leptin in lung function.

ii. A methods section (Chapter 2) that addresses in detail the study methodology for this work including the statistical procedures that were applied for the data analysis.

iii. A results section (Chapters 3, 4, 5) of three manuscripts, with each chapter comprising of a journal article that will be submitted to peer- reviewed journals for publication.

Manuscript 1. *LEPR* SNPs are associated with lung function: a longitudinal analysis

Manuscript 2. *LEP* SNPs and DNA methylation influence serum leptin levels in boys and girls: Two-stage model of epigenetic analysis

Manuscript 3. Leptin associated with lung function independent of body mass index

iv. Finally, a concluding discussion chapter (Chapter 6) that synthesizes the findings from the three journal articles.

v. The appendices include tables that serve as supplemental material for the articles.

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1.3 Literature Review

1.3.1 Brief Overview of Leptin and Leptin Receptor Proteins

Leptin is a 15 kDa protein that consists of 167 amino acids [1]. The protein is coded by the *LEP* (or *ob*) gene which is located on chromosome 7 in humans [1]. The *LEP* gene was first cloned in 1994 at the Jackson Laboratory following the observation of the random occurrence of mutant mice that weighed three times as much as the wild type mice [1]. These mice had a mutation of what was then named the *ob* gene that prevented the functional expression of a protein that is now called leptin. Because of this, leptin was initially suspected to only play a role in appetite regulation and obesity/adiposity.

While leptin is known to be mainly synthesized and secreted by white adipose tissue [3], it has also been found in human placenta [90], gastric fundic mucosa in rats [91], human pancreas [92], fetal baboon lung tissue [93], fetal rat lung fibroblasts[94], and in human peripheral lung tissue (bronchial epithelial cells, alveolar type II pneumocytes, and lung macrophages) [36,37]. The regulation of leptin secretion in the body also appeared to be straightforward at first. In mouse models, it was observed that food intake lead to decreased leptin levels while food consumption resulted in transient increases in leptin expression [21] suggesting leptin's role as an appetite regulator. This supported the initial observations mentioned above of leptin deficient mice having greater weight and lower resting metabolic rate. However, in humans it was observed that obesity was associated with increased leptin levels, suggesting leptin resistance in the body beyond a certain weight [95,96] and therefore challenging the previo usly understood linear relationship between weight and leptin observed in mouse populations. Since its discovery, leptin has

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also been found to play a role in the body's immune response [5,8-10,12-17,97], stimulation of insulin secretion and fatty acid oxidation [19], and reduction of cortisol synthesis [20].

The leptin receptor is a member of the class I cytokine receptor super-family and is coded by the *LEPR* (or *Ob-R*) gene [2]. Of particular interest for this dissertation is the expression of the *LEPR* gene in lung tissue in mice, rats, baboons, and other animals [93,94,98-104], contributing to the interest of the role of *LEPR* in human lung function.

Previous findings therefore support the idea that leptin and leptin receptor are involved with processes in the lung and could be linked to lung diseases such as asthma. Hence, a logical step is to explore the association of *LEP* and *LEPR* genes with lung function.

1.3.2 *LEP* AND *LEPR* SNPS AND LUNG FUNCTION

LEP SNPs (rs2167270, rs13228377, rs2167270) have generally been associated with lowered respiratory performance. One study in humans found an association between the A allele of LEP 19 G>A LEP SNP and decreased FEV_1 and FVC [52] while another study found an association between 3'UTR A/G and -2549 A/G LEP SNPs and asthma [105]. Ob/ob (leptin deficient mice) mutant mice were observed to have lower lung volume and alveolar surface area compared the wild type mice [106]. Ob/ob mice also developed rapid breathing patterns, altered baseline breathing patterns, and depressed hypercapnic ventilatory response. The latter was present before the mice became obese, demonstrating deteriorated respiratory control independent of body mass index (BMI). Total lung capacity and lung compliance were halved in ob/ob mutant mice [32].

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In humans, *LEPR* gene expression has been observed in human airway smooth muscle cells [107], epithelial cells, and submucosa of lung tissue obtained by bronchial biopsies [108]. In two human studies, there was no association found between the rs1137100 and rs1137101 *LEPR* SNPs and lung function and asthma respectively [52,105]. Hansel et al found that 21 *LEPR* SNPs (rs7531867, rs1805096, rs1892535, rs6588153, rs1938484, rs12564626, rs10443259, rs6691346, rs4655680, rs1137100, rs6702028, rs1782763, rs1171265, rs1171271, rs1782754, rs1171274, rs10889558, rs1327121, rs17412682, rs2025804, rs9436746) were associated with lung function decline in a subset of the Lung Health Study European Cohort [54].

1.3.3 *LEP* DNA Methylation

Current information on *LEP* DNA methylation is sparse. The literature shows a trend for increased DNA methylation in the promoter region correlating with poor health outcomes. Lesseur et al has found that *LEP* epigenetic control may be influenced by perinatal factors. For example, cord blood *LEP* methylation was found to be higher in small for gestational age babies and genotypes of rs2167270 *LEP* SNPs [109]. In a following study by Lesseur et al [70] *LEP* methylation for placental samples of healthy term babies showed negative correlation with *LEP* gene expression, only in male participants. Higher *LEP* DNA methylation was also associated with increased lethargy and hypotonicity, again, only in males in the study. In a small case-control study, mean DNA methylation of proximal *LEP* promoter was increased in low birth weight men [110].

Although DNA methylation can be stable throughout one's life, it can be modified by environmental factors. Data from the Dutch Famine Birth Cohort have

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reported higher blood *LEP* methylation levels in adult men who were prenatally exposed to war-time famine in 1944-45 compared to unexposed siblings [111]. This suggests that an outside variable may have influenced the *LEP* DNA methylation. It is therefore valuable to consider an environmental component in the analysis of the association between genetic variants, DNA methylation, and leptin concentrations. The specific choice of an environmental variable for this dissertation is one that is prenatal and influences prenatal development. During pregnancy the placenta is where maternal exposures, such as smoking, are translated and can affect fetal programming. Therefore, a variable that represents the time period of this exposure can provide insight onto how changed in *LEP* methylation can affect leptin levels in the body later in life.

1.3.4 Protein Leptin Levels, Immune Response, and Lung Function

Leptin's role in the body's immune response involves connections between food intake, metabolism, and immune homeostasis [3,83]. The hypothalamic-pituitary-adrenal (HPA) axis is one of the main structures that is responsible for this connection and this axis secretes hormones during inflammation to control the body's immune response. Leptin and leptin receptor are expressed in the hypothalamus and the pituitary gland while only the leptin receptor is expressed in the adrenal gland [112]. Leptin, in conjunction with leptin receptor, regulates the secretion of HPA hormones. Studies in mice have shown that leptin has both a direct and indirect effect on the body's immunity via the modulation of central or peripheral pathways [113]. Leptin's role in the body's immune response varies depending on how much serum leptin is circulating in the blood. If there are low amounts of leptin in the body (hypoleptinaemia) then Th1 cells are impaired and

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Treg cells proliferate, leading to increased infections and disease susceptibility (tuberculosis, candida, pneumonia). If the body has an excess of leptin (hyperleptinaemia), then Treg cells are inhibited and Th1 cells increase the secretion of proinflammatory cytokines, leading to increased autoimmune disease susceptibility (multiple sclerosis, rheumatoid arthritis). When the body has the appropriate moderate levels of leptin, there is Th1/Th2/Treg balance and the body has an optimal immune response.

Leptin affects both the body's innate and adaptive immunity. Studies on human and animal models have found the following associations between leptin and immune process in the body. Regarding innate immunity, leptin binds to it receptors in monocytes, macrophages, neutrophils, eosinophils, dendritic cells, and natural killer cells. In monocytes and macrophages, leptin is associated with increased phagocytosis and increased secretion of IL-1, IL-6, and TNFα. It is also linked to increased expression of activation markers, increased expression of surface markers, and increased eicosanoids, nitric oxide, leukotriene B4, cholesterol acyltransferase 1, and cyclooxygenase-2. In neutrophils, leptin seems to increase the formation and release of reactive oxygen species, increases chemotaxis, decreases apoptosis, and increases the expression of CD1 1b. In eosinophils, leptin is associated with increased expression of adhesion molecules, increased chemokinesis, and increased release of IL-1β, IL-6, and IL-8. In dendritic cells, leptin is associated with increased IL-1β, IL-6, IL-8, IL-12, and TNF-α. Lastly, in natural killer cells leptin plays a role in increased proliferation and maturation, increase differentiation activation, and cytoxicity [86].

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When looking at adaptive immune response, human and animal studies have found that leptin affects thymoctyes, naïve T cells, memory T cells, activates Th1 cells, inhibits Th2 cells, Treg cells, B cells, and Natural Killer T cells. In thymocytes leptin is associated with increased maturation and decreases apoptosis [86]. In Naïve T cells, leptin increase proliferation and decreases apoptosis. In memory T cells, leptin binds to its receptor and decreases proliferation. In Th1 cells activation, leptin increases the IgG2a switch, increases TNF-α, and increases TNF-γ. During Th2 cell inhibition, leptin decreases IgG1 switch, and increases secretion of IL-10 [5,22]. In Treg cells, leptin increases anergy (absence of immune response), and increases hyperresponsiveness. In B cells, leptin decreases apoptosis and increases secretion of IL-12, IL-6 and TNF-α. In natural killer cells, leptin increases proliferation.

Of the cytokines that leptin may affect, IL-10 and IL-12 are available in our dataset. Furthermore, IL-10 and IL-12 are potentially linked to lung function and asthma, In a prospective study, IL-10 polymorphisms were associated with obstructive lung function parameters, suggesting as important role if IL-10 in the development of lung function deficiency in early bronchitis patients [114]. Similarly, a longitudinal study of 379 firefighters with at least six annual FEV_1 measurements found that those with IL-10 SNPs had different rates of decline in lung function as they aged [115]

Studies in animals have suggested that leptin is involved in respiratory control. For example, when comparing mutant *LEP* mice to their wild type counterparts and even before the onset of obesity, the mutant mice have increased breathing frequency, minute ventilation and tidal volume, elevated P_aCO_2 and depressed hypercapnic ventilatory response [38]. Chronic leptin administration restores breathing rates and improves lung

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compliance. However, because leptin administration in mice also decreases weight, the improvement of breathing and lung compliance cannot be conclusively attributed to leptin therapy and not simply a result of lowered body mass index (BMI). Acute leptin replacement, on the other hand, has led to increased baseline ventilation independent of weight gain [116], and leptin injections in rat brain has led to increased pulmonary ventilation and respiratory volume [29,38]. Leptin has also been observed to be involved in the regulation of embryonic lung growth and maturation [106,117]. Specifically, administration of leptin to antenatal mice has led to improved lung development in the newborn [117], whereas leptin-deficient mice have been observed to have impaired alveolar formation and lower lung volumes at birth [106]. Additionally, postnatal leptin treatment of these same mice has resulted in increased alveolar surface area and lung volume [106].

Whereas the consensus in animal models is that higher leptin levels are correlated with improved respiratory response, human studies yield either null findings or an inverse association. Clinical studies have found that leptin is a predictor of lung function in asthma patients [35] and is negatively correlated to lung volumes in COPD patients [118] and clinical severity of asthma [119]. Van den Borst et al observed no association between FEV_1 and serum leptin levels but found that increased serum leptin levels were associated with decreased FVC in men but not in women [52]. There has also been an observed inverse association between serum leptin levels and lung function in an African American population [89] and a twin population [52]. Sin et al in a large sample (N=2808) also observed an inverse association between serum leptin levels and FEV_1 and FVC% predicted, independent of adipocytes in men but found no such pattern in women [88]. In a study involving pre-pubertal boys and girls, increased leptin levels were associated with asthma

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only in boys [48]. In an Indian population, increased plasma leptin was associated with decreased FEV_1 but no association was found with FVC.

While sleep apnea cannot be tested from the data available in this study, it is important to note that the increasing scope of leptin's influence in the respiratory system includes the hypothesis that obstructive sleep apnea syndrome (characterized by repeated episodes of partial or complete upper airway obstruction) is a leptin-resistant state. Impaired leptin activity in the central nervous system due to a down-regulation of leptin receptors (54, 76-78) can be responsible for the above hypothesis and is supported by findings of a *LEPR* SNP being associated with obstructive sleep apnea syndrome (79) but not confirmed by another group (80). Patients with untreated asthma have shown decreased leptin expression in their bronchial epithelium (*ex vivo*) [108].

Although there is strong evidence that leptin plays a role in the respiratory system, it is difficult to isolate this role from other factors, such as BMI. Hence there is a need to control BMI when assessing the risk related to lung function when assessing leptin. Furthermore, while animal studies show a consistent reaction to leptin levels in the body, human studies show an opposite effect that may be stratified by sex and endocrine changes during puberty

1.3.5 Gaps and Limitations in the Current Literatures

- Investigations into the associations between *LEP* SNPs and lung function only considered one SNP in the intron region of the gene [52,53]. Investigations into the associations between *LEPR* SNPs and lung function by Loos et al only looked at SNPs that consisted of benign missense mutations (mutations that results in genes that code for

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a different amino acid but still express the same protein) and mutations that occurred in the intron region of the gene[53]. Only one study has included SNPs that were in the promoter or 3' UTR region of the gene or investigated lung function measurements at multiple time points [54]. Lastly, no study has investigated this relationship stratified by sex or at multiple time points throughout puberty.

- The DNA methylation data used in this dissertation was collected with the Infinium Human Methylation 450 array, which is reported to have superior reproducibility and validity than other methods [120,121]. Of the studies that investigated an association between DNA methylation and leptin levels, none used this method of DNA methylation assessment.

- No studies have analyzed the association between methQTLs and DNA methylation or employed the two-step analysis for understanding the mechanism behind an association between *LEP* SNPs interacting with *in utero* smoking exposure and serum leptin levels.

- No studies have explored and association between serum leptin levels and lung function independent of BMI while stratifying by sex. Given that leptin is inhibited by testosterone but increased by ovarian sex steroids [5], any analysis involving leptin and BMI should consider boys and girls separately.

- Studies investigating the association between leptin levels and lung function, do not take into account the change in leptin levels during puberty [119].

1.4 Specific Aims and Hypotheses

This dissertation seeks to enhance our understanding of the association between *LEP* and *LEPR* SNPs and lung function as how this relationship is mediated by leptin.

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Furthermore, this dissertation aimed to demonstrate the *LEP* SNP →leptin→lung function mechanism while taking into consideration the role of epigenetics. In all analyses, boys and girls were either considered separately or sex was controlled as a confounder in the models.

Specific Aim 1: The association between *LEP* and *LEPR* SNPs and lung function at ages 10 and 18 years.

Hypothesis 1: LEP and *LEPR* SNPs are associated with lung function measures of FVC, FEV_1, and FEV_1/FVC at ages 10 and 18 and this association is gender-specific.

Specific Aim 2: Investigate *LEP* SNPs that are potential methQTLs interacting with *in utero* smoking exposure. DNA methylation sites that are identified in the methQTL analysis are associated with serum leptin concentrations.

Hypothesis 2a: *LEP* SNP interacting with *in utero* smoking exposure is associated with *LEP* DNA methylation at age 18 and this association is gender-specific.

Hypothesis 2b: LEP DNA methylation sites found in 2a are associated with protein leptin levels at age 18 and this association is gender-specific.

We also tested whether hypothesis 2a can be replicated in the subsequent generation of the Isle of Wight birth cohort (F2-generation).

Specific Aim 3: IL-10 and IL-12 mediate the association of leptin with lung function at ages 10 and 18.

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Hypothesis 3: Serum leptin levels at ages 10 and 18 are associated with lung function measures of FVC, FEV_1, and FEV_1/FVC at ages 10 and 18, using IL-10 and IL-12 as intervening variables in change models. These associations were stratified by sex.

Theoretical path model:

Leptin→IL-10, IL-12→FVC, FEV_1, FEV_1/FVC

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CHAPTER 2

METHODS

2.1 STUDY DESIGN AND PARTICIPANTS

A whole population birth cohort was established on the Isle of Wight, UK, in 1989 to prospectively study the natural history of allergies from birth to 18 years of age. Ethics approvals were obtained from the Isle of Wight Local Research Ethics Committee (now the National Research Ethics Service Committee South Central – Southampton B). Of the 1,536 children born between January 1, 1989, and February 28, 1990, written informed consent was obtained from parents to enroll 1,456 newborns. Children were followed up at the ages of 1 (n=1,167), 2 (n=1,174), 4 (n=1,218), 10 (n=1,373), and 18 years (n=1,313). The IOW birth cohort has been described in detail elsewhere [122]. Detailed interviews and examinations were completed for each child at each follow-up. When a visit was not possible, a telephone questionnaire was completed or a postal questionnaire was sent. Information on maternal smoking during pregnancy was obtained from mothers at the time of recruitment (birth).

2.2 LEPTIN CONCENTRATIONS

Leptin concentrations were obtained from blood samples collected at ages 10 and 18 years. Aliquots of blood serum that were isolated from the blood samples were used in enzyme-linked immunosorbent assays from Biokit, S.A. (Barcelona, Spain). The assays

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were conducted according to the manufacturer's kit instructions. Each sample, including standards and the blank, was assayed in duplicate. As part of the repeated follow ups, the original questionnaire-based information was updated and weight and height of the child were measured at age 18 years.

2.3 *LEP*, *LEPR*, AND *LEPROT* GENOTYPING AND DNA METHYLATION ANALYSIS

LEP SNPS (k=4) and *LEPR* SNPs (k=21) that tagged the *LEP*, *LEPR* and neighboring *LEPROT* genes were identified using Tagger implemented in Haploview. Leptin receptor overlapping transcript (*LEPROT*) was included in the analysis because it shares the same promoter and the first two exons as the *LEPR* gene. DNA was extracted from blood or saliva samples from cohort subjects (n=1,211). DNA samples were interrogated using Golden Gate Genotyping Assays (Illumina Inc, San Diego, CA) on the Bead Xpress Veracode platform (Illumina, Inc, San Diego, CA) per Illumina's protocol [120,121]. In brief, samples were fragmented and hybridized to the pool of allele-specific primer sets. Following an extension/ligation reaction the samples were then hybridized to the Veracode bead pool and processed on the Bead Xpress reader. Data were analyzed using the genotyping module of the GenomeStudio Software package (Illumina, Inc, San Diego, CA). DNA from each subject plus 37 replicate samples were analyzed. The quality threshold for allele determination was set at a GenCall score 0.25 (scores #0.25 were ''no calls'') with 98.3% retained for further analysis. Analysis of each locus included reclustering of genotyping data using our project data to define genotype cluster positions with additional manual reclustering to maximize both cluster separation and the

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50th percentile of the distribution of the GenCall scores across all genotypes (50% GC score).

For measuring methylation levels, DNA was extracted from whole blood collected at age 18 years from 245 female offspring[123] as well as 125 males, 100 pregnant women, and 130 F2 offspring. One microgram of DNA was bisulfite-treated for cytosine to thymine conversion using the EZ 96-DNA methylation kit (Zymo Research, CA, USA), following the manufacturer's standard protocol. Genome-wide DNA-M was assessed using the Illumina Infinium Human Methylation 450 Bead Chip (Illumina, Inc., CA, USA), which interrogates>484,000 CpG sites associated with approximately 24,000 genes. Arrays were processed using a standard protocol as described elsewhere [120], with multiple identical control samples assigned to each bisulphite conversion batch to assess assay variability and samples randomly distributed on microarrays to control against batch effects. The Bead Chips were scanned using a Bead Station, and the methylation level (beta value) calculated for each queried CpG locus using the Methylation Module of Bead Studio software.

Concentrations of IL-10 and IL-12 cytokines were measured using the electrochemiluminescence Meso Scale Delivery (MSD) Kit platform from blood samples collected at age 18 years (Meso Scale Discovery, Rockville, Maryland, USA), known for of higher sensitivity, lower level of detection, and wide dynamic range with the standard curve being linear within the range of experimental samples [124]. All experiments were performed according to the manufacturer's instructions with minimal modifications and optimization. Briefly, 50 µl of each 1:2 diluted sample was added to each well of the pre-coated 96-well plate and incubated at room temperature (RT) for 2.5 h with continuous

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counter-clock-wise shaking. The plates were then washed three times with 1× Wash Buffer (MSD) and Sulfotag Detection Antibody Cocktail (MSD) was then added to each well and the plates then incubated for an additional 2 h with shaking at RT. Finally, the plates were washed again, and were scanned by a SECTOR® Imager 6000 Reader (MSD) after adding 150 µl of 2× Read Buffer (MSD).

2.4 Statistical Analysis

2.4.1 SA1: Hypothesis 1

LEP and LEPR SNPs are associated with lung function measures of FVC, FEV_1, and FEV_1/FVC at ages 10 and 18 and this association is gender-specific.

To identify haplotype blocks, linkage disequilibrium analysis was performed on fourteen *LEPR* SNPs and seven *LEPROT* SNPs with Haploview 4.2 [125], using the Gabriel et al. method [126]. We then selected one SNP from each block based on what was previously chosen in Yousefi et al [61]. All four *LEP* SNPs were tested for their association with FVC and FEV_1 at age 18 years.

Linear mixed models were used to test the association between *LEP*, *LEPR*, and *LEPROT* SNPs and lung function at ages 10 and 18. With 10 *LEPR/LEPROT* SNPs and 4 *LEP* SNPs, and three measures of lung function (FVC, FEV_1, and FEV_1/FVC) this resulted in 42 individual tests. Each dependent lung function variable (FVC, FEV_1, FEV_1/FVC) was analyzed in a separate model and data from ages 10 and 18 years were analyzed in the same model. The following variables also had data at both years: age at follow up, BMI, and height. The full mixed model was controlled for the following confounders: age at follow up (years), sex (male, female), *in utero* smoking exposure

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(yes, no), parental asthma status (mother, father, both, or none), ever smoking (yes, no), SES group (low, middle, high), birth weight (kg), height (cm), BMI (kg/cm^2), and duration of breastfeeding (weeks). An autoregressive covariance structure was used. Manual backward elimination of confounder applying the 10% rule was used to determine the most appropriate model.

False discovery rate (FDR) was used to adjust for multiple testing [127]. FDR was applied separately, once with tests involving *LEPR* and *LEPROT* SNPs only and another time looking only at the *LEP* SNPs.

2.4.2 SA2A: Hypothesis 2A

LEP SNP interacting with in utero smoking exposure is associated with LEP DNA methylation at age 18 and this association is gender-specific. We will attempt to replicate this analysis in the F2 population.

Haplotype analysis was performed on LEP SNPs in the F1 and F2 generation of this cohort (note: Mukherjee et al has performed the analysis in the F1 cohort in a previous publication that we used in this study as well [128]). We used one SNP from each haplotype block and proceeded with the following analyses.

F1 population: To identify methQTLs modified by gestational maternal smoking, modeling was performed by using *LEP* SNPs and their interaction with in utero smoking exposure to predict the methylation of *LEP* CpG sites. Each CpG (M-values) was modeled against rs11763517, rs4731429, and rs10954176, with each SNP interacting with *in utero* smoking exposure. In addition, all models were controlled for cell-composition to ensure that we were indeed observing associations with DNA methylation

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un-confounded by the proportion of these cells. We controlled for levels of the following cells: B-cells, granulocytes, monocytes, natural killer cells, eosinophils, and CD4 T-cells. These cell counts were obtained from breast cancer patients from the Winship Cancer Institute [129], 61 subjects at baseline and 39 subjects at six-month-follow-up. We followed the method used by Kaushal et al [130] where 484,489 CpG sites were tested against cell types. The most parsimonious model was determined via backward elimination using the 10% rule, first by removing interaction terms followed by individual SNPs. False discovery rate was used to adjust for multiple testing.

F2 Population: The methQTL analysis was replicated in the F2 population (offspring of F1). Eighteen *LEP* SNPs were genotyped in the F2 population but only 5 SNPs (rs2167270, rs2278815, rs11760956, rs11763517, rs12706832) proved to have polymorphisms (the others were monomorph, with all or almost all the population having one of the possible genotypes). Haplotype analysis using Haploview software [125] revealed that SNPs rs2278815 and rs12706832 were in LD with each other while SNPs rs11763517 and rs11760956 were in LD with each other (Figure 4.1). Therefore, the methQTL analysis included the following SNPs interacting with in utero smoking exposure predicting DNA methylation levels: rs2278815, rs11763517, and rs2167270. While SNPs in the F1 populations were collected via selected probes, those from the F2 children were from a genome-wide product. Therefore, the following SNPs, rs2167270, rs2278815, rs11760956, and rs12706832 were not identical with the SNPs identified in the F1 population. Hence, in order to see if the findings in the F2 population are based on closely related SNPs, we generated a second LD plot using the International Haplotype

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Map (HapMap) project data from the Central European University database [131] (Figure 4.2).

2.4.3 SA2B: Hypothesis 2B

LEP DNA methylation sites found in 2a are associated with protein leptin levels at age 18 and this association is gender-specific.

The selected CpG sites (M-values) from hypothesis 2a were tested on whether they modified the association that *LEP* SNPs had with leptin serum concentrations at age 18. In this step, our focus was on the interaction effects and false discovery rate was applied to correct for multiple testing among the tests for interaction effects between CpG sites and genetic variants. For both sets of statistical analyses, the GLM procedure was used in SAS 9.4 (SAS, Gary, NC, USA). In addition, because leptin levels vary greatly between boys and girls following puberty, all models initially controlled for sex. As the proportion of girls in the subsample with DNA-methylation was higher than the proportion of boys, the analyses were weighted for sex. Since the same SNP (rs4731429) that was a methQTL for cg00840332 was also a genetic modifier in the association between DNA-M and leptin levels, we calculated the residuals from the association between rs4731429 and cg00840332 using linear regression analyses. These DNA-methylation residuals were then used in testing the association between DNA-M and leptin levels.

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2.4.4 SA3: Hypothesis 3

Hypothesis 3: Serum leptin levels at ages 10 and 18 are associated with lung function measures of FVC, FEV_1, and FEV_1/FVC at ages 10 and 18, using IL-10 and IL-12 as intervening variables in change models. These associations are stratified by sex.

Spearman correlations were used to initially check for associations between the cytokines (IL-10 and IL-12) and leptin at ages 10 and 18 and all lung function measures at ages 10 and 18. The cytokines were not associated with the exposures or the outcome so path analysis could not be performed and IL-10 and Il-12 were not used in subsequent analyses. Linear models were used the test the association between leptin and lung function (FVC, FEV_1, and FEV_1/FVC). Two sets of analyses were performed: 1) Concurrent models tested the association between leptin at age 10 and lung function at age 10 and leptin at age 10 and lung function at age 18 years. 2) Change in leptin levels from ages 10 to 18 were employed to predict lung function at age 18 years (time-delayed model). Since prior findings suggested variations of the associations by the sex of the child, the subgroup of boys and girls were analyzed separately. In the concurrent models, we controlled for height and BMI at the respective ages. In the delayed model, we controlled for height at age 18, change in height between ages 10 and 18, and change in standardized BMI from ages 10 to 18.

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CHAPTER 3

RESULTS I - MANUSCRIPT #1
LEPR SNPS ARE ASSOCIATED WITH LUNG FUNCTION: A LONGITUDINAL ANALYSIS[1]

3.1 INTRODUCTION

Leptin is an extensively studied pleiotropic cytokine that is commonly known for its association with body mass index (BMI) and energy regulation in the body[4,21,22]. However, it is also recognized to play an important role in the body's host inflammatory response, although the mechanism is complex and poorly understood [87,132,133]. Specifically, with regards to obstructive lung diseases such as asthma, leptin may contribute by activating the Janus Kinase 2/Signal Transducer and Activator of Transcript 3 (JAK2/STAT3) signaling pathway [134-139]. It has been proposed that the leptin protein binds to the leptin receptor, enabling the JAK2 to bind to the protein-receptor complex. This in turn phosphorylates tyrosine residues on the leptin receptor, leading to the initiation of the JAK2/STAT3 signaling pathway. This pathway subsequently results in the release of pro-inflammatory cytokines, such as Th1, TNF-α, IFN-γ, IL-2, IL-6, and IL-12 [86], that can lead to airway obstruction diseases. Because of leptin's involvement

[1] M. Yousefi, W. Karmaus, H. Zhang, M Torres, J Burch. *LEPR* SNPs are associated with lung function: A longitudinal analysis. To be submitted.

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with immune response, changes in leptin and leptin receptor protein structures (resulting from genetic variation) may influence respiratory diseases.

Only a few studies have investigated the relationship between *LEP* SNPs and respiratory outcomes. Animal studies have observed characteristics in mice that have a mutation that result in no leptin protein expression (i.e., leptin mutant mice, ob/ob knockout mice, leptin deficient mice). These mice have been observed to have lower lung volume and alveolar surface area compared to the wild type mice [106], rapid breathing patterns, and depressed hypercapnic ventilatory response. The latter has been observed before the mice became obese, demonstrating deteriorated respiratory control independent of body mass index (BMI). Total lung capacity and lung compliance are also halved in ob/ob mutant mice [52]. In humans 3'UTR A/G and -2549 A/G *LEP* SNPs have been associated with asthma [105].

To date, only two studies have investigated the relationship between *LEP* SNPs and lung function specifically. Van den Borst et al found that the AA genotype of rs2167270 was associated with declined force expiratory volume in one second (FEV_1) and forced vital capacity (FVC) compared to the other genotypes[52]. Mukherjee et al found that rs11763517 was associated with decreased FEV_1 and FEV_1/FVC; however, only with increased DNA methylation of the *LEP* gene [128]. This study was the first to explore the longitudinal association between *LEP* SNPs (rs10249476, rs11763517, rs4731429, and rs10954176) and FVC, FEV_1, FEV_1/FVC.

The leptin protein must bind to the leptin receptor to initiate the pathway that is involved in the body's host inflammatory response. Therefore, it is important to also consider the *LEPR* gene when understanding the role of leptin in the body. Evidence that

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LEPR gene expression has been observed in human airway smooth muscle cells [107], epithelial cells, and submucosa of lung tissue obtained by bronchial biopsies [108], suggests that it may play a role in respiratory illnesses. However, in a human study no association was detected between *LEPR* SNP rs1137101 and asthma [105]. In two human studies, no association was detected between the rs1137100 and rs1137101 *LEPR* SNPs and lung function and asthma respectively [52,105] (Table 3.1). Hansel et al (N=429) found that 21 *LEPR* SNPs (rs7531867, rs1805096, rs1892535, rs6588153, rs1938484, rs12564626, rs10443259, rs6691346, rs4655680, rs1137100, rs6702028, rs1782763, rs1171265, rs1171271, rs1782754, rs1171274, rs10889558, rs1327121, rs17412682, rs2025804, rs9436746) were associated with lung function decline in a subset of the Lung Health Study European Cohort [54]. However, when looking at two of the same SNPs investigated by Hansel it al (rs1137100 and rs1137101) , van den Borst et al found no association with and FEV_1 or FVC [52]. It is important to note that both studies had a similar sample size and a longitudinal analysis.

Another gene assisting our understanding in leptin and inflammation is the leptin receptor overlapping transcript gene, *LEPROT*. This gene overlaps with the *LEPR* (as the name suggests), and aids in cell surface expression of the leptin receptor protein.

When investigating the relationship between *LEP*, *LEPR*, and *LEPROT* SNPs and lung function, it is important to control for potential confounders. It is well documented that birthweight is associated with adult lung function. Linear mixed models and path analysis in a study by Balte et al revealed that increased birthweight was associated with increased FEV_1 and FVC at 18 years of age after adjusting for potential confounders [140]. Other studies have also found a positive linear relationship between birthweight and lung

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function in both adults and children [141-143]. Maternal smoking during pregnancy has been directly associated with FEF_{25-75} in the Isle of Wight cohort and should therefore be tested as a potential confounder [140]. Height and age are used to predict lung function against spirometry tests and should therefore be included as well. Likewise body mass index (BMI) is known to be associated with leptin protein [144,145], *LEP* and *LEPR* genes [105,146,147], and lung function[148]. It is known that leptin levels are different between men and women [55,149,150], so all analyses need to take sex into account. In a previous path analysis using samples from Isle of Wight cohort that was also analyzed in this work, duration of breastfeeding was directly positively associated with FVC at age 10 and indirectly with FVC at age 18 [151].

The genetic data from the Isle of Wight Birth Cohort Study [152] provides a unique opportunity to investigate the relationship between leptin (*LEP*) and (*LEPR*) single nucleotide polymorphisms (SNPs) and lung function. We tested the association of *LEP*, *LEPROT*, and *LEPR* SNPs with lung function measured by FVC, FEV_1, and FEV_1/FVC. FVC quantifies lung volume, FEV_1 reveals airway obstruction, and the ratio of FEV_1/FVC shows if there is respiratory distress. In the case of asthma and other obstructive lung diseases, there is lower than normal FEV_1/FVC. This was only the second study to investigate the relationship between these *LEP* SNPs and lung function, and the first to do so in a longitudinal analysis.

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3.2 Methods

3.2.1 Population Characteristics

A whole population birth cohort was established on the Isle of Wight, UK, in 1989 to prospectively study the natural history of allergies from birth to 18 years of age. Ethics approvals were obtained from the Isle of Wight Local Research Ethics Committee (now the National Research Ethics Service Committee South Central – Southampton B). Of the 1,536 children born between January 1,1989, and February 28, 1990, written informed consent was obtained from parents to enroll 1,456 newborns. Children were followed up at the ages of 1 (n=1,167), 2 (n=1,174), 4 (n=1,218), 10 (n=1,373), and 18 years (n=1,313). The IOW birth cohort has been described in detail elsewhere [153]. Detailed interviews and examinations were completed for each child at each follow-up. When a visit was not possible, a telephone questionnaire was completed or a postal questionnaire was sent. Information on maternal smoking during pregnancy was obtained from mothers at the time recruitment (birth).

3.2.2 Lung Function Measurements

FVC and FEV_1 were measured at ages 10 and 18 years. Lung function testing was performed using the Koko spirometry software package on a portable desktop device (PDS Instrumentation, Louisville, KY, USA) [154]. Tests were performed in accordance with American Thoracic Society and European Respiratory Society spirometry guidelines [6]. Children were required to be free from respiratory infection for 14 days and not taking systemic oral steroids. In addition, subjects were asked to abstain from any beta-agonist medication for at least 6 hours and from caffeine intake for at least 4 hours prior to

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testing. Measurements were made in a standardized manner with the subject standing without a nose clip [155]. Forced vital capacity (FVC) was recorded in liters as the best of three consecutive forced expiratory maneuvers. Participants that exhibited clinical symptoms consistent with either current infection or a recent (within two weeks) asthma exacerbation, which required antibiotics or oral steroid within the preceding two weeks, were rescheduled for spirometry testing.

3.2.3 Confounders

We considered the following as potential confounders: age at follow-up, sex, *in utero* exposure to maternal smoking, history of parental asthma, birth weight, height at ages 10 and 18 years, duration of breastfeeding in weeks, if the child ever smoked, and socioeconomic status (SES).

Age at follow-up was calculated by subtracting date of follow-up from date of birth. Information on birthweight was obtained from hospital records. Information on whether the mother smoked while pregnant, parental history of asthma, and the sex of the child was collected after delivery. Breastfeeding duration was ascertained at ages 1 and 2 years. Socio-economic status was based on three variables: 1) the British socioeconomic classes (1–6) derived from parental occupation reported at birth; 2) the number of children in the index child's bedroom (collected at age four years); and 3) family income at age 10 years. Height and weight were measured before the spirometric tests at age 10 and 18 years. Height was ascertained via the standard height measurement (cm) used at the allergy clinic for those that visited the center. BMI was calculated. Ever smoking status was determined by whether the child reported that they currently smoked at age 10

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or 18 follow up or if the child reported to have ever started smoking (even if they no longer smoked at the follow up times).

The full mixed model was controlled for the following variables: age at follow up (years), sex (male, female), *in utero* smoking exposure (yes, no), parental asthma status (mother, father, both, or none), ever smoking (yes, no), SES group (low, middle, high), birth weight (kg), height (cm), BMI (kg/cm^2), and duration of breastfeeding (weeks).

3.2.4 SNP Collection and Selection

DNA was extracted from blood or saliva samples from cohort subjects (n=1,211). DNA samples were interrogated using Golden Gate Genotyping Assays (Illumina Inc, San Diego, CA) on the Bead Xpress Veracode platform (Illumina, Inc, San Diego, CA) per Illumina's protocol [45, 46]. In brief, samples were fragmented and hybridized to the pool of allele-specific primer sets. Following an extension/ligation reaction the samples were then hybridized to the Veracode bead pool and processed on the Bead Xpress reader. Data were analyzed using the genotyping module of the GenomeStudio Software package (Illumina, Inc, San Diego, CA). DNA from each subject plus 37 replicate samples were analyzed. The quality threshold for allele determination was set at a GenCall score 0.25 (scores #0.25 were ''no calls'') with 98.3% retained for further analysis. Analysis of each locus included reclustering of genotyping data using our project data to define genotype cluster positions with additional manual reclustering to maximize both cluster separation and the 50th percentile of the distribution of the GenCall scores across all genotypes (50% GC score). Reference groups or genotypes were based on the one that presented the highest frequency.

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All four *LEP* SNPs were selected to be used in the analysis. To identify haplotype blocks, linkage disequilibrium analysis was performed on fourteen LEPR SNPs and seven LEPROT SNPs with Haploview 4.2 [125],using the Gabriel et al method [126]. This resulted in the same haplotype blocks found in Yousefi et al [61], so we selected the same SNPs from each block.

3.2.5 Statistical Analysis

Linear mixed models were used to test the association between *LEP*, *LEPR*, and *LEPROT* SNPs and lung function at ages 10 and 18. With 10 *LEPR/LEPROT* SNPs and 4 *LEP* SNPs, and three measures of lung function (FVC, FEV_1, and FEV_1/FVC) this resulted in 42 individual tests. Each dependent lung function variable (FVC, FEV_1, FEV_1/FVC) was analyzed in separate model and data from ages 10 and 18 years were analyzed in the same model. The following variables also had data at both years: age at follow up, BMI, and height. The full mixed model was controlled for the confounders mentioned above. An autoregressive covariance structure was used. Manual backward elimination of confounder applying the 10% rule was used to determine the most appropriate model. False discovery rate (FDR) was used to adjust for multiple testing [127]. FDR was applied separately, once with tests involving *LEPR* and *LEPROT* SNPs only and another time looking only at the *LEP* SNPs.

3.3 Results

Of the 1,373 children who followed up at age 10 years, n=981 and n=980, children had measures on FVC and FEV_1, respectively. Of the 1,313 children who

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followed up at age 18 years, FVC and FEV_1 were collected on n=838 and n=839 children, respectively (Table 3.2). Height, BMI, FVC, and FEV_1 all increased from ages 10 to 18 years. Boys and girls had about the same average FVC and FEV_1 at age 10 years (FVC~2.3 L, FEV_1~2.0 L) but at age 18, lung function in boys nearly doubled while that in girls increased by half the amount (data not shown). At age 18, FVC in boys increased by 3 L (STD: 0.5) and increased by 1.7 L (STD=0.4) in girls. Similarly, FEV_1 in boys increased by 2.5 L (STD: 0.4) while only increasing by 1.5 L (STD: 0.4) in girls (stratified data by sex not shown). The FEV_1/FVC ratio was the same at both ages (Table 3.2). About a quarter of the children (25.25%) were exposed *in utero* to maternal smoking, almost half of the children in the study (45.46%) reported ever having smoked; 9.3 % of mothers and 10.3 % of fathers were reported to have asthma (Table 3.2).

The four *LEP* SNPs that were examined in this study spanned the length of the leptin gene, with one in the promoter region (5'UTR), one in the gene's intron, and two located in the 3'UTR. In our data, the first *LEPROT* SNP we have is in the intron region and is preceded by the *LEPR* 5'UTR rs3896318 SNP. Two more *LEPROT* SNPs are in the intron region, followed by four located in the flanking 3'UTR (Table 3.4). This is followed by three more *LEPR* SNPs that are in the promoter region of the gene, three in the codon region, and six in the intron region. The last *LEPR* SNP is in the 3'UTR region.

Linkage disequilibrium analysis of the 14 *LEPR* and 7 *LEP*ROT genes resulted in 5 haplotype blocks and 5 SNPs that did not fit into any blocks (Figure 3.1). SNPs from the blocks were chosen based on what was used previously by our team [61] and the five individual SNPs were also considered. These ten SNPs included rs3806318, rs9436740,

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rs9436301, rs17412175, rs6669354, rs7526141, rs12059300, rs1137101, rs3762274, and rs8179183 (Figure 3.1).

After adjustment for multiple testing via FDR (p-value cut-off of 0.05) [127], linear mixed models indicated that three SNPs (rs6669354, rs1137101, rs3762274) were associated with lowered lung function in the repeated measurement analyses (Table 3.5). In the flanking 3'UTR region of the *LEPROT* SNP, those with the AC genotype of rs6669354 had 0.092 L lower FVC and 0.10 L lower FEV_1 than those with the AA genotype (Adjusted P=0.015 for both tests, Table 3.5). Maternal smoking during pregnancy, whether the child ever smoked, birth weight, duration breastfeeding, and socioeconomic status did not confound the association. The *LEPR* SNPs rs1137101 and rs3762274 were associated with lower FEV_1/FVC (Table 3.5). Whether the child smoked and breastfeeding duration were significant factors in the association between rs1137101 and FEV_1/FVC, while breastfeeding duration did not confound the association when considering the rs3762274 SNP (Table 3.5). None of the *LEP* SNPs were associated with FVC, FEV_1, or FEV_1/FVC (data not shown).

Since sex was a significant confounder in all the models, we investigated interaction effects of sex and age of follow-up in the four significant models (Table 3.6). We found that in all the four models, there was a significant difference in the rate of change in lung function over time between girls and boys. Girls had a lesser increase in FVC, FEV_1, and FEV_1/FVC compared to boys (Table 3.6). For example, when considering rs6669354, girls had an increase in FVC that was -0.090 liters lower than the increase in FVC in boys. Sex did not mediate the association between any of the *LEP* SNPs and any of the lung function values (data not shown). In addition, the child ever

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smoking or duration of breastfeeding also did not mediate the relationship between any of the *LEP* SNPs and any of the lung function values.

3.4 Discussion

The findings of this study suggest that single nucleotide polymorphisms (SNPs) of the leptin receptor play an important role in the body's lung volume and in airway obstruction. Our findings agree with Hansel et al that found an association between *LEPR* SNPs and lung function decline [54], although they tested different SNPs. Our findings, however, are not in agreement with van den Borst et al [52] who looked at one of our same significant *LEPR* SNPs (rs1137101) and found no association with FEV_1 or FVC. *LEPROT* and *LEPR* SNPs were associated with decreased lung function in boys and girls between ages 10 and 18 years. Lung function increased in both boys and girls with increased age; however, those with certain genotypes experienced less of an increase. For example, 207 children with the AC genotype of rs6669354 (19.5% for the SNP) had lower FVC and FEV_1 than those with the AA genotype (Tables 3.4 and 3.5). Likewise, 347 (30.2%) children with AA genotype of rs1137101 and 421 (37.8%) children with AA genotype of rs3762274 had lower FEV_1/FVC than those with the AG genotype. Furthermore, girls experienced significantly less increase in all lung function measures when compared to boys. None of the *LEP* SNPs were associated with lung function.

The leptin receptor protein is known to exist in six isoforms, LEPRa, LEPRb, LEPRc, LEPRd, LEPRe, and LEPRf. LEPRb is the [156] is the long form of the leptin receptor and is the one that the leptin protein attaches to and starts the JAK/STAT pathway that is involved in inflammation. It is not possible while looking at just *LEPR*

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SNPs, to determine what splicing will occur, subsequently resulting in the different forms of the leptin receptor. For this reason, it would be valuable also to consider the leptin receptor protein.

There were no differences between the whole population cohort and the sample used in this analysis when considering the confounding variables (Table 3.3). However, there was a significant difference in missing data in the follow-up data for socioeconomic status, maternal smoking during pregnancy, and whether the child ever smoked. The proportion of missing data is lower in the samples with lung function, hence it is unlikely that a non-response bias affected the analyses of the data. The significant difference in the missing data suggests that those who did not provide information on socioeconomic status, maternal smoking during pregnancy, and whether the child ever smoked, were also less likely to follow up with their lung function measurements later in the study. FVC and FEV_1 were collected under standard clinic conditions at ages 10 and 18-year visits, reducing the possibility for information bias. Due to the link between leptin and body mass index (BMI), we tested that BMI at ages 10 and 18 and the SNPs were not associated (Appendix A).

Understanding leptin's role in the body is difficult because the protein is involved in both the pro-inflammatory and anti-inflammatory response. Leptin up-regulates the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-12 [86,157], but also increases chemotaxis and natural killer cell functions [83]. The observed association between mutations at the coding and intron region of the *LEPR* gene being associated with lower FEV_1/FVC, suggest that these SNPs may affect the body's pro-inflammatory response. Leptin has to bind to its receptor in order to start the chain of events that lead

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the body's secretion of various cytokines [134,135,137,138,158]. An illuminating step would be to explore associations between these *LEPR* and *LEPROT* SNPs and the cytokines in the JAK-STAT pathway.

3.5 Conclusion

Our findings show that the *LEPROT* SNP rs6669354 and the *LEPR* SNPs rs1137101 and rs3762274 are associated with decreased lung function and that between the ages of 10 and 18 years, girls experience a smaller increase in lung function that boys. However, no difference was found between the SNP-lung function association between boys and girls. This shows the differences observed between the sexes in leptin and lung function between the ages of 10 and 18 may not be explained on the genetic level. It would be valuable to look directly at protein leptin receptor. It could also be important to consider associated between these *LEPROT* and *LEPR* SNPs and cytokines involved in the JAK/STAT pathway. Lastly, in our data LEP SNPs were not found to be associated with lung function values, suggesting that *LEPR* SNPs play a more important role in lung function.

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Table 3.1. Literature review of associations between SNPs in this study and lung function

***LEPR* SNP**	**Article**	**Findings**
rs8179183	Hansel, N.N., et al., *Leptin receptor polymorphisms and lung function decline in COPD.* Eur Respir J, 2009. **34**(1): p. 103-10.	Associated with reduced FEV_1.
	Loos, R.J., et al., *Polymorphisms in the leptin and leptin receptor genes in relation to resting metabolic rate and respiratory quotient in the Quebec Family Study.* Int J Obes (Lond), 2006. **30**(1): p. 183-90.	Homozygote had a lower respiratory quotient than the other genotypes.
rs1137100	van den Borst, B., et al., *Genetics of maximally attained lung function: a role for leptin?* Respir Med, 2012. **106**(2): p. 235-42.	No association found with FEV_1 or FVC.
	Hansel, N.N., et al., *Leptin receptor polymorphisms and lung function decline in COPD.* Eur Respir J, 2009. **34**(1): p. 103-10.	Associated with reduced FEV_1.
	Loos, R.J., et al., *Polymorphisms in the leptin and leptin receptor genes in relation to resting metabolic rate and respiratory quotient in the Quebec Family Study.* Int J Obes (Lond), 2006. **30**(1): p. 183-90.	Homozygote had a lower respiratory quotient than the other genotypes.
rs1137101	van den Borst, B., et al., *Genetics of maximally attained lung function: a role for leptin?* Respir Med, 2012. **106**(2): p. 235-42.	No association found with FEV_1 or FVC.
rs1782754	Hansel, N.N., et al., *Leptin receptor polymorphisms and lung function decline in COPD.* Eur Respir J, 2009. **34**(1): p. 103-10.	Associated with reduced FEV_1 in COPD patients.
rs3828934	Hansel, N.N., et al., *Leptin receptor polymorphisms and lung function decline in COPD.* Eur Respir J, 2009. **34**(1): p. 103-10.	Associated with reduced FEV_1 in COPD patients.

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Table 3.2. Population Characteristics

Variable	N	Mean	Median	p5, p95
Birth weight (kg)	1511	3.39	3.41	2.57, 4.20
Duration breastfeeding (weeks)	1345	14.28	8.00	0.00, 40.00
FVC at age 10 (L)	981	2.29	2.28	1.76, 2.88
FVC at age 18 (L)	838	4.61	4.47	3.22, 6.28
FEV_1 at age 10 (L)	980	2.03	2.01	1.58, 2.56
FEV_1 at age 18 (L)	839	4.01	3.91	2.85, 5.41
FEV_1/FVC at age 10	980	0.89	0.89	0.79, 0.97
FEV_1/FVC at age 18	839	0.87	0.88	0.75, 0.98
Height at age 10 (cm)	1043	138.93	138.70	129.10, 149.50
Height at age 18 (cm)	994	171.21	171.00	156.50, 187.00
BMI at age 10 (kg/m^2)	1043	18.11	17.35	14.71, 23.95
BMI at age 18 (kg/m^2)	964	23.19	22.15	18.20, 32.14
Age at 10-year follow up (years)	1536	10.02	10.00	9.57, 10.55
Age at 18-year follow up (years)	1255	17.91	17.72	17.17, 19.13
		N	%	
Sex	Male	786	51.17	
	Female	750	48.83	
Socio-economic status	High	111	8.18	
	Mid	1037	76.42	
	Low	209	15.40	
Parental asthma status	None	1208	80.37	
	Father	140	9.31	
	Mother	155	10.31	
In utero smoking exposure	Yes	384	25.25	
Child ever smoked	Yes	581	45.46	

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Table 3.3 Whole population cohort vs. sample with lung function data at ages 10 or 18

Variable		Whole Cohort	Sample with FEV_1 data at ages 10 or 18 years	P-Value
Variable		N, Mean; STD	N, Mean, STD	P-Value
Birth weight (kg)		1511, 3.39; 0.54	1102, 3.41, 0.51	0.34
Missing		25	18	
Duration breastfeeding (weeks)		1345, 14.28; 14.8	1031, 15.08, 14.96	0.19
Missing		191	89	
Height at age 10 (cm)		1043, 138.93; 6.18	1025, 138.90, 6.16	1.00
Missing		493	95	
Height at age 18 (cm)		994, 171.21; 9.45	918, 170.98, 9.30	1.00
Missing		542	202	
BMI at age 10 (kg/m^2)		1043, 18.11; 2.98	1025, 18.12, 2.98	1.00
Missing		493	95	
BMI at age 18 (kg/m^2)		964, 23.19; 4.33	896, 23.21, 4,33	0.92
Missing		572	224	
		N (%)	N(%)	
Sex	Male	786 (51.17)	557 (49.73)	0.48
	Female	750 (48.83)	563 (50.27)	
Socio-economic status	High	111 (7.23)	85 (7.59)	<.0000001*
	Mid	1037 (67.51)	849 (75.80)	
	Low	209 (13.61)	160 (14.29)	
	Missing	179 (11.65)	26 (2.32)	
Parental asthma status	None	1208 (78.65)	892 (79.64)	0.37
	Father	140 (9.11)	104 (9.20)	
	Mother	155 (10.09)	110 (9.82)	
	Missing	33 (2.15)	15 (1.34)	
In utero smoking exposure	Yes	384 (25.00)	253 (22.59)	0.05*
	Missing	15 (0.98)	4 (0.36)	
Child ever smoked	Yes	581 (37.83)	449 (40.09)	<.0000001*
	Missing	258 (16.80)	93 (8.30)	

*This significant difference is due to differences in frequency of missing data for these variables, not differences between the other groups.

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Table 3.4: *LEP*, *LEPR*, and *LEPROT* SNP characteristics

Gene	SNP	Genotype	N (%)	Location	Coordinate
		AA	156 (13.55)		
LEP	rs10249476	AC	542 (47.09)	Flanking_5UTR	128236973
		CC	453 (39.36)		
		AA	293 (25.46)		
LEP	rs11763517	AG	569 (49.44)	Intron	128250009
		GG	289 (25.11)		
		AA	238 (20.66)		
LEP	rs4731429	AG	582 (50.52)	Flanking_3UTR	128263486
		GG	332 (28.82)		
		AA	338 (29.57)		
LEP	rs10954176	AG	534 (46.72)	Flanking_3UTR	128267266
		GG	271 (23.71)		
		AA	580 (50.88)		
LEPR	rs3806318	AG	465 (40.79)	Flanking 5'UTR	65885357
		GG	95 (8.33)		
		AA	10 (0.86)		
LEPROT	rs9436738	AG	241 (20.81)	Intron	65888560
		GG	907 (78.32)		
		AA	101 (8.83)		
LEPROT	rs9436740	AT	446 (38.99)	Intron	65891901
		TT	597 (52.19)		
		AA	658 (57.17)		
LEPROT	rs9436301	AG	431 (37.45)	Intron	65895927
		GG	62 (5.39)		
		AA	243 (21.11)		
LEPROT	rs17412175	AT	560 (48.65)	Flanking 3'UTR	65904886
		TT	348 (30.23)		
		AA	175 (15.07)		
LEPROT	rs9436747	AG	512 (44.10)	Flanking 3'UTR	65911607
		GG	474 (40.83)		
		AA	214 (18.71)		
LEPROT	rs9436748	AC	556 (48.60)	Flanking 3'UTR	65911672
		CC	374 (32.69)		
		AA	836 (78.65)		
LEPROT	rs6669354	AC	207 (19.47)	Flanking 3'UTR	65925349
		CC	20 (1.88)		
LEPR	rs7526141	AA	256 (22.20)	Flanking 5'UTR	65975275
		AG	556 (48.22)		

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		GG	341 (29.58)		
		AA	32 (2.78)		
LEPR	rs1171275	AG	334 (29.04)	Flanking 5'UTR	65982633
		GG	784 (68.17)		
		AA	590 (50.82)		
LEPR	rs1782754	AG	473 (40.74)	Flanking 5'UTR	65993348
		GG	98 (8.44)		
		AA	592 (51.30)		
LEPR	rs1137100	AG	465 (40.29)	Coding	66036441
		GG	97 (8.41)		
		AA	580 (50.70)		
LEPR	rs3790424	AG	457 (39.95)	Intron	66044013
		GG	107 (9.35)		
		AA	791 (69.45)		
LEPR	rs10493380	AC	317 (27.83)	Intron	66046117
		CC	31 (2.72)		
		AA	37 (3.20)		
LEPR	rs12059300	AG	378 (32.70)	Intron	66047072
		GG	741 (64.10)		
		AA	347 (30.15)		
LEPR	rs1137101	AG	558 (48.48)	Coding	66058513
		GG	246 (21.37)		
		AA	777 (67.80)		
LEPR	rs3828034	AG	330 (28.80)	Intron	66062325
		GG	39 (3.40)		
		AA	421 (37.83)		
LEPR	rs3762274	AG	525 (47.17)	Intron	66064113
		GG	167 (15.00)		
		CC	37 (3.23)		
LEPR	rs8179183	CG	320 (27.97)	Coding	66075952
		GG	787 (68.79)		
		AA	159 (13.96)		
LEPR	rs6678033	AG	533 (46.80)	Intron	66077624
		GG	447 (39.24)		
		AA	38 (3.27)		
LEPR	rs17415296	AC	332 (28.55)	3' UTR	66099013
		CC	793 (68.19)		

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Table 3.5. *LEPR* and *LEPROT* SNPs associated with lung function measures

Variable	Genotype	Estimate	Standard Error	F-test P-value	FDR adjusted P-value
Associated with FVC (Liters)					
rs6669354[a]	AC	-0.092	0.028		
	CC	0.16	0.097	0.0010	0.01500
	AA	REF	REF		
Associated with FEV_1 (Liters)					
Variable	Genotype	Estimate	Standard Error	F-test P-value	FDR adjusted P-value
rs6669354[b]	AC	-0.10	0.026		
	CC	-0.021	0.090	0.0007	0.01500
	AA	REF	REF		
Associated with FEV_1/FVC (Liters)					
Variable	Genotype	Estimate	Standard Error	F-test P-value	FDR adjusted P-value
rs1137101[c]	AA	-0.012	0.0046		
	GG	0.0053	0.0051	0.0055	0.04125
	AG	REF	REF		
rs3762274[d]	AA	-0.013	0.0042		
	GG	0.0031	0.0057	0.0024	0.02400
	AG	REF	REF		

a, b: Controlled for age at each follow up, sex, height at ages 10 and 18, and BMI at ages 10 and 18.

c: Controlled for age at each follow up, sex, whether the child ever smoked, height and BMI at ages 10 and 18, and the duration of breastfeeding.

d: Controlled for age at each follow up, sex, whether the child ever smoked, and height and BMI at ages 10 and 18.

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Table 3.6. Interaction effect of sex and age of follow up on lung function measures

	Variable	Genotype	Estimate	Standard Error	F-test P-value	FDR adjusted P-value
Associated with FVC (Liters)						
Model 1	rs6669354[a]	AC vs AA	-0.098	0.027	0.00017	
		CC vs AA	0.18	0.093		
	Age at follow up (years)		0.15	0.0085	<0.0000001	
	Sex Female vs. Male		0.76	0.065	<0.0000001	
	Age*Sex (Female vs. Male)		-0.090	0.0045	<0.0000001	<0.0000001
Associated with FEV_1 (Liters)						
Model 2	rs6669354[b]	AC vs AA	-0.11	0.025	0.0001	
		CC vs AA	-0.0020	0.086		
	Age at follow up (years)		0.13	0.0077	<0.0000001	
	Sex Female vs. Male		0.71	0.058	<0.0000001	
	Age*Sex (Female vs. Male)		-0.078	0.0044	<0.0000001	<0.0000001
Associated with FEV_1/FVC (Liters)						
Model 3	rs1137101[c]	AA vs AG	-0.012	0.0046	0.0041	
		GG vs AG	0.0053	0.0051		
	Age at follow up (years)		0.0032	0.0014	0.0483	

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	Sex Female vs. Male		0.037	0.010	0.0004	
	Age*Sex (Female vs. Male)		-0.0018	0.00077	0.0199	0.0199
Model 4	rs3762274[d]	AA vs AG	-0.013	0.0042	0.0017	
		GG vs AG	0.0027	0.0057		
	Age at follow up (years)		0.0037	0.0014	0.0166	
	Sex Female vs. Male		0.037	0.010	0.0003	
	Age*Sex (Female vs. Male)		-0.0019	0.00075	0.0127	0.0199

a, b: Also controlled for height at ages 10 and 18, and BMI at ages 10 and 18.
c: Also controlled for whether the child ever smoked, height and BMI at ages 10 and 18, and the duration of breastfeeding.
d: Also controlled for whether the child ever smoked, and height and BMI at ages 10 and 18.

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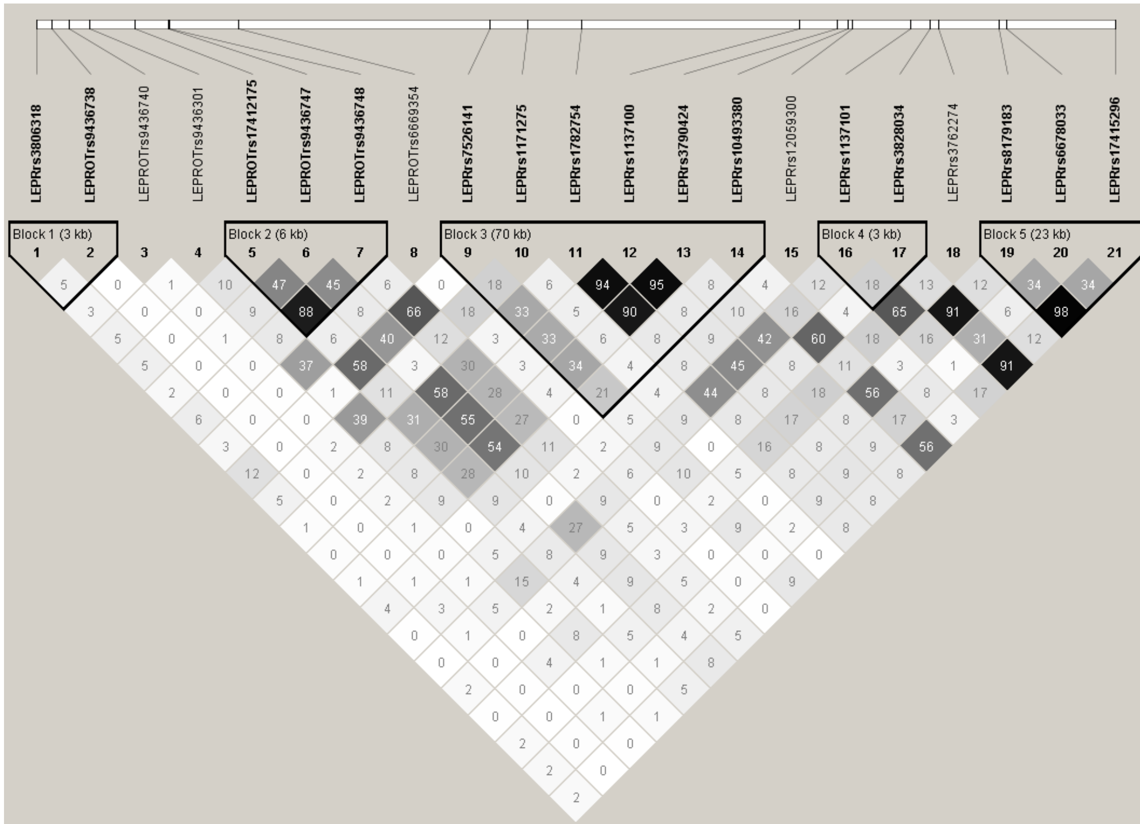

Figure 3.1 Haplotype analysis of *LEPR* and *LEPROT* genes (r^2)

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CHAPTER 4

RESULTS II – MANUSCRIPT #2

LEP SNPS AND DNA METHYLATION INFLUENCE SERUM LEPTIN LEVELS IN BOYS AND GIRLS: TWO-STAGE MODEL OF EPIGENETIC ANALYSIS[2]

4.1 INTRODUCTION

Leptin is a 16kDa pleiotropic cytokine that plays a role in many physiological processes in the body [159-162] and is intimately linked to the body's host inflammatory response [132,133,163]. The levels of circulating leptin in the body affect subsequent biological pathways. For example, with moderate leptin levels Th1/Th2/Treg cells are in equilibrium indicating an optimal immune response. However, when the body has low amounts of leptin (hypoleptinaemia), Th1 cells are impaired and Treg cells proliferate, leading to suppressed immune response and increased infections and disease susceptibility (such as tuberculosis, candida, and pneumonia). If the body has an excess of leptin (hyperleptinaemia), Treg cells are reduced and Th2 cells increase secretion of proinflammatory cytokines, which can influence obstructive lung diseases such as asthma [5,22]. It is therefore important to understand what factors can influence leptin levels in the body.

Genetic variations in the leptin gene have been associated with protein leptin levels. Numerous candidate gene studies looking at single nucleotide polymorphisms

[2] M. Yousefi, W. Karmaus, H. Zhang, M Torres, J Burch, E Schauberger. *LEP* SNPs and DNA methylation influence serum leptin levels in boys and girls: Two-Stage model of epigenetic analysis. To be submitted.

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(SNPs) have detected associations between G-2548A (also known as rs7799039, located in the promoter region of the gene) and decreased leptin levels [164-169]. Conversely, Fourati et al found that two *LEP* SNPs in the promoter region of the gene (H1328084 and A19G) were associated with increased leptin levels [62]. In a genome-wide meta-analysis, questions arose about the association of *LEP* SNPs with circulating leptin levels. The *LEP* SNP, rs10487505 (located in the intron region), was associated with decreased leptin levels, but was not associated with *LEP* messenger RNA expression in the omental or subcutaneous adipose tissue, liver, lymphocytes, or the brain. This suggested that other mechanisms may be at play in mediating the association between *LEP* SNPs and leptin levels [170].

A possible such mediator (and effect modifier) could be DNA methylation of *LEP* gene, which may influence the expression of leptin protein. Few studies have looked at DNA methylation in the leptin gene and only one study has investigated the association between *LEP* DNA methylation and leptin protein levels. Hillemacher et al found that *LEP* DNA methylation in the promoter region of the gene was associated with increasing serum leptin levels in alcohol dependent patients who were going through withdrawal [68].

Since the process of DNA methylation begins *in utero*, it is helpful to look at environmental exposures that occur during pregnancy. In a previous study using data from the Isle of Wight birth cohort study, the interaction between maternal smoking during pregnancy and leptin receptor (*LEPR*) SNPs was associated with DNA methylation in the offspring [61]. We followed the same analytical steps using *LEP* SNPs and *LEP* DNA methylation levels. Specifically, we used data from the Isle of Wight Birth Cohort Study to investigate a two-stage explanatory model: First, *LEP* SNPs have the

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potential to constitute methylation quantitative trait loci (methQTLs), i.e., specific genotypes of SNPs may influence the susceptibility of specific gene loci to change the level of DNA-methylation. In addition, this susceptibility may be modified by environmental exposures. Thus, SNPs may interact, for instance, with *in utero* smoking exposure. Second, we tested to see if the DNA methylation sites (CpG sites: locations on a DNA sequence where hydrogen groups are replaced with a methyl group) that are identified as being related to methQTLs were associated with serum leptin concentrations at age 18 years. We also examined whether the methQTLs findings could be replicated in the F2 population of the three generation Isle of Wight birth cohort. This was the first study to test the association of *LEP* methylation sites with *LEP* SNPs and leptin protein levels.

4.2 METHODS

4.2.1 STUDY POPULATION AND CHARACTERISTICS

4.2.1A F1 POPULATION

Between January 1989 and February 1990, 1536 F1-children born on the Isle of Wight (IOW), UK were recruited and interviewed with 1456 available for further follow-up in a longitudinal study. The local research ethics committee approved the study and informed written parental consent was obtained from all participants at recruitment and subsequently at each follow-up. The IOW birth cohort has been described in detail elsewhere [61,152]. Briefly, upon delivery, birth weight was measured and data from birth records and questionnaires were collected, including information on maternal smoking during pregnancy. Children were followed up at the ages of 1 (n=1167, 80.2%), 2

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(n=1174, 80.6%), 4 (n=1218, 83.7%), 10 (n=1373, 94.3%) and 18 years (n=1313, 90.2%). Data was collected at each follow up time via interviews and examinations. When a visit was not possible, a telephone questionnaire was completed or a postal questionnaire was sent. Information on maternal smoking during pregnancy was obtained from mothers at the time of birth.

Leptin concentrations were obtained from blood samples collected from F1-children at age 18 years. Aliquots of blood serum isolated from the blood samples were used in enzyme-linked immunosorbent assays from Biokit, S.A. (Barcelona, Spain). The assays were conducted according to the manufacturer's kit instructions. Each sample, including standards and the blank, was assayed in duplicate.

DNA was extracted from blood or saliva samples from cohort subjects (n=1,211). Details of the procedure have been described elsewhere [61,171]. Four *LEP* SNPs (rs4731429, rs10249476 rs10954176, and rs11763517) were genotyped in the F1 population. For measuring methylation levels, DNA was extracted from whole blood collected at age 18 years from 245 female offspring and 125 male offspring. DNA was extracted from peripheral blood samples collected from the F_1 generation at age 18, during pregnancy, and from cord blood of F_2 at birth. DNA methylation levels were measured using the Illumina 450K platform and quantified using β values that present the proportion of methylated (M) over the sum of methylated and unmethylated (U) allele intensities (β=M/ [c+M+U]), where c is a constant to prevent zero in the denominator [172]. The β values were employed as predictors of asthma or wheezing; however, for methylation analysis we used M-values (logit-transformed β values) as the response variable since β values are often heteroscedastic.[173]

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4.2.1B F2 POPULATION

These F_1 participants themselves became pregnant or fathered a child, who was then enrolled in a follow-up study. Cord blood was collected from the F2-offspring for DNA methylation; however, cord blood samples could not be collected in all deliveries. SNPs in the F_2 generation were genotyped on Illumina's HumanOmniExpressExome beadchip (v1.2) for more than 960k loci. Arrays were processed at the Wellcome Trust Centre for Human Genetics (Oxford, UK). Genotypes were extracted from image data using GenomeStudio software (Illumina). SNPs located in or near IL1RL1 were identified using the Illumina annotations from the genotyping chip's annotation file (available at: http://support.illumina.com/downloads/humanomniexpressexome-8v1-2_product_support_files.html).

4.2.2 STATISTICAL ANALYSIS

4.2.2A F1 POPULATION

Haplotype analysis performed on the *LEP* SNPs in the F1 generation of this cohort in a previous study by Mukherjee et al [128] showed that two of the SNPs present in the F1 generation data had been in LD with each other: rs10249476 and rs11763517. Therefore, in our analysis involving *LEP* SNPs in the F1 generation we included: rs11763517, rs4731429, and rs10954176.

After cleaning the DNA-M data, beta (β) values were presented as the proportion of methylated (M) over methylated (M) and unmethylated (U) sites ($\beta = M/[c+M+U]$, with c being constant to prevent dividing by zero) were used to estimate the effect of DNA-M

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[51]. M-values were then calculated using the logit-transformed β values ($\log_2 \beta/(1-\beta)$). The M-values of the methylation of seven CpG sites (six at the start and one at the end of the *LEP* gene) were analyzed in this study.

In Stage 1, to identify methQTLs modified by gestational maternal smoking, modeling was performed by using *LEP* SNPs and their interaction with in utero smoking exposure to predict the methylation of *LEP* CpG sites. Each CpG (M-values) was modeled against rs11763517, rs4731429, and rs10954176, with each SNP interacting with *in utero* smoking exposure. In addition, all models were controlled for cell-composition to ensure that we were indeed observing associations with DNA methylation un-confounded by the proportion of these cells. We controlled for levels of the following cells: b cells, granulocytes, monocytes, natural killer cells, eosinophils, and CD4 T-cells. These cell counts were obtained from breast cancer patients from the Winship Cancer Institute [129], 61 subjects at baseline and 39 subjects at six-month-follow-up. We followed the method used by Kaushal et al [130] where 484,489 CpG sites were tested against cell types. The most parsimonious model was determined via backward elimination using the 10% rule, first by removing interaction terms followed by individual SNPs. False discovery rate was used to adjust for multiple testing.

Stage 2: Next, the selected CpG sites (M-values) were tested on whether they modified the association that *LEP* SNPs had with leptin serum concentrations at age 18. In this step, our focus was on the interaction effects and false discovery rate was applied to correct for multiple testing among the tests for interaction effects between CpG sites and genetic variants. For both sets of statistical analyses, the GLM procedure was used in SAS 9.4 (SAS, Gary, NC, USA). In addition, because leptin levels vary greatly between

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boys and girls following puberty, all models initially controlled for sex as well. As the proportion of girls in the subsample with DNA-methylation was higher than the proportion of, the analyses were weighted for sex. Since the same SNP (rs4731429) that was a methQTL for cg00840332 was also a genetic modifier in the association between DNA-M and leptin levels, we calculated the residuals from the association between rs4731429 and cg00840332 using linear regression analyses. These residuals are no longer affected by rs4731429 and can be used as independent variable to test interaction. The residuals were then used in testing direct and interactive effects of DNA-M on leptin levels.

4.2.2B F2 POPULATION

The methQTL analysis was replicated in the F2 population. Eighteen *LEP* SNPs were genotyped in the F2 population but only 5 SNPs (rs2167270, rs2278815, rs11760956, rs11763517, rs12706832) proved to have polymorphisms (the others were monomorph, with all or almost all the population having one of the possible genotypes). Haplotype analysis using Haploview software [125] revealed that SNPs rs2278815 and rs12706832 were in LD with each other while rs11763517 and rs11760956 were in LD with each other (Figure 4.1). Therefore, the methQTL analysis included the following SNPs interacting with in utero smoking exposure predicting DNA methylation levels: rs2278815, rs11763517, and rs2167270. While the SNPs in the F1 population were collected via selected probes, a commercial genome wide product was used in the F2 population. This led to the following SNPs, rs2167270, rs2278815, rs11760956, and rs12706832 not being identical with the SNPs identified in the F1 population. Hence, in order to see if the findings in the F2 population are based on closely related SNPs, we

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generated a second LD plot using the International Haplotype Map (HapMap) project data from the Central European University database [131] (Figure 4.2).

4.3 Results

4.3.1 F1 Generation Cohort

Of the 1,152 participants from the whole birth cohort who had SNP data, 370 also had DNA methylation data. There was no difference in the frequency of *LEP* SNP alleles between the sub and whole populations (Table 4.1). There was also no difference in the percentage of participants who were exposed to *in utero* smoking or in leptin levels at age 18. Because of interest of the parent study in the female line, more female participants with DNA-methylations were selected for DNA-methylation analysis (66.2%). Girls had higher leptin levels at age 18 (19.2 ng/mL) than boys (4.0 ng/mL, Table 4.1).

The four *LEP* SNPs in the F1 generation spanned the length of the leptin gene, with one SNP in the flanking 5'UTR region, one in the intron region, and two in the Flanking 3'UTR region (Table 4.3). *LEP* SNPs rs10249476 and rs11753517 were in linkage disequilibrium with each other [128]. Therefore, the models predicting DNA methylation levels used the following SNPs: rs11763517, rs4731429, and rs10954176.

There were seven DNA methylation sites that were examined in the F1 generation, which were located at the start and end of the leptin gene, but none in the middle of the gene (Table 4.4). All but two of the CpG sites presented high DNA methylation levels (β-levels >0.90) while cg00840332 and cg00666422 showed low (β-levels: average 0.16) and medium (average 0.46) methylation, respectively (Table 4.4).

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In utero smoking exposure did not play a role in the association between *LEP* SNPs and *LEP* DNA methylation levels.

Three CpG sites were observed to have methylation quantitative trait loci (methQTL) (Table 4.5). Those with the AA and AG alleles of the rs4731429 SNP were associated with a higher proportion of DNA methylation at the cg00666422 and cg00840332 sites than those with the GG allele. Both the cg00840332 and cg00666422 methylation sites are located at the start of the *LEP* gene (TS200 CpG island and 5'UTR region respectively, Table 4.4) while the rs4731429 SNP is located at the 3'UTR region of the gene (Table 4.3a). Both of these methylation sites are also in cis association with rs4731429 since they are less than 1Mb from each other [82], Tables 4.2 and 4.3. *LEP* SNP rs4731429 also acted as a methQTL for cg24862443 (located at 3'UTR, Table 4.4), but the association is in the opposite direction: those with the AA and AG genotype of the SNP are associated with decreased proportion of methylation at that CpG site. The same direction of association was observed for rs11763157, which was also a methQTL for cg24862448. Sex only played a role in the cg00666422 methylation site. Boys had 0.17 higher proportion of methylation (β-levels) than girls at the cg00666422 methylation site (Table 4.5). Because of the higher proportion of girls than boys in this subsample, sex was weighted in the analysis.

One CpG site associated with a methQTL, cg00840332, was associated with leptin levels at age 18 years. This association was modified by rs4731429, which was the same SNPs that was a methQTL for the cg00840332 CpG site. Therefore, we calculated the residuals from the association between rs4731429 and cg00840332, and used those residuals in place of the cg0040332 in the model predicting leptin levels at age 18 (Table

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4.7). Among those with the AA genotype of the rs4731429 and rs11763517 SNPs, increased methylation at cg00840332 was associated with decreased protein leptin levels by 20.4ng/mL and 16.4 ng/mL respectively (Table 4.7).

4.3.2 Replication Analysis of MethQTLs in F2 Population Cohort

Regarding the 419 children enrolled thus far in the F2 population cohort, cord blood samples could not be collected from all children. Of the F2-children 139 also had SNP data, and 125 people had SNP and DNA methylation data. There was no difference in *LEP* SNP frequencies, sex, and *in utero* smoking exposure between the whole population cohort and the children who had SNP data (Appendix B). There was also no difference between the population with only SNP data and the population with SNP and DNA methylation data (Appendix B). After removing monomorphic genetic markers (no genetic polymorphisms), there were 5 *LEP* SNPs in the F2 population, with most of the children having the heterozygous genotypes (Table 4.2). The SNPs in the F2 cohort consisted of one in the exon region and the rest in the intron region of the gene (Table 4.3). Of these 5 SNPs, rs2278815 and rs1276832 were in linkage disequilibrium with each other, rs11763517 and rs11760956 also were in linkage disequilibrium, (Figure 4.1). Even though only one of the *LEP* SNPs (rs11763517) in the F2 generation was the same as those analyzed in the F1 generation, other F2 and F1 SNPs were in linkage disequilibrium with each other (Figure 4.2). Namely, rs4731429 and rs11760956 were in the same haplotype block (Figure 4.2). Therefore, we could test whether we could replicate the methQTL results found in the F1-generation. The results show that those with the AA an AG genotypes of rs11760956 had a higher proportion of methylation at

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cg00840332 than those with the GG genotype. This mirrors the findings of rs4731429 being a methQTL for the same CpG site in the F1 population.

4.4 Discussion

First, our findings show an association between *LEP* SNPs and DNA-methylation in the F1-generation that could (partially) be replicated in the F2-generation. Those with the AA and AG genotypes of the rs4731429 SNP were more susceptible to DNA methylation at the cg00666422 and cg00840332 sites of the leptin gene (promoter regions of the gene). Conversely, those with the AA and AG genotypes of the rs4731429 were less susceptible to DNA methylation at the 3'UTR region of the gene, CpG site cg24862443 (Table 4.5). The findings at the cg00840332 CpG site were replicated in the subsequent generation (Table 4.6). As DNA methylation increased at the cg00840332 site, those with AA genotype of the rs11763517 SNP had lower leptin levels at age 18 (Table 4.7). Second, our results show that associations between genetic polymorphisms and protein leptin levels are modified by *LEP* DNA methylation. The F2 population did not have data on leptin levels, so the second part of the analysis could not be replicated in the subsequent generation. When comparing models of SNPs and CPG sites predicting protein leptin levels at age 18, SNPs and proportion of methylation indicate the same level of fit (R^2=0.17) while the SNP-CPG interaction model shows a slightly stronger fit (R^2=0.20).

It is unlikely that selection bias occurred in this study because the 370 participants in the F1 cohort and the 125 participants in the F2 cohort were selected due to study interests and do not deviate from their respective whole populations. For instance, there

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were no differences between the frequencies of *LEP* SNPs between the sample and whole populations in both the F1 and F2 generations. There were also no differences in the proportion of people who were exposed to *in utero* smoking. Using the Infinium Human Methylation 450 array in the collection of DNA methylation data reduces the possibility of information bias because it has strong reproducibility and validity [120,121].

As expected, leptin levels were higher in girls than boys at age 18 years (Table 4.1). However, this difference was not overwhelmingly reflected in DNA methylation levels. We did not see a significant association when testing the interaction of SNPs and sex on DNA methylation. Only one of the models for methQTL analysis showed that sex had a significant effect on the association between *LEP* SNPs and LEP DNA methylation: girls had lower DNA methylation at cg00840332 than boys. Hence, sex was a confounder in the association between *LEP* DNA methylation and protein leptin levels, but this association was not modified by sex. The investigated *LEP* SNPs are likely to be representative of the leptin gene since they spanned the length of the gene (Table 4.3a) and were in linkage disequilibrium with SNP data used in the International HapMap project (Figure 4.2). However, the CpG sites used were mostly from the promotor region of the region, with only one site at the 3'UTR region and no sites from coding on intron regions of the gene (Table 4.4). It would be helpful for subsequent studies to investigate the effects of DNA methylation on more regions of the *LEP* gene.

4.5 Conclusion

This study suggests that genetic variants of the LEP gene alter LEP DNA methylation patterns in two consecutive generations, and that these methylation patterns

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in turn alter protein leptin levels in the F1 generation. Our findings show that SNPs and DNA-M equally explain the variations in protein leptin levels but do not explain the difference in leptin levels between boys and girls. When leptin binds to the leptin receptor, it activates the Janus Kinas 2/Signal transducer and activator of transcription (JAK2/STAT) pathway that leads to the release of cytokines involved in the body's immune response. A next step would be to investigate if there are other genes involved in the JAK/STAT pathway and in protein leptin levels that could explain the differences observed between boys and girls.

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Table 4.1. Population characteristics for F1 generation cohort

		Sub population with DNA methylation data	Whole cohort population with SNP data	P-Value
		N=370	N=1152	
SNP	**Genotype**	**N (%)**	**N (%)**	
rs4731429	AA	76 (22.1)	238 (20.7)	0.50
	AG	174 (50.6)	582 (50.5)	
	GG	94 (27.3)	332 (28.8)	
rs10249476	AA	49 (14.2)	156 (13.6)	0.19
	AC	177 (51.3)	542 (47.1)	
	CC	119 (34.5)	453 (39.4)	
rs10954176	AA	93 (27.11)	338 (29.6)	0.50
	AG	164 (47.81)	534 (46.7)	
	GG	86 (25.07)	271 (23.7)	
rs11763517	AA	76 (21.9)	293 (25.5)	0.25
	AG	179 (51.6)	569 (49.4)	
	GG	92 (26.5)	289 (25.1)	
Variable		**N (Mean, Median; 5, 95%)**	**N (Mean, Median; 5, 95%)**	**P-Value**
Sex	Male	125 (33.8)	577 (50.1)	<.0000001
	Female	245 (66.2)	575 (49.9)	
In utero smoking exposure (Yes)		75 (20.4)	266 (23.2)	0.26
Leptin at age 18 in boys (ng/mL)		120 (4.0, 1.2; 0.40, 16.0)	270 (4.0, 1.2; 0.40, 14.0)	
Leptin at age 18 in girls (ng/mL)		239 (19.2, 13.1; 2.8, 54.6)	245 (18.7, 13.1; 2.4, 54.3)	

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Table 4.2. Population characteristics for F2 generation cohort

	Sub population with SNP and DNA methylation data	
	N=125	
SNP	**Genotype**	**N (%)**
rs2167270	AA	9 (7.2)
	AG	61 (48.8)
	GG	55 (44.0)
rs2278815	AA	45 (36.0)
	AG	62 (49.6)
	GG	18(14.4)
rs11760956	AA	9 (7.2)
	AG	63 (50.4)
	GG	53 (42.4)
rs11763517	AA	34 (27.2)
	AG	64 (51.2)
	GG	27 (21.6)
rs12706832	AA	18 (14.4)
	AG	62 (49.6)
	GG	45 (36.0)
Variable		**N (%)**
Sex	Male	64 (51.2)
	Female	61 (48.8)
In utero smoking exposure	No	77 (64.2)
	Yes	43 (35.8)

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Table 4.3. Location of *LEP* SNPs in F1 and F2 generation cohorts

F1 generation			F2 generation		
SNP	Location	Chromosome: Coordinate	SNP	Location	Chromosome: Coordinate
rs10249476	Flanking 5'UTR	7: 127877026	rs2167270	Exon	7: 127881349
rs11763517	Intron	7: 127890062	rs2278815	Intron	7: 127881851
rs4731429	Flanking 3'UTR	7: 127903539	rs12706832	Intron	7: 127887139
rs10954176	Flanking 3'UTR	7: 127907319	rs11763517	Intron	7: 127890062
			rs11760956	Intron	7: 127891087

*Using coordinates from the GRCh37 assembly

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Table 4.4. Distribution of DNA methylation of CPG sites on the *LEP* gene for F1 and F2 generations (β methylation levels)

			F1 generation				F2 generation			
CpG site	Location	Chromosome: Coordinate	N	Mean	5%	95%	N	Mean	5%	95%
cg14734794	TSS1500	7:127879921	370	0.96	0.95	0.97	125	0.92	0.90	0.95
cg00840332	TSS200	7:127881270	370	0.16	0.10	0.23	125	0.084	0.049	0.13
cg00666422	5'UTR	7:127881441	370	0.46	0.37	0.54	125	0.44	0.38	0.50
cg12083122	5'UTR	7:127883820	370	0.95	0.94	0.97	125	0.91	0.89	0.93
cg25435800	5'UTR	7:127890194	370	0.93	0.90	0.96	125	0.89	0.85	0.93
cg25730670	5'UTR	7:127891366	370	0.95	0.93	0.96	125	0.90	0.87	0.92
cg24862443	3'UTR	7:127896860	370	0.94	0.92	0.95	125	0.88	0.86	0.90

*Using coordinates from the GRCh37 assembly

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Table 4.5. Methylation quantitative trait loci analysis for F1 generation cohort (M-values)

Variable		Estimate	STD	T -test P-value	F-test P-value	FDR Adjusted P-Value
			*Predicting methylation site: cg00666422**			
rs11763517	(AA vs GG)	-0.12	0.06	0.036	0.10	0.10
	(AG vs GG)	-0.05	0.05	0.24		
rs4731429	(AA vs GG)	0.13	0.06	0.03	0.005	0.006
	(AG vs GG)	0.14	0.04	0.001		
			Predicting methylation site: cg00840332			
rs4731429	(AA vs GG)	0.31	0.07	0.000002	0.00001	0.00005
	(AG vs GG)	0.15	0.06	0.006		
Sex	Male vs. Female	0.17	0.06	0.01	0.007	N/A
			*Predicting methylation site: cg24862443**			
rs11763517	(AA vs GG)	-0.14	0.04	0.001	0.005	0.006
	(AG vs GG)	-0.05	0.03	0.095		
rs4731429	(AA vs GG)	-0.14	0.04	0.0014	0.0046	0.006
	(AG vs GG)	-0.08	0.03	0.01		

*Models controlled for concentrations of the following cells: b cells, granulocytes, monocytes, natural killer cells, eosinophils, and CD4t cells.

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Table 4.6. Methylation quantitative trait loci analysis for F2 generation cohort (M-values)

Variable		Estimate	STD	T -test P-value	F-test P-value
*Predicting methylation site: cg00666422**					
rs11760956	(AA vs GG)	0.20	0.08	0.01	0.03
	(AG vs GG)	0.13	0.06	0.02	
rs2167270	(AA vs GG)	0.23	0.10	0.02	0.006
	(AG vs GG)	0.17	0.05	0.002	
*Predicting methylation site: cg00840332**					
rs11760956	(AA vs GG)	0.51	0.16	0.0019	0.0002
	(AG vs GG)	0.30	0.08	0.0004	

*Models controlled concentrations of the following cells: b cells, granulocytes, monocytes, natural killer cells, eosinophils, and CD4t cells.

Note rs11760956 in the F2-generation and rs4731429 in the F1 generation are in linkage disequilibrium, presenting the same haplotype block of the *LEP* gene.

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Table 4.7. *LEP* DNA methylation predicting protein leptin levels (ng/mL) at age 18 in the F1 generation*

		Estimate	STD	T- test P-value	F-test P-value	FDR Adjusted P-value
Cg00840332**		11.3	8.3	0.18	0.016	N/A
rs11763517	AA vs GG	7.7	3.7	0.04	0.11	N/A
	AG vs GG	3.2	2.8	0.25		
Cg00840332** rs11763517	AA vs GG	-16.4	7.8	0.037	0.01	0.02
	AG vs GG	-0.20	6.8	0.98		
rs4731429	AA vs GG	7.7	3.6	0.03	0.07	N/A
	AG vs GG	1.4	2.7	0.6		
Cg00840332** rs4731429	AA vs GG	-20.4	8.9	0.02	0.028	0.028
	AG vs GG	-14.7	5.9	0.01		
Sex	Male vs. Female	-17.5	2.4	<.0000001	<.0000001	N/A

**This model is also controlled for the following cells: b cells, granulocytes, monocytes, natural killer cells, eosinophils, and CD4t cells.
**We are using the residuals of DNA-methylation subtracting the effects of methQTL SNPs

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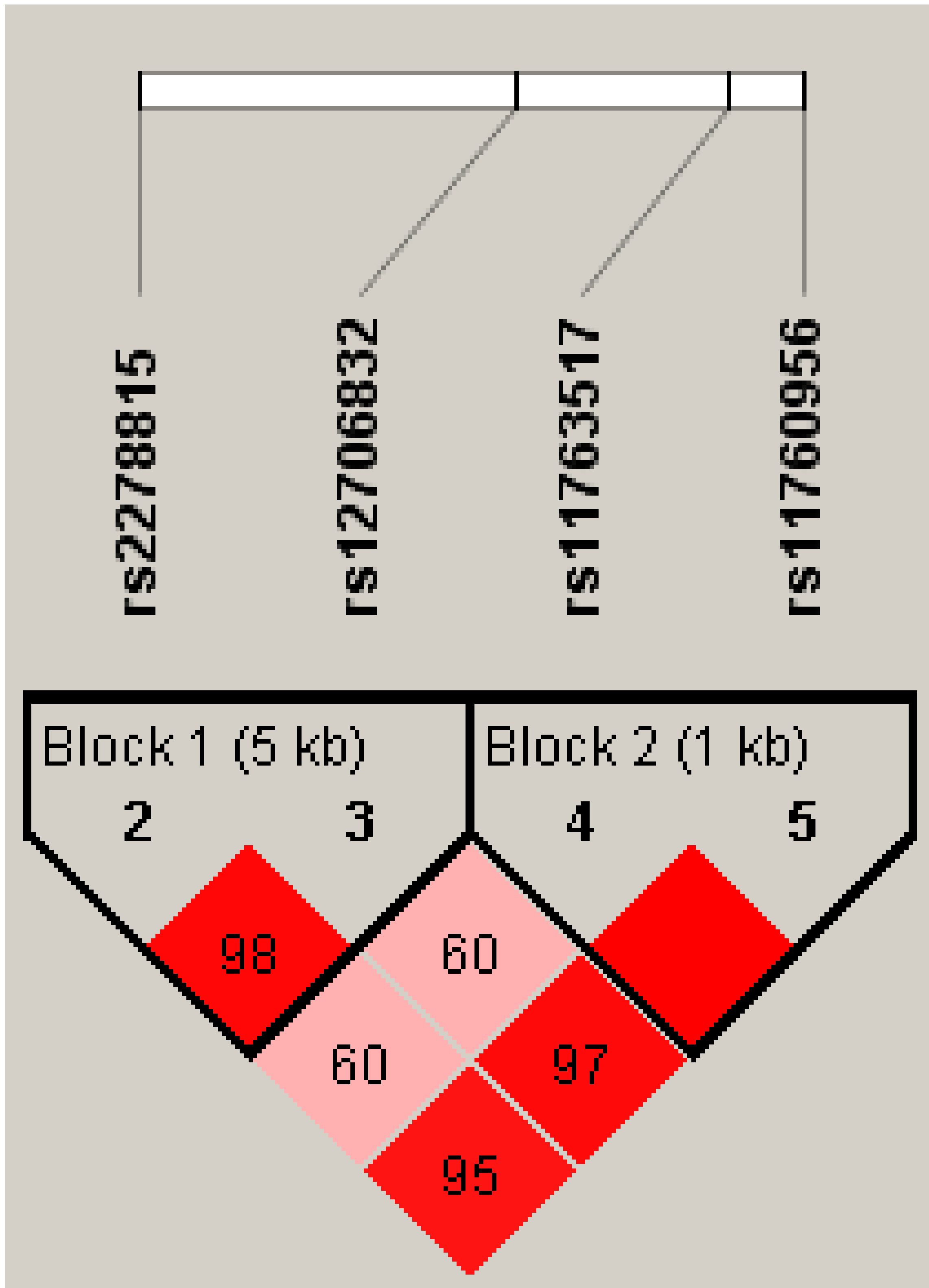

Figure 4.1. Linkage disequilibrium plot of *LEP* SNPs in F2 cohort, D' values

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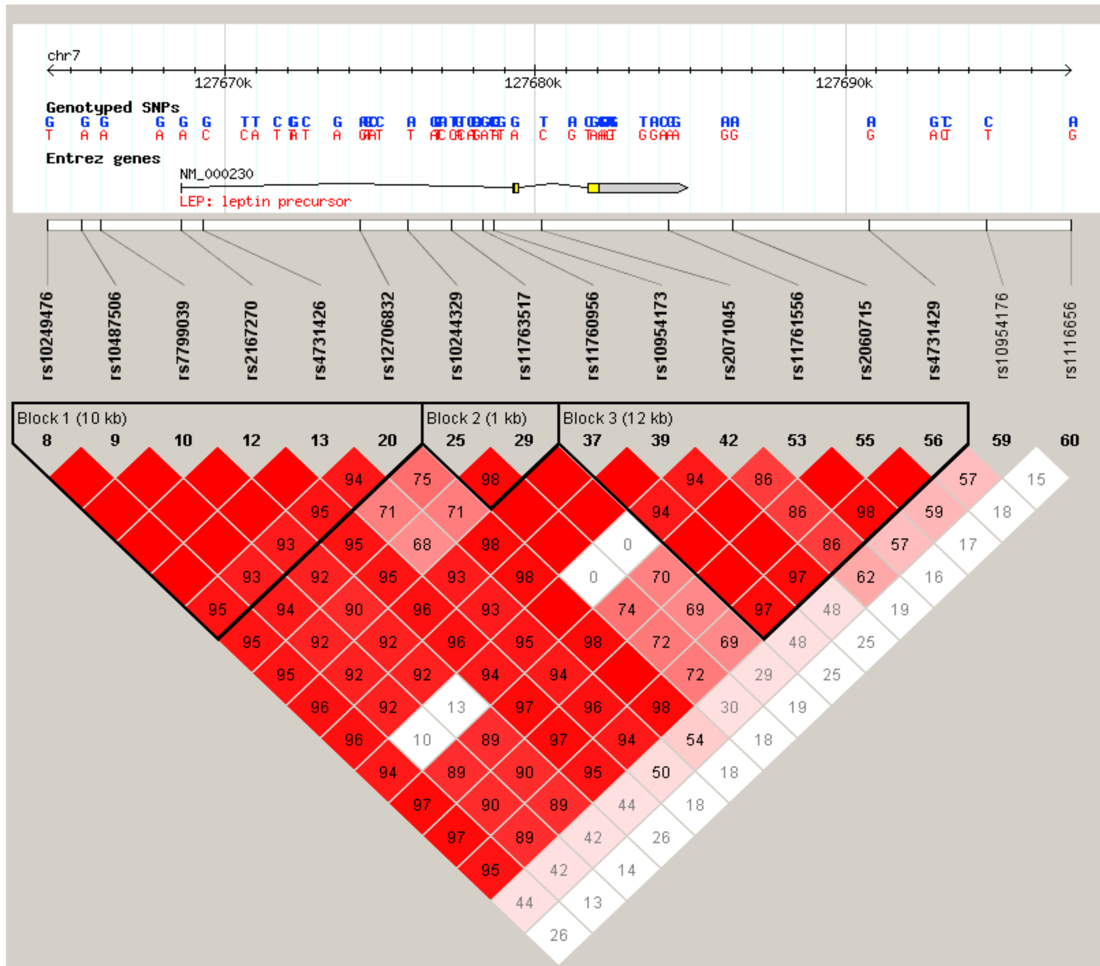

Figure 4.2. Linkage disequilibrium plot of *LEP* SNPs from HapMap data

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CHAPTER 5

RESULTS III - MANUSCRIPT #3

LEPTIN ASSOCIATED WITH LUNG FUNCTION INDEPENDENT OF BODY MASS INDEX[3]

5.1 INTRODUCTION

Historically leptin has been identified as regulator of appetite and energy expenditure and is known for its relationship with obesity [1]. However, over time it has been established as a pleiotropic hormone that is responsible for many processes in the body [3-20,22,24]. Leptin was initially believed to be secreted only from adipose tissue, but in consequent years it has also been detected in lower concentrations in peripheral lung tissue in mice (bronchial epithelial cells, alveolar type II pneumocytes, and lung macrophages) [174]. Leptin is also thought to play a key role in lung maturation and to be a stimulant of ventilation [29,31,38]. In addition, the adipose tissue, initially believed to be inert, is now known to be the site of release of numerous pro-inflammatory and anti-inflammatory cytokines [28]. Therefore, leptin is now considered to play a role in the body's host inflammatory response, both in relationship to adiposity and other inflammatory pathways.

Given leptin's involvement with inflammation, a few studies have investigated a potential link between leptin and lung function. Studies in animals have suggested that

[3] [3]M. Yousefi, W. Karmaus, H. Zhang, M Torres, J Burch. Leptin associated with lung function independent of body mass index. To be submitted.

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leptin is involved in respiratory control. For example, when comparing mutant *LEP* mice to their wild type counterparts and even before the onset of obesity, the mutant mice have increased breathing frequency, minute ventilation and tidal volume, elevated P_aCO_2 and depressed hypercapnic ventilatory response. Chronic leptin administration restores breathing rates and improves lung compliance [30,32]. However, because leptin administration in mice also decreases weight, the improvement of breathing and lung compliance cannot be conclusively attributed to leptin therapy and may not simply a result of lowered body mass index (BMI). Acute leptin replacement, on the other hand, has led to increased baseline ventilation independent of weight gain, and leptin injections in rat brain has led to increased pulmonary ventilation and respiratory volume [29]. Leptin has also been observed to be involved in the regulation of embryonic lung growth and maturation [106,117]. Specifically, administration of leptin to antenatal mice has led to improved lung development in the newborn [117], whereas leptin-deficient mice have been observed to have impaired alveolar formation and lower lung volumes at birth [106]. Additionally, postnatal leptin treatment of these same mice has resulted in increased alveolar surface area and lung volume [106].

Whereas the consensus in animal models is that higher leptin levels are correlated with improved respiratory response, human studies yield either null findings or an inverse association. Clinical studies have found that leptin is a predictor of lung function in asthmatic children [35] and is negatively correlated to lung volumes in patients with chronic obstructive pulmonary disease [118] and clinical severity of asthma [119]. Van den Borst et al, 2012 observed no association between FEV_1 and serum leptin levels but found that increased serum leptin levels were associated with decreased FVC in men but not in

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women [52]. There has also been an observed inverse association between serum leptin levels and lung function in an African American population [89] and in a twin population [52]. Specifically, Hickson et al detected an inverse association between serum leptin levels and predicted FEV_1 and predicted FVC % independent of adiposity (total body weight, waist circumference, and BMI) in an African American population women of a normal body weight [89]. The explanatory models were also adjusted for age, education, smoking status, pack-years of cigarette smoking, respiratory medication use, and menopausal status in women. Sin et al in a large sample (N=2808) also observed and inverse association between serum leptin levels and FEV_1 and FVC% predicted, independent of adipocytes in men but found no such pattern in women [88]. However, in a study involving prepubertal boys and girls, increased leptin levels were associated with asthma only in boys [48]. In an Indian population, increased plasma leptin was associated with decreased FEV_1 but no association was found for FVC.

Although these studies suggest a role of leptin in lung function, their designs lack the appropriate temporal sequence. The above-mentioned studies are cross-sectional or case-control studies and either focus on the stage of childhood or well into middle age. The current study provided a longitudinal analysis addressing a time-order of exposure to leptin with later measurements of lung function, particularly during the transition from childhood to adulthood.

Using data from the Isle of Wight Birth Cohort, we tested the association of leptin at ages 10 and 18 years with FVC, FEV_1, and FEV_1/FVC at ages 10 and 18 years. We tested concurrent and effect-delayed models, controlling for BMI and analyzed boys and girls separately.

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5.2 Methods

5.2.1 Population Characteristics

A whole population birth cohort was established on the Isle of Wight, UK, in 1989 to prospectively study the natural history of allergies starting from birth. Ethics approvals were obtained from the Isle of Wight Local Research Ethics Committee (now the National Research Ethics Service Committee South Central – Southampton B). Of the 1,536 children born between January 1, 1989, and February 28, 1990, written informed consent was obtained from parents to enroll 1,456 newborns. Children were followed up at the ages of 1 (n=1,167), 2 (n=1,174), 4 (n=1,218), 10 (n=1,373), and 18 years (n=1,313). The IOW birth cohort has been described in detail elsewhere [153]. Detailed interviews and examinations were completed for each child at each follow-up, including BMI. When a visit was not possible, a telephone questionnaire was completed or a postal questionnaire was sent. However, blood draw and the spirometric tests required that the child visited the David Hide Asthma and Allergy Research Centre.

5.2.2 Leptin Collection

Leptin concentrations were obtained from blood samples collected from at ages 10 and 18 years. Aliquots of blood serum isolated from the blood samples were used in enzyme-linked immunosorbent assays from Biokit, S.A. (Barcelona, Spain). The assays were conducted according to the manufacturer's kit instructions. Each sample, including standards and the blank, was assayed in duplicate. Leptin that was collected at age 10 was analyzed in two different labs by two different investigators. Adjustments were made for differences in scale of leptin values at age 10 between the two sets of measurements.

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Leptin levels that were collected at age 18 were determined by one of the same investigator and same laboratory that collected leptin at age 10, providing some consistency.

5.2.3 Lung Function Measurements

FVC and FEV_1 were measured at ages 10 and 18 years. Lung function testing was performed using the Koko spirometry software package on a portable desktop device (PDS Instrumentation, Louisville, KY, USA) [154]. Tests were performed in accordance with American Thoracic Society and European Respiratory Society spirometry guidelines [175]. Children were required to be free from respiratory infection for 14 days and not taking systemic oral steroids. In addition, subjects were asked to abstain from any beta-agonist medication for at least 6 hours and from caffeine intake for at least 4 hours prior to testing. Measurements were made in a standardized manner with the subject standing without a nose clip [155]. Forced vital capacity (FVC) was recorded in liters as the best of three consecutive forced expiratory maneuvers. Participants that exhibited clinical symptoms consistent with either current infection or a recent (within two weeks) asthma exacerbation, which required antibiotics or oral steroid within the preceding two weeks, were rescheduled for spirometry testing.

5.2.4 Statistical Analysis

Linear models were used the test the association between leptin and lung function (FVC, FEV_1, and FEV_1/FVC). First, concurrent models tested the association between leptin at age 10 and lung function at age 10 and leptin at age 10 and lung function at age

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18 years. Second, change in leptin levels from ages 10 to 18 were employed to predict lung function at age 18 years (time-delayed model). Since prior findings suggested variations of the associations by the sex of the child, boys and girls were analyzed separately. In the concurrent models, we controlled for height and BMI at the respective ages. In the delayed model, we controlled for height at age 18, change in height between ages 10 and 18, and change in standardized BMI from ages 10 to 18.

Lastly, we tested the association between change in leptin levels interacting with sex and lung function at age 18, stratifying the analysis by categorized BMI levels of low, normal, and high. All analyses were done using SAS 9.4 (Gary, NC).

5.3 RESULTS

Of the 1,313 participants who were followed-up at age 18, 132 had data on leptin, lung function, BMI, and height at ages 10 and 18 years. There was no statistical difference between the cohort that followed up at age 18 and the sample of people that were used in this study (Table 5.2). Girls had significantly higher levels of leptin at ages 10 and 18 years than boys (Table 5.1). Conversely, boys had significantly higher FVC and FEV_1 at both ages compared to girls. On average, between the ages of 10 and 18 years, leptin levels in boys increased by 1.3 ng/mL while that in girls increased by 12.9 ng/mL, this difference in change between the sexes was statistically significant. In some boys, leptin levels dropped from ages 10 to 18. When investigating this change in leptin levels throughout puberty and the relationship it could have on lung function at age 18, we observed that the pattern in boys and girls was markedly different (Figure 5.1). In boys, FVC and FEV_1 were observed across a wide range while changes in leptin levels

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remained low, whereas in girls, FVC and FEV_1 at age 18 remained the same across a range of levels of change in leptin.

Controlling for BMI, concurrent models revealed that in boys, increased leptin levels were associated with decreased FVC and FEV_1 at age 10 (Table 5.3). A similar pattern was observed in girls but only when analyzing the concurrent relationship between leptin and FEV_1 at age 10 (Table 5.3). No association was found between leptin at age 10 and FEV_1/FVC at age 10 in either boys or girls. In addition, concurrent models at age 18 did not reveal any significant associations with leptin considering any of the lung function measurements in boys or girls. This a cross-sectional analysis did not allow for an evaluation of the time order between leptin and lung function.

Given the changes in leptin serum concentrations, to further investigate the time order of exposure and effect, we tested whether there was an association between a change in leptin levels (from ages 10 and 18) and lung function at age 18. In boys only, for every 1 ng/mL increase in the change in leptin from age 10 to 18 years, FVC at age 18 decreased by 0.016 L. A similar pattern was observed in FEV_1 in boys where, for every 1 ng/mL increase in the change in leptin from ages 10 to 18 years, FEV_1 decreased by 0.014 L. (Table 5.4). No significant associations were detected in girls when testing the association between change in leptin levels and lung function measurements at age 18.

The analysis of an interaction between change in leptin levels and sex with lung function at age 18 revealed that in normal BMI only (N=234), for every 1 ng/mL increased change in leptin between ages 10 and 18, boys had 0.02 L higher FEV_1 at age 18 than girls.

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5.4 DISCUSSION

At age 10, our study shows an association between leptin and lung function that exists even after controlling for body mass index (BMI). Both in boys and girls, increased leptin levels were associated with decreased lung function. However, we observed different patterns of leptin and lung function between boys and girls, both at ages 10 and 18 years. In girls, the increase in leptin between ages 10 and 18 was significantly higher than the increase seen in boys (mean +1.3 in boys versus +12.9 in girls, p-Value: <0.0000001). Conversely, boys had nearly double the increase in FVC (in liters) following adolescence than girls (mean +3.0 in boys vs +1.7 in girls, p-Value: <0.0000001). In boys only, the greater the increase in leptin between ages 10 and 18, the lower FVC and FEV_1 at age 18. Therefore, the change in leptin levels during puberty (from age 10 to 18) was associated with FVC and FEV_1 levels at age 18, controlling for BMI and height changes. In those with normal BMI, boys who had a greater change in leptin between ages 10 and 18 had 0.02 L higher FEV_1 (P-value=0.03) than girls.

Of those participants who were followed-up at ages 10 and 18, data on lung function measures, height, BMI, and leptin was available in a sample of 307 children. There were no statistically significant differences in demographic and personal characteristics between the sample population used in the analysis and the entire cohort (Table 5.2), minimizing the possibility of selection bias in the study. When considering the pubertal period between ages 10 and 18, we saw a significant association between the change in leptin-sex interaction and lung function, only in normal weight participants (N=234). However, the smaller sample size in the low (N=18) and high (N=47) BMI groups may not have allowed identifying weaker associations. Trained physicians or

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nurses measured lung function, height, and weight, reducing the possibility of information bias. There was a possibility of information bias when considering leptin samples at age 10 because they were collected by two different investigators in different laboratories. However, we standardized these values. In addition, after standardization, we also controlled for the lab effect in all our models and found that it is did not have a significant effect on our analysis.

Since leptin is involved in inflammatory pathways in the body, it would be helpful to consider relevant cytokines that could play a role in the association between leptin and lung function. It is thought that when leptin binds to the leptin receptor, a down-stream JAK2/STAT pathway is activated [134-139,176-179] which results in the release of pro-inflammatory cytokines, such as Th1, TNF-α, IFN-γ, IL-2, IL-6, IL-12, and IL-10 [86]. Among these cytokines, we had collected IL-10 and IL-12 in our study, and we tested their association with FVC, FEV_1, and FEV_1/FVC at ages 10 and 18 with null results (Appendix C and D).

In other studies, it has been observed that testosterone inhibits the expression of leptin in the body while ovarian sex steroids have the opposite effect [55,59,60]. This is supported by our data that shows significantly higher leptin levels in girls at both ages, as well as a significantly higher increase between ages in girls compared the change observed in boys (Table 5.1). Since we did not have data on sex steroid levels in our participants we used the proxy measure of controlling for pubertal events in our models. These pubertal events included ages of onset of breast growth and menarche in girls, age of growth spurt in boys, average age of pubertal onset, and duration of onset of pubertal events. Detailed information on these variables can be found in a previous puberty study

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by our team [153]. We found that none of the pubertal events were significant in any of our models.

5.5. Conclusion

The role of leptin in lung function is still not completely clear and the different trends observed in boys and girls has not been explained. Although notable changes occur in leptin and lung function level during the transition between childhood to adulthood, looking at the onset of pubertal events does not explain the associations observed between these two factors. It would be important to explore more direct variables, such as sex steroid levels or relevant genes that play a role in sex steroid synthesis. Furthermore, other cytokines that are more closely related to leptin, such as IL-6, should be evaluated.

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Table 5.1 Population characteristics

	Male				Female				
	N	Mean	Median	p5, p95	N	Mean	Median	p5, p95	P-value
Leptin at age 10 (ng/mL)	150	3.19	1.55	0.4, 12.2	246	8.2	4.2	0.6, 29.2	<.0000001
Leptin at age 18 (ng/mL)	288	3.99	1.22	0.4, 13.1	265	18.9	13.1	2.4, 54.6	<.0000001
Change in leptin between ages 10 and 18 (ng/mL)	145	1.3	0	-3.9, 10.0	174	12.9	9.3	-4.0, 44.8	<.0000001
Height at age 10 (cm)	488	2.35	2.33	1.8, 2.9	493	2.2	2.2	1.7, 2.8	0.8099200
Height at age 18 (cm)	395	5.3	5.3	4.2, 6.6	443	4.0	4.0	3.1, 4.8	<.0000001
Change in height between ages 10 and 18 (cm)	391	38.9	39.0	31.6, 46.5	436	25.7	26.2	15.8, 33.9	<.0000001
Forced vital capacity (FVC) at age 10 (L)	488	2.35	2.33	1.8, 2.9	493	2.2	2.2	1.7, 2.8	<.0000001
FVC at age 18 (L)	395	5.3	5.3	4.2, 6.6	443	4.0	4.0	3.1, 4.8	<.0000001
Change in FVC between ages 10 and 18 (L)	326	3.0	3.0	2.1, 3.9	372	1.7	1.7	1.1, 2.4	<.0000001
Forced expiratory volume in one second (FEV_1) at age 10 (L)	488	2.06	2.04	1.6, 2.6	492	2.00	2.00	1.6, 2.5	0.0011217
FEV_1 (L) at age 18	396	4.62	4.55	3.6, 5.6	443	3.47	3.48	2.7, 4.2	<.0000001
Change in FEV_1 between ages 10 and 18 (L)	327	2.5	2.5	1.9, 3.2	372	1.5	1.5	0.9, 2.0	<.0000001
FEV_1/FVC at age 10	488	0.88	0.88	0.8, 0.97	492	0.90	0.90	0.8, 0.98	0.0000003
FEV_1/FVC at age 18	396	0.87	0.87	0.7, 0.98	443	0.88	0.88	0.8, 0.98	0.0060977
Change in FEV_1/FVC between ages 10 and 18	327	-0.01	-0.01	-0.01, 0.09	372	-0.02	-0.01	-0.1, 0.07	0.2687622
Standardized BMI at age 10 (kg/m^2)	516	-0.2	-0.4	-1.2, 1.6	527	0.19	-0.05	-1.1, 2.4	<.0000001
Standardized BMI at age 18 (kg/m^2)	465	-0.2	-0.3	-1.2, 1.6	499	0.15	-0.08	-1.1, 2.4	0.0000005
Change in standardized BMI between ages 10 and 18 (kg/m^2)	382	0.03	0.05	-1.28, 1.15	424	0.03	0.10	-1.2, 1.3	0.9143892

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Table 5.2 Population Characteristics in sample and whole cohort at age 18

	Cohort at age 18 follow up				Sample used in analysis				
	N	Mean	Median	P5, P95	N	Mean	Median	P5, P95	P-Value
Leptin at age 18 (ng/mL)	553	11.12	4.92	0.40, 43.0	317	12.65	6.78	0.40, 48.96	0.25
FVC at age 18 (L)	838	4.61	4.47	3.22, 6.28	307	4.66	4.46	3.34, 6.41	0.44
FEV_1 at age 18 (L)	839	4.01	3.91	2.85, 5.41	308	4.07	3.97	3.01, 5.42	0.25
FEV_1/FVC at age 18	839	0.87	0.88	0.75, 0.98	308	0.88	0.88	0.76, 0.98	0.54
Standardized BMI at age 18 (kg/m^2)	964	5.6E-15	-0.24	-1.15, 2.07	317	0.09	-0.17	-1.03, 2.24	0.21
Height at age 18 (cm)	994	171.2	171.0	156.5, 187.0	317	171.0	171.0	156.0, 186.0	0.83
	N	%			N	%		P-Value	
Female — Male	786	51.17			144	45.4			0.60
Female — Female	750	48.83			173	54.6			

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Table 5.3 Concurrent models at age 10 years in girls and boys

Variable	Estimate	STD	P-Value
FVC at age 10 in BOYS (Liters)			
Leptin at age 10 (ng/mL)	-0.016	0.007	0.02
Standardized BMI at age 10 (kg/m^2)	0.077	0.041	0.07
Height at age 10 (cm)	0.038	0.004	<.0000001
FEV_1 at age 10 in BOYS (Liters)			
Leptin at age 10 (ng/mL)	-0.014	0.006	0.03
Standardized BMI at age 10 (kg/m^2)	0.048	0.037	0.20
Height at age 10 (cm)	0.030	0.003	<.0000001
FEV_1 at age 10 in GIRLS (Liters)			
Leptin at age 10 (ng/mL)	-0.005	0.002	0.02
Standardized BMI at age 10 (kg/m^2)	0.050	0.020	0.013
Height at age 10 (cm)	0.032	0.002	<.0000001

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Table 5.4. Change in leptin levels between ages 10 to 18 predicting FVC and FEV1 at age 18 years in girls and boys

Variable	Estimate	STD	P-Value
FVC at age 18 in BOYS (Liters)			
Change in leptin from ages 10 to 18 years (ng/mL)	-0.017	0.0072	0.018
Change in standardized BMI from ages 10 to 18 years (kg/m^2)	0.402	0.095	0.000045
Change in height from ages 10 to 18 years (cm)	-0.025	0.014	0.082
Height at age 18 (cm)	0.071	0.009	<0.0000001
FEV_1 at age 18 in GIRLS (Liters)			
Change in leptin from ages 10 to 18 years (ng/mL)	-0.013	0.0060	0.029
Change in standardized BMI from ages 10 to 18 years (kg/m^2)	0.235	0.079	0.0037
Change in height from ages 10 to 18 years (cm)	-0.021	0.012	0.069
Height at age 18 (cm)	0.063	0.0074	<0.0000001

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Table 5.5 Interaction of change in leptin levels between 10 and 18 years with sex associated with FEV1 at age 18, stratified by low, normal, and high BMI categories

Variable	**Estimate**	**STD**	**P-Value**
BMI=low; N=18			
Delta Leptin(ng/mL)[1]	0.01	0.05	0.83
Sex (Male vs Female)	1.20	0.40	0.01
Delta Leptin (ng/mL) * sex (Male vs Female)	-0.13	0.09	0.16
Delta Height[2] (cm)	-0.04	0.03	0.16
Height at age 18 (cm)	0.03	0.02	0.17
BMI=normal; N=234			
Delta Leptin(ng/mL)[1]	-0.003	0.004	0.35
Sex (Male vs Female)	0.63	0.10	<0.0000001
Delta Leptin (ng/mL) * sex (Male vs Female)	0.02	0.009	0.03
Delta Height[2] (cm)	-0.009	0.007	0.19
Height at age 18 (cm)	0.05	0.005	<0.0000001
BMI=high; N=47			
Delta Leptin(ng/mL)[1]	-0.005	0.004	0.18
Sex (Male vs Female)	0.38	0.24	0.13
Delta Leptin (ng/mL) * sex (Male vs Female)	-0.007	0.007	0.30
Delta Height[2] (cm)	-0.006	0.01	0.63
Height at age 18 (cm)	0.049	0.008	0.0000006

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Figure 5.1. Lung function versus the difference in leptin levels between ages 10 and 18

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Chapter 6

Conclusion and Final Remarks

This dissertation contributes to knowledge of the role of leptin in the body's host inflammatory response by showing an association between *LEPR* SNPs and lung function, and presenting a possible mechanism of this association. We found that *LEPR* SNPs were associated with decreased FVC, FEV_1 and FEV_1/FVC in a repeated measurement analysis (SA1). Using data from the F1 generation Isle of Wight birth cohort, we then demonstrated a two-stage model of epigenetic analysis (SA2) by first identifying methQTLs in the association between LEP SNPs and LEP DNA methylation and second, finding an association between an LEP CpG sites (that were identified in stage 1) and serum leptin levels. We replicated the stage 1 findings in the F2 generation cohort. Lastly, we found that increases in leptin levels from ages 10 to 18 were associated with decreased lung function in boys as girls; and that in those with normal BMI, boys who had a greater change in leptin between the ages of 10 and 18 years had a higher FEV_1 than girls (SA3).

6.1 Summary of Aim 1

Our longitudinal analysis of repeated measurements of FVC, FEV_1, and FEV_1/FVC identified one *LEPROT* and two *LEPR* SNPs that were associated with lung function, while reporting no significant associations between lung function and *LEP*

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SNPs. To our knowledge, we were the first to explore the longitudinal association between *LEP* SNPs (rs10249476, rs11763517, rs4731429, and rs10954176) and FVC, FEV_1, FEV_1/FVC. More studies need to be conducted on *LEP* SNPs (the ones used in this study as well as other genetic variations) for comprehensive understanding of the role of *LEP* and *LEPR* SNPs.

Regarding LEPR SNPs, the CC genotype of rs6669354 was associated with decreased FVC and FEV_1 when compared to the AA genotype. Similarly, the AA genotype of rs1137101 was associated with decreased FEV_1/FVC when compared to the GG genotype. Despite leptin and lung function values being significantly different between boys and girls, especially the change between ages 10 and 18 years, we did not find that sex modified the relationship of these SNPs with lung function. We also did not find that the child ever smoking or breastfeeding duration modified the relationship.

A potential shortcoming in looking at *LEPR* SNPs is that we cannot known what splicing will occur. Of the leptin receptor's six isoforms, it is the long form for the leptin receptor, LEPRb, that circulating leptin binds to which starts the inflammatory pathway that can lead to airway obstruction. Therefore, it may be more useful to investigate the leptin receptor protein. In addition, there are other genes that could influence leptin's relationship with lung function.

6.2 Summary of Aim 2

We used the two-stage epigenetic model of analysis to first identify methQTLs (specifically *LEP* SNPs rs11763517 and rs4731429 associated with *LEP* DNA methylation) and second to test whether *LEP* DNA methylation and serum leptin levels at

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age 18 were associated. We did not find an interaction between maternal smoking during pregnancy and LEP SNPs in predicting DNA methylation levels. We successfully replicated our methQTL analysis in the F2 population cohort. Specifically, we found that in the F1 population, *LEP* SNP rs4731429 was associated with increased DNA-M at cg00666422 and cg00840332 and decreased DNA-M at cg24862443 on the *LEP* gene; rs11763517 was associated with decreased DNA-M at the cg24862443 site. In the F2 population, rs11760956 (in LD with rs4731429) is associated with increased DNA methylation at cg00666422 and cg00840332.

Given that the leptin receptor is a requirement for the initiation of inflammatory pathways involving leptin, a necessary next step is to further investigate the leptin receptor protein as well as *LEPR* DNA methylation. It would also be interesting to see how the leptin receptor and leptin proteins and genes interact with each other in these associations.

6.3 Summary of Aim 3

We found that in both boys and girls, the higher the increase in leptin serum concentrations between ages 10 and 18, the lower the lung function measurements. In those with normal BMI, for every one ng/mL increase in the change in leptin between ages 10 and 18, boys had 0.02 L higher FEV_1 than girls. Investigating at both ages 10 and 18, we did not find any associations between cytokines IL-10 and IL-12 with FVC, FEV_1, or FEV_1/FVC. Regarding pulmonary function, we observed that the increase in FVC and FEV_1 between ages 10 and 18 in boys nearly doubled compared to the increase in girls. Conversely, leptin increased in girls between those ages at much higher rates that than

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observed in boys. These differential changes that occur during the pubertal transition suggest that puberty may play a role in this difference between the sexes. Furthermore, testosterone is known to suppress leptin while ovarian sex hormones are associated with increased leptin. However, when testing markers of puberty onset in our dataset (such as the age of onset of menarche or the age of onset of growth spurt), we found no associations in our models. We were not able to explain this difference between boys and girls. Improved markers for pubertal changes, such as levels of sex steroid hormones, or additional genetic markers may provide further insights.

We successfully demonstrated an association between leptin protein and lung function measurements that existed even after adjusting for BMI. However, although leptin is associated with obesity, BMI is not the most accurate approximation of adiposity. Nevertheless, future studies should investigate whether co-variation of BMI and leptin may be improved when using other variables for adiposity. In addition, we only had two cytokines (IL-10 and IL-12) in our dataset that were relevant to leptin and the body's immune response. When looking at the relationship between leptin and lung function, the association could be mediated by other cytokines that are more directly related to this inflammatory process. A few relevant cytokines to be considered are IL-1β, IL-6, IL-8, IL-12, TNF-α, and TNF-γ. Lastly, when looking at the protein-to-lung function association, the leptin receptor protein must also be included. There could be a more direct relationship between concentrations of LEPRb (the long isoform of the leptin receptor) and lung function measures.

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6.4 Final Remarks

Leptin clearly plays a role in inflammation and is associated with lung function, but this association still needs to be better explored. Our longitudinal analysis of SNPs and lung function revealed that *LEPR* SNPs (but not *LEP* SNPs) were associated with FVC, FEV_1, and FEV_1/FVC. Therefore, the leptin receptor may play a more prominent role in lung function than leptin and more studies should consider the leptin receptor protein when investigating leptin's relationship with lung function and obstructive pulmonary diseases. When possible, studies should specifically look at the longform of the leptin receptor (LEPRb) because it is that isoform that, when bound to the leptin protein, is involved with the JAK/STAT pathway.

A weakness of our study could be that we only had data on four *LEP* SNPs, although these SNPs did span the length of the leptin gene. In our data CpG sites on the leptin gene were mostly located in the promoter region, with only one in the 3' UTR region and none in the coding or intron regions. It might be illuminating to perform the 2-stage analysis demonstrated in SA2 with different LEP SNPs and CpG sites. We also did not have data on cytokines that were more closely linked to leptin or lung function, which prevented us from doing a path analysis in SA3 (testing the theoretical path of protein leptin→cytokine→lung function measurement). Lastly, there was a possibility of information bias in the collection of leptin levels at age 10 since the values were collected by two different investigators in two different labs. However, we corrected for this by standardizing the leptin values and controlling for the different labs in the models.

A strength of this dissertation is that in all three specific aims, the possibility of selection bias was unlikely. In addition, the reproducibility and validity of our DNA

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methylation data was superior to other methods, and we successfully replicated our methQTL analysis of the F1 population in the F2 generation cohort (SA2). We found an association between *LEPR* SNPs and lung function and presented a potential mechanism for this association via *LEP* DNA methylation and serum leptin protein.

A consistent theme throughout this dissertation was the importance of considering boys and girls separately when looking at leptin and lung function (and by extension, other pulmonary disorders). Although we could not explain the differences between boys and girls in leptin and lung function levels at the genetic level, we did see a difference between boys and girls when investigating the interaction effect of change in leptin and sex on lung function at age 18. More studies need to investigate the mechanism behind these observed changes so that potential clinical attempts to treat suboptimal lung function with leptin administration can consider differentiated dosages based on sex.

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REFERENCES

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature.* 1994;372(6505):425-432.
2. Fruhbeck G. Intracellular signalling pathways activated by leptin. *Biochem J.* 2006;393(Pt 1):7-20.
3. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature.* 1998;395(6704):763-770.
4. Widjaja A, Schurmeyer TH, Von zur Muhlen A, Brabant G. Determinants of serum leptin levels in Cushing's syndrome. *The Journal of clinical endocrinology and metabolism.* 1998;83(2):600-603.
5. La Cava A, Alviggi C, Matarese G. Unraveling the multiple roles of leptin in inflammation and autoimmunity. *Journal of molecular medicine.* 2004;82(1):4-11.
6. Pelleymounter MA, Cullen MJ, Baker MB, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science.* 1995;269(5223):540-543.
7. Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology.* 2003;144(9):3765-3773.
8. Matarese G, Moschos S, Mantzoros CS. Leptin in immunology. *Journal of immunology.* 2005;174(6):3137-3142.
9. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature reviews Immunology.* 2006;6(10):772-783.
10. Otero M, Lago R, Gomez R, et al. Towards a pro-inflammatory and immunomodulatory emerging role of leptin. *Rheumatology.* 2006;45(8):944-950.
11. Cao R, Brakenhielm E, Wahlestedt C, Thyberg J, Cao Y. Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98(11):6390-6395.
12. Dunbar JC, Hu Y, Lu H. Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. *Diabetes.* 1997;46(12):2040-2043.
13. Fruhbeck G. Peripheral actions of leptin and its involvement in disease. *Nutrition reviews.* 2002;60(10 Pt 2):S47-55; discussion S68-84, 85-47.
14. Kiguchi N, Maeda T, Kobayashi Y, Fukazawa Y, Kishioka S. Leptin enhances CC-chemokine ligand expression in cultured murine macrophage. *Biochemical and biophysical research communications.* 2009;384(3):311-315.

المنارة للاستشارات
www.manaraa.com

15. Bouloumie A, Drexler HC, Lafontan M, Busse R. Leptin, the product of Ob gene, promotes angiogenesis. *Circ Res.* 1998;83(10):1059-1066.
16. Frank S, Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J. Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair. *The Journal of clinical investigation.* 2000;106(4):501-509.
17. Gordeladze JO, Reseland JE. A unified model for the action of leptin on bone turnover. *Journal of cellular biochemistry.* 2003;88(4):706-712.
18. Keisler DH, Daniel JA, Morrison CD. The role of leptin in nutritional status and reproductive function. *J Reprod Fertil Suppl.* 1999;54:425-435.
19. Muoio DM, Lynis Dohm G. Peripheral metabolic actions of leptin. *Best Pract Res Clin Endocrinol Metab.* 2002;16(4):653-666.
20. Kruse M, Bornstein SR, Uhlmann K, Paeth G, Scherbaum WA. Leptin down-regulates the steroid producing system in the adrenal. *Endocr Res.* 1998;24(3-4):587-590.
21. Saladin R, De Vos P, Guerre-Millo M, et al. Transient increase in obese gene expression after food intake or insulin administration. *Nature.* 1995;377(6549):527-529.
22. La Cava A, Matarese G. The weight of leptin in immunity. *Nature reviews Immunology.* 2004;4(5):371-379.
23. Zhang HH, Kumar S, Barnett AH, Eggo MC. Tumour necrosis factor-alpha exerts dual effects on human adipose leptin synthesis and release. *Mol Cell Endocrinol.* 2000;159(1-2):79-88.
24. Otero M, Lago R, Lago F, et al. Leptin, from fat to inflammation: old questions and new insights. *FEBS letters.* 2005;579(2):295-301.
25. Kallen CB, Lazar MA. Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. *Proceedings of the National Academy of Sciences of the United States of America.* 1996;93(12):5793-5796.
26. Popa C, Netea MG, Radstake TR, van Riel PL, Barrera P, van der Meer JW. Markers of inflammation are negatively correlated with serum leptin in rheumatoid arthritis. *Ann Rheum Dis.* 2005;64(8):1195-1198.
27. Raguso CA, Guinot SL, Janssens JP, Kayser B, Pichard C. Chronic hypoxia: common traits between chronic obstructive pulmonary disease and altitude. *Curr Opin Clin Nutr Metab Care.* 2004;7(4):411-417.
28. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *The Journal of allergy and clinical immunology.* 2005;115(5):911-919; quiz 920.
29. O'Donnell C P, Schaub CD, Haines AS, et al. Leptin prevents respiratory depression in obesity. *American journal of respiratory and critical care medicine.* 1999;159(5 Pt 1):1477-1484.
30. Tankersley CG, O'Donnell C, Daood MJ, et al. Leptin attenuates respiratory complications associated with the obese phenotype. *Journal of applied physiology.* 1998;85(6):2261-2269.
31. Groeben H, Meier S, Brown RH, O'Donnell CP, Mitzner W, Tankersley CG. The effect of leptin on the ventilatory responseto hyperoxia. *Exp Lung Res.* 2004;30(7):559-570.

المنارة للاستشارات
www.manaraa.com

32. Tankersley C, Kleeberger S, Russ B, Schwartz A, Smith P. Modified control of breathing in genetically obese (ob/ob) mice. *Journal of applied physiology.* 1996;81(2):716-723.
33. Inyushkin AN, Inyushkina EM, Merkulova NA. Respiratory responses to microinjections of leptin into the solitary tract nucleus. *Neurosci Behav Physiol.* 2009;39(3):231-240.
34. Caro JF, Kolaczynski JW, Nyce MR, et al. Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. *Lancet.* 1996;348(9021):159-161.
35. Kim KW, Shin YH, Lee KE, Kim ES, Sohn MH, Kim KE. Relationship between adipokines and manifestations of childhood asthma. *Pediatr Allergy Immunol.* 2008;19(6):535-540.
36. Vernooy JH, Drummen NE, van Suylen RJ, et al. Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers. *Thorax.* 2009;64(1):26-32.
37. Bruno A, Pace E, Chanez P, et al. Leptin and leptin receptor expression in asthma. *The Journal of allergy and clinical immunology.* 2009;124(2):230-237, 237 e231-234.
38. O'Donnell CP, Tankersley CG, Polotsky VP, Schwartz AR, Smith PL. Leptin, obesity, and respiratory function. *Respiration physiology.* 2000;119(2-3):163-170.
39. Campo A, Fruhbeck G, Zulueta JJ, et al. Hyperleptinaemia, respiratory drive and hypercapnic response in obese patients. *The European respiratory journal.* 2007;30(2):223-231.
40. Phillips BG, Kato M, Narkiewicz K, Choe I, Somers VK. Increases in leptin levels, sympathetic drive, and weight gain in obstructive sleep apnea. *American journal of physiology Heart and circulatory physiology.* 2000;279(1):H234-237.
41. Barcelo A, Barbe F, Llompart E, et al. Neuropeptide Y and leptin in patients with obstructive sleep apnea syndrome: role of obesity. *American journal of respiratory and critical care medicine.* 2005;171(2):183-187.
42. Shimizu K, Chin K, Nakamura T, et al. Plasma leptin levels and cardiac sympathetic function in patients with obstructive sleep apnoea-hypopnoea syndrome. *Thorax.* 2002;57(5):429-434.
43. Schols AM, Creutzberg EC, Buurman WA, Campfield LA, Saris WH, Wouters EF. Plasma leptin is related to proinflammatory status and dietary intake in patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine.* 1999;160(4):1220-1226.
44. Creutzberg EC, Wouters EF, Vanderhoven-Augustin IM, Dentener MA, Schols AM. Disturbances in leptin metabolism are related to energy imbalance during acute exacerbations of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine.* 2000;162(4 Pt 1):1239-1245.
45. Kythreotis P, Kokkini A, Avgeropoulou S, et al. Plasma leptin and insulin-like growth factor I levels during acute exacerbations of chronic obstructive pulmonary disease. *BMC Pulm Med.* 2009;9:11.
46. Johnston RA, Theman TA, Lu FL, Terry RD, Williams ES, Shore SA. Diet-induced obesity causes innate airway hyperresponsiveness to methacholine and

المنارة للاستشارات
www.manaraa.com

enhances ozone-induced pulmonary inflammation. *Journal of applied physiology.* 2008;104(6):1727-1735.
47. Shore SA, Schwartzman IN, Mellema MS, Flynt L, Imrich A, Johnston RA. Effect of leptin on allergic airway responses in mice. *The Journal of allergy and clinical immunology.* 2005;115(1):103-109.
48. Guler N, Kirerleri E, Ones U, Tamay Z, Salmayenli N, Darendeliler F. Leptin: does it have any role in childhood asthma? *The Journal of allergy and clinical immunology.* 2004;114(2):254-259.
49. Sood A, Ford ES, Camargo CA, Jr. Association between leptin and asthma in adults. *Thorax.* 2006;61(4):300-305.
50. Ribeiro R, Araujo AP, Coelho A, et al. A functional polymorphism in the promoter region of leptin gene increases susceptibility for non-small cell lung cancer. *Eur J Cancer.* 2006;42(8):1188-1193.
51. Carpagnano GE, Spanevello A, Curci C, et al. IL-2, TNF-alpha, and leptin: local versus systemic concentrations in NSCLC patients. *Oncol Res.* 2007;16(8):375-381.
52. van den Borst B, Souren NY, Loos RJ, et al. Genetics of maximally attained lung function: a role for leptin? *Respiratory medicine.* 2012;106(2):235-242.
53. Loos RJ, Rankinen T, Chagnon Y, Tremblay A, Perusse L, Bouchard C. Polymorphisms in the leptin and leptin receptor genes in relation to resting metabolic rate and respiratory quotient in the Quebec Family Study. *Int J Obes (Lond).* 2006;30(1):183-190.
54. Hansel NN, Gao L, Rafaels NM, et al. Leptin receptor polymorphisms and lung function decline in COPD. *The European respiratory journal.* 2009;34(1):103-110.
55. Blum WF, Englaro P, Hanitsch S, et al. Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. *The Journal of clinical endocrinology and metabolism.* 1997;82(9):2904-2910.
56. Garcia-Mayor RV, Andrade MA, Rios M, Lage M, Dieguez C, Casanueva FF. Serum leptin levels in normal children: relationship to age, gender, body mass index, pituitary-gonadal hormones, and pubertal stage. *The Journal of clinical endocrinology and metabolism.* 1997;82(9):2849-2855.
57. Hickey MS, Israel RG, Gardiner SN, et al. Gender differences in serum leptin levels in humans. *Biochemical and molecular medicine.* 1996;59(1):1-6.
58. Hickey MS, Houmard JA, Considine RV, et al. Gender-dependent effects of exercise training on serum leptin levels in humans. *The American journal of physiology.* 1997;272(4 Pt 1):E562-566.
59. Mantzoros CS, Moschos S, Avramopoulos I, et al. Leptin concentrations in relation to body mass index and the tumor necrosis factor-alpha system in humans. *The Journal of clinical endocrinology and metabolism.* 1997;82(10):3408-3413.
60. Castracane VD, Kraemer RR, Franken MA, Kraemer GR, Gimpel T. Serum leptin concentration in women: effect of age, obesity, and estrogen administration. *Fertility and sterility.* 1998;70(3):472-477.

المنارة للاستشارات
www.manaraa.com

61. Yousefi M, Karmaus W, Zhang H, Ewart S, Arshad H, Holloway JW. The methylation of the LEPR/LEPROT genotype at the promoter and body regions influence concentrations of leptin in girls and BMI at age 18 years if their mother smoked during pregnancy. *International journal of molecular epidemiology and genetics.* 2013;4(2):86-100.
62. Fourati M, Mnif M, Kharrat N, et al. Association between Leptin gene polymorphisms and plasma leptin level in three consanguineous families with obesity. *Gene.* 2013;527(1):75-81.
63. Llanos AA, Brasky TM, Mathew J, et al. Genetic variation in adipokine genes and associations with adiponectin and leptin concentrations in plasma and breast tissue. *Cancer Epidemiol Biomarkers Prev.* 2014;23(8):1559-1568.
64. Su PH, Yang SF, Yu JS, Chen SJ, Chen JY. Study of the leptin levels and its gene polymorphisms in patients with idiopathic short stature and growth hormone deficiency. *Endocrine.* 2012;42(1):196-204.
65. Furusawa T, Naka I, Yamauchi T, et al. The Q223R polymorphism in LEPR is associated with obesity in Pacific Islanders. *Human genetics.* 2010;127(3):287-294.
66. Su PH, Yang SF, Yu JS, Chen SJ, Chen JY. Study of leptin levels and gene polymorphisms in patients with central precocious puberty. *Pediatric research.* 2012;71(4 Pt 1):361-367.
67. Kim EY, Chin HM, Park SM, et al. Susceptibility of gastric cancer according to leptin and leptin receptor gene polymorphisms in Korea. *J Korean Surg Soc.* 2012;83(1):7-13.
68. Hillemacher T, Weinland C, Lenz B, et al. DNA methylation of the LEP gene is associated with craving during alcohol withdrawal. *Psychoneuroendocrinology.* 2015;51:371-377.
69. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nature reviews Genetics.* 2007;8(4):253-262.
70. Lesseur C, Armstrong DA, Murphy MA, et al. Sex-specific associations between placental leptin promoter DNA methylation and infant neurobehavior. *Psychoneuroendocrinology.* 2014;40:1-9.
71. Robins JC, Marsit CJ, Padbury JF, Sharma SS. Endocrine disruptors, environmental oxygen, epigenetics and pregnancy. *Front Biosci (Elite Ed).* 2011;3:690-700.
72. Nelissen EC, van Montfoort AP, Dumoulin JC, Evers JL. Epigenetics and the placenta. *Hum Reprod Update.* 2011;17(3):397-417.
73. Schubring C, Kiess W, Englaro P, et al. Levels of leptin in maternal serum, amniotic fluid, and arterial and venous cord blood: relation to neonatal and placental weight. *The Journal of clinical endocrinology and metabolism.* 1997;82(5):1480-1483.
74. Vatten LJ, Nilsen ST, Odegard RA, Romundstad PR, Austgulen R. Insulin-like growth factor I and leptin in umbilical cord plasma and infant birth size at term. *Pediatrics.* 2002;109(6):1131-1135.
75. Shekhawat PS, Garland JS, Shivpuri C, et al. Neonatal cord blood leptin: its relationship to birth weight, body mass index, maternal diabetes, and steroids. *Pediatric research.* 1998;43(3):338-343.

المنارة للاستشارات
www.manaraa.com

76. Shoemaker R, Deng J, Wang W, Zhang K. Allele-specific methylation is prevalent and is contributed by CpG-SNPs in the human genome. *Genome Res.* 2010;20(7):883-889.
77. Gibbs JR, van der Brug MP, Hernandez DG, et al. Abundant quantitative trait loci exist for DNA methylation and gene expression in human brain. *PLoS Genet.* 2010;6(5):e1000952.
78. Bell JT, Pai AA, Pickrell JK, et al. DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. *Genome Biol.* 2011;12(1):R10.
79. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. *Nature reviews Genetics.* 2011;12(8):529-541.
80. Kerkel K, Spadola A, Yuan E, et al. Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation. *Nature genetics.* 2008;40(7):904-908.
81. Hellman A, Chess A. Extensive sequence-influenced DNA methylation polymorphism in the human genome. *Epigenetics Chromatin.* 2010;3(1):11.
82. Zhang D, Cheng L, Badner JA, et al. Genetic control of individual differences in gene-specific methylation in human brain. *American journal of human genetics.* 2010;86(3):411-419.
83. Procaccini C, Jirillo E, Matarese G. Leptin as an immunomodulator. *Molecular aspects of medicine.* 2012;33(1):35-45.
84. Mandel MA, Mahmoud AA. Impairment of cell-mediated immunity in mutation diabetic mice (db/db). *Journal of immunology.* 1978;120(4):1375-1377.
85. Ozata M, Ozdemir IC, Licinio J. Human leptin deficiency caused by a missense mutation: multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction indicate new targets for leptin action, greater central than peripheral resistance to the effects of leptin, and spontaneous correction of leptin-mediated defects. *The Journal of clinical endocrinology and metabolism.* 1999;84(10):3686-3695.
86. Paz-Filho G, Mastronardi C, Franco CB, Wang KB, Wong ML, Licinio J. Leptin: molecular mechanisms, systemic pro-inflammatory effects, and clinical implications. *Arquivos brasileiros de endocrinologia e metabologia.* 2012;56(9):597-607.
87. Faggioni R, Feingold KR, Grunfeld C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2001;15(14):2565-2571.
88. Sin DD, Man SF. Impaired lung function and serum leptin in men and women with normal body weight: a population based study. *Thorax.* 2003;58(8):695-698.
89. Hickson DA, Burchfiel CM, Petrini MF, et al. Leptin is inversely associated with lung function in African Americans, independent of adiposity: the Jackson Heart Study. *Obesity.* 2011;19(5):1054-1061.
90. Masuzaki H, Ogawa Y, Sagawa N, et al. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Nature medicine.* 1997;3(9):1029-1033.

المنارة للاستشارات
www.manaraa.com

91. Bado A, Levasseur S, Attoub S, et al. The stomach is a source of leptin. *Nature.* 1998;394(6695):790-793.
92. Larsson H, Ahren B. Short-term dexamethasone treatment increases plasma leptin independently of changes in insulin sensitivity in healthy women. *The Journal of clinical endocrinology and metabolism.* 1996;81(12):4428-4432.
93. Henson MC, Swan KF, Edwards DE, Hoyle GW, Purcell J, Castracane VD. Leptin receptor expression in fetal lung increases in late gestation in the baboon: a model for human pregnancy. *Reproduction.* 2004;127(1):87-94.
94. Torday JS, Sun H, Wang L, Torres E, Sunday ME, Rubin LP. Leptin mediates the parathyroid hormone-related protein paracrine stimulation of fetal lung maturation. *Am J Physiol Lung Cell Mol Physiol.* 2002;282(3):L405-410.
95. Ahima RS. Central actions of adipocyte hormones. *Trends Endocrinol Metab.* 2005;16(7):307-313.
96. Bjorbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol Cell.* 1998;1(4):619-625.
97. Cao GY, Considine RV, Lynn RB. Leptin receptors in the adrenal medulla of the rat. *The American journal of physiology.* 1997;273(2 Pt 1):E448-452.
98. Hoggard N, Mercer JG, Rayner DV, Moar K, Trayhurn P, Williams LM. Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization. *Biochemical and biophysical research communications.* 1997;232(2):383-387.
99. Hoggard N, Hunter L, Duncan JS, Williams LM, Trayhurn P, Mercer JG. Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(20):11073-11078.
100. Bergen HT, Cherlet TC, Manuel P, Scott JE. Identification of leptin receptors in lung and isolated fetal type II cells. *American journal of respiratory cell and molecular biology.* 2002;27(1):71-77.
101. Bellmeyer A, Martino JM, Chandel NS, Scott Budinger GR, Dean DA, Mutlu GM. Leptin resistance protects mice from hyperoxia-induced acute lung injury. *American journal of respiratory and critical care medicine.* 2007;175(6):587-594.
102. Chelikani PK, Glimm DR, Kennelly JJ. Short communication: Tissue distribution of leptin and leptin receptor mRNA in the bovine. *J Dairy Sci.* 2003;86(7):2369-2372.
103. Lollmann B, Gruninger S, Stricker-Krongrad A, Chiesi M. Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. *Biochemical and biophysical research communications.* 1997;238(2):648-652.
104. Lin J, Barb CR, Matteri RL, et al. Long form leptin receptor mRNA expression in the brain, pituitary, and other tissues in the pig. *Domest Anim Endocrinol.* 2000;19(1):53-61.
105. Szczepankiewicz A, Breborowicz A, Sobkowiak P, Popiel A. Are genes associated with energy metabolism important in asthma and BMI? *J Asthma.* 2009;46(1):53-58.

المنارة للاستشارات
www.manaraa.com

106. Huang K, Rabold R, Abston E, et al. Effects of leptin deficiency on postnatal lung development in mice. *J Appl Physiol.* 2008;105(1):249-259.
107. Nair P, Radford K, Fanat A, Janssen LJ, Peters-Golden M, Cox PG. The effects of leptin on airway smooth muscle responses. *American journal of respiratory cell and molecular biology.* 2008;39(4):475-481.
108. Bruno A, Chanez P, Chiappara G, et al. Does leptin play a cytokine-like role within the airways of COPD patients? *The European respiratory journal.* 2005;26(3):398-405.
109. Lesseur C, Armstrong DA, Paquette AG, Koestler DC, Padbury JF, Marsit CJ. Tissue-specific Leptin promoter DNA methylation is associated with maternal and infant perinatal factors. *Mol Cell Endocrinol.* 2013;381(1-2):160-167.
110. Schultz NS, Broholm C, Gillberg L, et al. Impaired leptin gene expression and release in cultured preadipocytes isolated from individuals born with low birth weight. *Diabetes.* 2014;63(1):111-121.
111. Tobi EW, Lumey LH, Talens RP, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Human molecular genetics.* 2009;18(21):4046-4053.
112. Malendowicz LK, Rucinski M, Belloni AS, Ziolkowska A, Nussdorfer GG. Leptin and the regulation of the hypothalamic-pituitary-adrenal axis. *International review of cytology.* 2007;263:63-102.
113. Zhang Y, Wilsey JT, Frase CD, et al. Peripheral but not central leptin prevents the immunosuppression associated with hypoleptinemia in rats. *The Journal of endocrinology.* 2002;174(3):455-461.
114. Lauhkonen E, Koponen P, Terasjarvi J, et al. IL-10 Gene Polymorphisms Are Associated with Post-Bronchiolitis Lung Function Abnormalities at Six Years of Age. *PloS one.* 2015;10(10):e0140799.
115. Burgess JL, Fierro MA, Lantz RC, et al. Longitudinal decline in lung function: evaluation of interleukin-10 genetic polymorphisms in firefighters. *Journal of occupational and environmental medicine.* 2004;46(10):1013-1022.
116. Pho H, Hernandez AB, Arias RS, et al. The effect of leptin replacement on sleep-disordered breathing in the leptin-deficient ob/ob mouse. *Journal of applied physiology.* 2016;120(1):78-86.
117. Kirwin SM, Bhandari V, Dimatteo D, et al. Leptin enhances lung maturity in the fetal rat. *Pediatric research.* 2006;60(2):200-204.
118. Vondracek SF, Voelkel NF, McDermott MT, Valdez C. The relationship between adipokines, body composition, and bone density in men with chronic obstructive pulmonary disease. *International journal of chronic obstructive pulmonary disease.* 2009;4:267-277.
119. Tanju A, Cekmez F, Aydinoz S, Karademir F, Suleymanoglu S, Gocmen I. Association between clinical severity of childhood asthma and serum leptin levels. *Indian J Pediatr.* 2011;78(3):291-295.
120. Bibikova M, Barnes B, Tsan C, et al. High density DNA methylation array with single CpG site resolution. *Genomics.* 2011;98(4):288-295.
121. Sandoval J, Heyn H, Moran S, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics.* 2011;6(6):692-702.

المنارة للاستشارات
www.manaraa.com

122. Kurukulaaratchy RJ, Fenn MH, Waterhouse LM, Matthews SM, Holgate ST, Arshad SH. Characterization of wheezing phenotypes in the first 10 years of life. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2003;33(5):573-578.
123. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988;16(3):1215.
124. Fichorova RN, Richardson-Harman N, Alfano M, et al. Biological and technical variables affecting immunoassay recovery of cytokines from human serum and simulated vaginal fluid: a multicenter study. *Analytical chemistry.* 2008;80(12):4741-4751.
125. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21(2):263-265.
126. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. *Science.* 2002;296(5576):2225-2229.
127. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Statistics in medicine.* 1990;9(7):811-818.
128. Mukherjee N, Lockett GA, Merid SK, et al. DNA methylation and genetic polymorphisms of the Leptin gene interact to influence lung function outcomes and asthma at 18 years of age. *International journal of molecular epidemiology and genetics.* 2016;7(1):1-17.
129. Smith AK, Conneely KN, Pace TW, et al. Epigenetic changes associated with inflammation in breast cancer patients treated with chemotherapy. *Brain, behavior, and immunity.* 2014;38:227-236.
130. Kaushal A, Zhang H, Karmaus WJJ, et al. Comparison of different cell type correction methods for genome-scale epigenetics studies. *BMC bioinformatics.* 2017;18(1):216.
131. International HapMap C. The International HapMap Project. *Nature.* 2003;426(6968):789-796.
132. Fernandez-Riejos P, Najib S, Santos-Alvarez J, et al. Role of leptin in the activation of immune cells. *Mediators of inflammation.* 2010;2010:568343.
133. Conus S, Bruno A, Simon HU. Leptin is an eosinophil survival factor. *The Journal of allergy and clinical immunology.* 2005;116(6):1228-1234.
134. Banks AS, Davis SM, Bates SH, Myers MG, Jr. Activation of downstream signals by the long form of the leptin receptor. *The Journal of biological chemistry.* 2000;275(19):14563-14572.
135. White DW, Kuropatwinski KK, Devos R, Baumann H, Tartaglia LA. Leptin receptor (OB-R) signaling. Cytoplasmic domain mutational analysis and evidence for receptor homo-oligomerization. *The Journal of biological chemistry.* 1997;272(7):4065-4071.
136. Bates SH, Stearns WH, Dundon TA, et al. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature.* 2003;421(6925):856-859.
137. Jiang L, You J, Yu X, et al. Tyrosine-dependent and -independent actions of leptin receptor in control of energy balance and glucose homeostasis. *Proceedings of the National Academy of Sciences of the United States of America.* 2008;105(47):18619-18624.

المنارة للاستشارات
www.manaraa.com

138. Piper ML, Unger EK, Myers MG, Jr., Xu AW. Specific physiological roles for signal transducer and activator of transcription 3 in leptin receptor-expressing neurons. *Molecular endocrinology.* 2008;22(3):751-759.
139. Gao Q, Wolfgang MJ, Neschen S, et al. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(13):4661-4666.
140. Balte P, Karmaus W, Roberts G, Kurukulaaratchy R, Mitchell F, Arshad H. Relationship between birth weight, maternal smoking during pregnancy and childhood and adolescent lung function: A path analysis. *Respiratory medicine.* 2016;121:13-20.
141. Edwards CA, Osman LM, Godden DJ, Campbell DM, Douglas JG. Relationship between birth weight and adult lung function: controlling for maternal factors. *Thorax.* 2003;58(12):1061-1065.
142. Hancox RJ, Poulton R, Greene JM, McLachlan CR, Pearce MS, Sears MR. Associations between birth weight, early childhood weight gain and adult lung function. *Thorax.* 2009;64(3):228-232.
143. Lawlor DA, Ebrahim S, Davey Smith G. Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis. *Thorax.* 2005;60(10):851-858.
144. Falorni A, Bini V, Molinari D, et al. Leptin serum levels in normal weight and obese children and adolescents: relationship with age, sex, pubertal development, body mass index and insulin. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 1997;21(10):881-890.
145. Kirel B, Dogruel N, Akgun N, Kilic FS, Tekin N, Ucar B. Serum leptin levels during childhood and adolescence: relationship with age, sex, adiposity and puberty. *The Turkish journal of pediatrics.* 1999;41(4):447-455.
146. Duarte SF, Francischetti EA, Genelhu VA, Cabello PH, Pimentel MM. LEPR p.Q223R, beta3-AR p.W64R and LEP c.-2548G>A gene variants in obese Brazilian subjects. *Genetics and molecular research : GMR.* 2007;6(4):1035-1043.
147. Saeed S, Bonnefond A, Manzoor J, et al. Genetic variants in LEP, LEPR, and MC4R explain 30% of severe obesity in children from a consanguineous population. *Obesity.* 2015;23(8):1687-1695.
148. Wang R, Custovic A, Simpson A, Belgrave DC, Lowe LA, Murray CS. Differing associations of BMI and body fat with asthma and lung function in children. *Pediatric pulmonology.* 2014;49(11):1049-1057.
149. Chan JL, Bluher S, Yiannakouris N, Suchard MA, Kratzsch J, Mantzoros CS. Regulation of circulating soluble leptin receptor levels by gender, adiposity, sex steroids, and leptin: observational and interventional studies in humans. *Diabetes.* 2002;51(7):2105-2112.
150. Roemmich JN, Clark PA, Berr SS, et al. Gender differences in leptin levels during puberty are related to the subcutaneous fat depot and sex steroids. *The American journal of physiology.* 1998;275(3 Pt 1):E543-551.

المنارة للاستشارات
www.manaraa.com

151. Soto-Ramirez N, Alexander M, Karmaus W, et al. Breastfeeding is associated with increased lung function at 18 years of age: a cohort study. *The European respiratory journal.* 2012;39(4):985-991.
152. Ziyab AH, Karmaus W, Yousefi M, et al. Interplay of filaggrin loss-of-function variants, allergic sensitization, and eczema in a longitudinal study covering infancy to 18 years of age. *PloS one.* 2012;7(3):e32721.
153. Yousefi M, Karmaus W, Zhang H, et al. Relationships between age of puberty onset and height at age 18 years in girls and boys. *World journal of pediatrics : WJP.* 2013;9(3):230-238.
154. Ferraris Respiratory I. KoKo Spirometer & KoKo DigiDoser Windows Operations Guide

Appendix A: Normal Equations. 2002.

155. Dolan CM, Fraher KE, Bleecker ER, et al. Design and baseline characteristics of the epidemiology and natural history of asthma: Outcomes and Treatment Regimens (TENOR) study: a large cohort of patients with severe or difficult-to-treat asthma. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.* 2004;92(1):32-39.
156. Lago R, Gomez R, Lago F, Gomez-Reino J, Gualillo O. Leptin beyond body weight regulation--current concepts concerning its role in immune function and inflammation. *Cellular immunology.* 2008;252(1-2):139-145.
157. Lord GM. Leptin as a proinflammatory cytokine. *Contributions to nephrology.* 2006;151:151-164.
158. Bates SH, Myers MG. The role of leptin-->STAT3 signaling in neuroendocrine function: an integrative perspective. *Journal of molecular medicine.* 2004;82(1):12-20.
159. Allison MB, Myers MG, Jr. 20 years of leptin: connecting leptin signaling to biological function. *The Journal of endocrinology.* 2014;223(1):T25-35.
160. Farooqi IS, O'Rahilly S. 20 years of leptin: human disorders of leptin action. *The Journal of endocrinology.* 2014;223(1):T63-70.
161. O'Rahilly S. 20 years of leptin: what we know and what the future holds. *The Journal of endocrinology.* 2014;223(1):E1-3.
162. Chehab FF. 20 years of leptin: leptin and reproduction: past milestones, present undertakings, and future endeavors. *The Journal of endocrinology.* 2014;223(1):T37-48.
163. Conde J, Scotece M, Abella V, et al. An update on leptin as immunomodulator. *Expert review of clinical immunology.* 2014;10(9):1165-1170.
164. Ben Ali S, Kallel A, Ftouhi B, et al. Association of G-2548A LEP polymorphism with plasma leptin levels in Tunisian obese patients. *Clin Biochem.* 2009;42(7-8):584-588.
165. Hager J, Clement K, Francke S, et al. A polymorphism in the 5' untranslated region of the human ob gene is associated with low leptin levels. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity.* 1998;22(3):200-205.
166. Chavarria-Avila E, Vazquez-Del Mercado M, Gomez-Banuelos E, et al. The Impact of LEP G-2548A and LEPR Gln223Arg Polymorphisms on Adiposity,

المنارة للاستشارات
www.manaraa.com

Leptin, and Leptin-Receptor Serum Levels in a Mexican Mestizo Population. *BioMed research international.* 2015;2015:539408.

167. Hinuy HM, Hirata MH, Forti N, et al. Leptin G-2548A promoter polymorphism is associated with increased plasma leptin and BMI in Brazilian women. *Arquivos brasileiros de endocrinologia e metabologia.* 2008;52(4):611-616.
168. Shabana, Hasnain S. Leptin promoter variant G2548A is associated with serum leptin and HDL-C levels in a case control observational study in association with obesity in a Pakistani cohort. *Journal of biosciences.* 2016;41(2):251-255.
169. Mammes O, Betoulle D, Aubert R, Herbeth B, Siest G, Fumeron F. Association of the G-2548A polymorphism in the 5' region of the LEP gene with overweight. *Annals of human genetics.* 2000;64(Pt 5):391-394.
170. Kilpelainen TO, Carli JF, Skowronski AA, et al. Genome-wide meta-analysis uncovers novel loci influencing circulating leptin levels. *Nature communications.* 2016;7:10494.
171. Ziyab AH, Ewart S, Lockett GA, et al. Expression of the filaggrin gene in umbilical cord blood predicts eczema risk in infancy: A birth cohort study. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2017.
172. Kuan PF, Wang S, Zhou X, Chu H. A statistical framework for Illumina DNA methylation arrays. *Bioinformatics.* 2010;26(22):2849-2855.
173. Andreev VP, Dwivedi RC, Paz-Filho G, et al. Dynamics of plasma proteome during leptin-replacement therapy in genetically based leptin deficiency. *The pharmacogenomics journal.* 2011;11(3):174-190.
174. Vernooy JH, Bracke KR, Drummen NE, et al. Leptin modulates innate and adaptive immune cell recruitment after cigarette smoke exposure in mice. *Journal of immunology.* 2010;184(12):7169-7177.
175. Society AT. Standardization of Spirometry, 1994 Update. *Am J Respir Crit Care Med.* 1995;152(3):p. 1107-1136.
176. Baumann H, Morella KK, White DW, et al. The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proceedings of the National Academy of Sciences of the United States of America.* 1996;93(16):8374-8378.
177. Chen H, Charlat O, Tartaglia LA, et al. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell.* 1996;84(3):491-495.
178. Taga T. The signal transducer gp130 is shared by interleukin-6 family of haematopoietic and neurotrophic cytokines. *Annals of medicine.* 1997;29(1):63-72.
179. Hekerman P, Zeidler J, Bamberg-Lemper S, et al. Pleiotropy of leptin receptor signalling is defined by distinct roles of the intracellular tyrosines. *The FEBS journal.* 2005;272(1):109-119.

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APPENDIX A

ASSOCIATION OF *LEP*, *LEPR*, AND *LEPROT* SNPS WITH BMI AT AGES 10 AND 18 YEARS

Table A.1

SNP	**BMI age 10 (P-value)**	**BMI age 18 (P-value)**
LEPrs4731429	0.0102	0.0892
LEPrs10249476	0.0166	0.3117
LEPrs10954176	0.4254	0.3054
LEPrs11763517	0.0327	0.7317
LEPRrs3806318	0.5306	0.5477
LEPROTrs17412175	0.3387	0.3867
LEPRrs7526141	0.3480	0.9443
LEPRrs1137101	0.6604	0.8446
LEPROTrs9436740	0.0518	0.2136
LEPROTrs9436301	0.2881	0.1414
LEPROTrs6669354	0.3264	0.2773
LEPRrs12059300	0.4537	0.9881
LEPRrs3762274	0.6499	0.1330
LEPRrs8179183	0.2096	0.6693

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Appendix B

Population Characteristics in F2 Cohort

Table B.1

SNP	Genotype	Whole Population Cohort N=419 N (%)	Sub population with SNP data N=139 N (%)	Sub population with SNP and DNA methylation data N=125 N (%)	P value (Whole population vs Sub population with SNP data)	P value (sub population with SNP data vs sub population with SNP and DNA-M data)
rs2167270	AA		12 (8.63)	9 (7.2)		
	AG		64 (46.04)	61 (48.8)	N/A	0.86
	GG		63 (45.32)	55 (44.0)		
rs2278815	AA		50 (35.97)	45 (36.0)		
	AG		67 (48.20)	62 (49.6)	N/A	0.94
	GG		22 (15.83)	18(14.4)		
rs11760956	AA		11 (7.91)	9 (7.2)		
	AG		67 (48.20)	63 (50.4)	N/A	0.93
	GG		61 (43.88)	53 (42.4)		
rs11763517	AA		40 (28.78)	34 (27.2)		
	AG		69 (49.64)	64 (51.2)	N/A	0.96
	GG		30 (21.58)	27 (21.6)		
rs12706832	AA		22 (15.83)	18 (14.4)		
	AG		67 (48.20)	62 (49.6)	N/A	0.94
	GG		50 (35.97)	45 (36.0)		
Variable		**N (%)**	**N (%)**	**N (%)**		
Sex	Male	231 (55.8)	72 (51.8)	64 (51.2)	0.41	0.92
	Female	183 (44.2)	67 (48.2)	61 (48.8)		
In utero smoking		244 (62.72)	86 (64.66)	77 (64.2)	0.69	0.93
	Yes	145 (37.28)	47 (35.34)	43 (35.8)		

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Appendix C

Boys: Spearman Correlations of Leptin and Lung Function Values at Ages 10 and 18 with IL-10 and IL-12 at Ages 10 and 18

Table C.1

ρ P-value N	Leptin at age 10	Leptin at age 18	FVC at age 10	FVC at age 18	FEV_1 at age 10	FEV_1 at age 18	FEV_1/FVC at age 10	FEV_1/FVC at age 18
IL10 at age 10	0.08889	-0.03494	-0.11012	0.03311	-0.10069	0.09228	-0.01514	0.04649
	0.3816	0.6989	0.2273	0.7129	0.2698	0.3041	0.8685	0.6052
	99	125	122	126	122	126	122	126
IL10 at age 18	0.06051	-0.00604	-0.00810	0.01669	-0.00306	0.01518	-0.01733	-0.07470
	0.5437	0.9348	0.9201	0.8246	0.9698	0.8397	0.8299	0.3189
	103	186	156	179	156	180	156	180
IL12 at age 10	-0.01374	-0.03137	-0.10226	0.09142	-0.01880	0.15777	0.18853	0.11286
	0.8926	0.7284	0.2624	0.3086	0.8371	0.0777	0.0376	0.2083
	99	125	122	126	122	126	122	126
IL12 at age 18	0.02876	0.00681	-0.15366	-0.11231	-0.14123	-0.09637	0.02054	0.00484
	0.7730	0.9265	0.0555	0.1344	0.0786	0.1981	0.7991	0.9486
	103	186	156	179	156	180	156	180

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APPENDIX D

GIRLS: SPEARMAN CORRELATIONS OF LEPTIN AND LUNG FUNCTION VALUES AT AGES 10 AND 18 WITH IL-10 AND IL-12 AT AGES 10 AND 18

Table D.1

ρ P-value N	Leptin at age 10	Leptin at age 18	FVC at age 10	FVC at age 18	FEV_1 at age 10	FEV_1 at age 18	FEV_1/FVC at age 10	FEV_1/FVC at age 18
IL10 at age 10	0.03127 0.7292 125	0.18895 0.0238 143	0.03754 0.6597 140	-0.05066 0.5479 143	0.01062 0.9009 140	-0.02968 0.7249 143	-0.06265 0.4621 140	0.09196 0.2747 143
IL10 at age 18	0.02250 0.7911 141	0.04611 0.5136 203	0.01695 0.8233 176	0.08449 0.2319 202	-0.02361 0.7557 176	0.10732 0.1284 202	-0.09080 0.2307 176	0.02745 0.6981 202
IL12 at age 10	0.08109 0.3687 125	0.05273 0.5317 143	-0.00614 0.9426 140	-0.02898 0.7311 143	0.00938 0.9124 140	-0.03221 0.7025 143	-0.00237 0.9779 140	0.04518 0.5921 143
IL12 at age 18	-0.05995 0.4801 141	0.00836 0.9058 203	0.04535 0.5500 176	0.10146 0.1508 202	0.09102 0.2296 176	0.18607 0.0080 202	0.06606 0.3837 176	0.09727 0.1685 202

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