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Effect of different environmental factors on epigenetic modification in allergy and asthma

Inaugural-Dissertation zur Erlangung des Doktorgrades der Humanbiologie

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1. Introduction

1.1. Epigenetics

Epigenetics is a very old word that has been used either to describe the cell fate and system dynamics (Hall 2004) or in developmental psychology to describe psychological development as the result of an ongoing, bi-directional interchange between heredity and the environment. Furthermore, epigenetics is the study of the mechanisms of temporal and spatial control of gene activity during development of complex organisms (Holliday 1990). In biology, and specifically genetics, epigenetics is mostly the study of changes in gene activity which can be transmitted though mitosis and/or to meiosis but are not caused by changes in the DNA sequence. This makes epigenetic changes not only a temporary changes but it can lead to a permanent change in the activity status of the gene, either by gene silencing or by gene activation, which can be inherited to daughter cells. (Kadauke and Blobel 2013). To a lesser extent, epigenetics also describes the study of stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable (Issa and Baylin 1996).

1.2. Epigenetic mechanisms

1.2.1. DNA methylation

DNA methylation is a biochemical process by the addition of a methyl group to the DNA cysteine or adenine. It is considered one of the epigenetic processes that leads mainly to gene silencing and inhibition of the gene transcription (Zhou and Lu 2008). DNA methylation mainly occurs on the different CpGs clustered as islands in the promoter regions of different genes. Once these islands are methylated no gene transcription is allowed (Nan et al. 1998). On the other hands, once these CpG islands are unmethylated, an active promoter is there and an active transcription of different genes is enabled (Nan et al. 1998). The major regulatory enzymes of DNA methylation are DNA methyltransferases known as DNMT. There are different DNMTs that play different roles in the DNA methylation process (Cox 1986). DNMT1 is the major DNA methyltransferase and it plays

a very important role in the maintenance of DNA methylation status of a gene. This is very important to retain any gene silencing in its usually normal state (Gaudet et al. 1998). Moreover DNMT3a and DNMT3b are the main players for De novo methylation of different genes. DNMT3a and DNMT3b can mediate methylation-independent gene repression. DNMT3a can co-localize with heterochromatin protein (HP1) and methyl-CpG-binding protein (MeCBP) (Bachman et al. 2001). They can also interact with DNMT1, which might be a co-operative event during DNA methylation (Hsieh 1999) (**Figure 2.2.1.1.).**

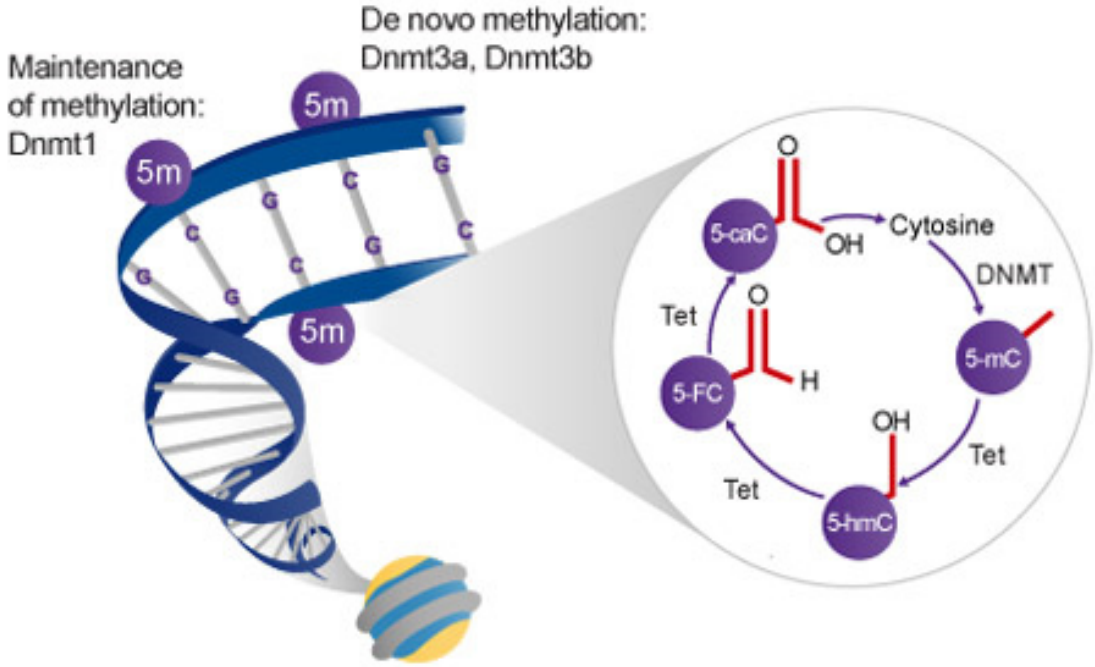


Figure 2.2.1. 1. DNA methylation at the cytosine in the different CpG islands showing different enzymes responsible for either De novo methylation (DNMT3a, DNMT3b) or maintenance of the methylation status (DNMT1) (Bachman et al. 2001)..

In T-cells DNA methylation play a very important role in both the development of T-cells and later on in the differentiation of distinct T cell subtypes. Demethylation of the FOXP3 region in the T-cell can cause the development of the T-cell toward a regulatory T-cell (Kehrmann et al. 2014). Furthermore, there are different methylation statuses for the $CD4^+$ cells in different diseases when compared with the control groups (Graves et al. 2013; Park et al. 2013). The development of T-cells toward different types of T-helper or effector cells

undergoes a very extensive epigenetics regulation. The zinc finger protein Th-POK regulates the development of $CD4^+$ cells and inhibits the development of the $CD8^+$ cells through different DNA methylation of different CD8-associated genes (He et al. 2005). Furthermore, displacement of the polycomb protein through STAT-6 regulation causes a long maintenance of the GATA3 transcription factor and maintenance of Th2 cells (Onodera et al. 2010). On the other hand, Dnmt1 and DNA methylation are necessary to prevent Th2 cytokine expression in $CD8^+$ T-cells (Makar and Wilson 2004a).

1.2.2. Histone modifications

DNA is usually wrapped around two copies of the core histones H2A, H2B, H3 and H4 (Wilkinson and Gozani 2014). The main mechanism of regulating the chromatin is by post-translational modifications of these histones (Bannister and Kouzarides 2011). These modifications can influence gene activation (Araki et al. 2008) and/or gene silencing (Akbarian and Huang 2009) and can have some DNA repair functions as well (Rossetto et al. 2012).

The major histone modifications are acetylation, methylation, phosphorylation, ubiquitination and sumoylation (**Figure 2.2.2.1.**).

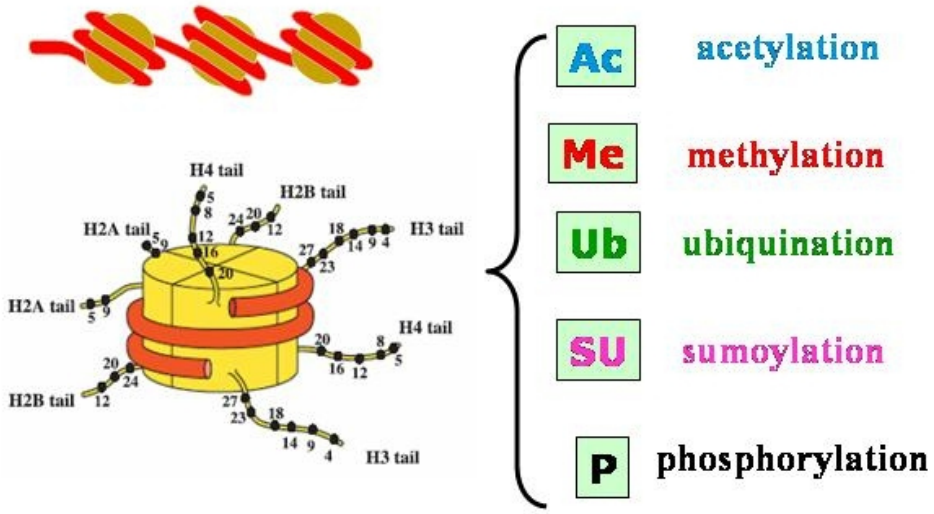


Figure 2.2.2. 1. The figure illustrates the major posttranslational modifications which play essential role in gene expression regulation. (Picture is courtesy of integratedhealthcare.eu)

Histone acetylation of different lysine residues can lead to activation of the transcription (Barnes et al. 2005). This activation is catalyzed by histone acetyltransferases (HATs). HATs transfer the acetyl group from the acetyl-CoA cofactor to the Nζ nitrogen of a lysine side chain within histones. Structural, biochemical, mutational, and enzymatic analyses have provided insights into the catalytic mechanism of these enzymes. A remarkable outcome of these studies is that each HAT subfamily uses a different catalytic strategy for acetyl transfer. This is unusual for a superfamily of enzymes that catalyze the same chemical reaction, but perhaps not so surprising for these enzymes because the transfer of an acetyl group from a thioester to an amine is not a chemically demanding reaction, thus allowing different HAT subfamilies to use different chemical strategies to mediate acetyl transfer (Marmorstein and Zhou 2014; Zhang et al. 2014c). On the other hand histone methylation proved to be more complicated. For example, histone methylation on the lysine residue number 4, 79 and arginine number 17 (K4, K79, R17) can lead to an active transcription of the gene (Benard et al. 2014), whereas histone methylation on other lysine residues like K9 and K27 renders the promoter region inactive and causes a silencing of the genes (Snowden et al. 2002). Furthermore, Phosphorylation of serine residues at histone H3 is a highly dynamic process that creates together with acetylation and methylation marks at neighboring lysine residues specific combinatorial patterns that are read by specific detector proteins (Bannister and Kouzarides 2011) (**Figure 2.2.2.2.**). Histone modifications in the T-cells play a very important role in both the development of the T-cell toward different T-cell subsets as well as the activation or inhibition of different effector genes in various diseases. For examples, gene-specific targeting of H3K9 methylation can cause an efficient gene silencing in many effector Th1 or Th2 genes (Snowden et al. 2002). Furthermore, Th-POK the primary $CD4^+$ transcription factor recruits different histone deacetylases (HDACs) causing silencing of the CD8 genes pushing toward the development of a $CD4^+$ cells (Rui et al. 2012). Lymphocyte development is controlled by either repression or activations of different genes. These activation/inhibition programs controlled by the assembly of Ag receptor genes by V(D)J recombination. Genes expression and the changes in the V(D)J recombination is affected by different epigenetic modifications primarily in histones, altering the chromatin folding rendering it inaccessible for nuclear factors. G9a a H3K9 methyltransferase causes the

development of B-lymphocytes to their mature phase by H3K9 methylation (Thomas et al. 2008). All the results suggested a role for histone methylation at most and overlook the effect of acetylation as a histone modification and its effect on T-cell epigenetics. There are some evidence that histone acetylation can directly affect different T-cell function and this was suggested by Han S et al. showing that HDAC4 and P300 build a complex with GATA3 causing deacetylation on the IL5 promoter suppressing the production of IL5 (Han et al. 2006). A detailed description of the epigenetics of T-cells is further discussed elsewhere.

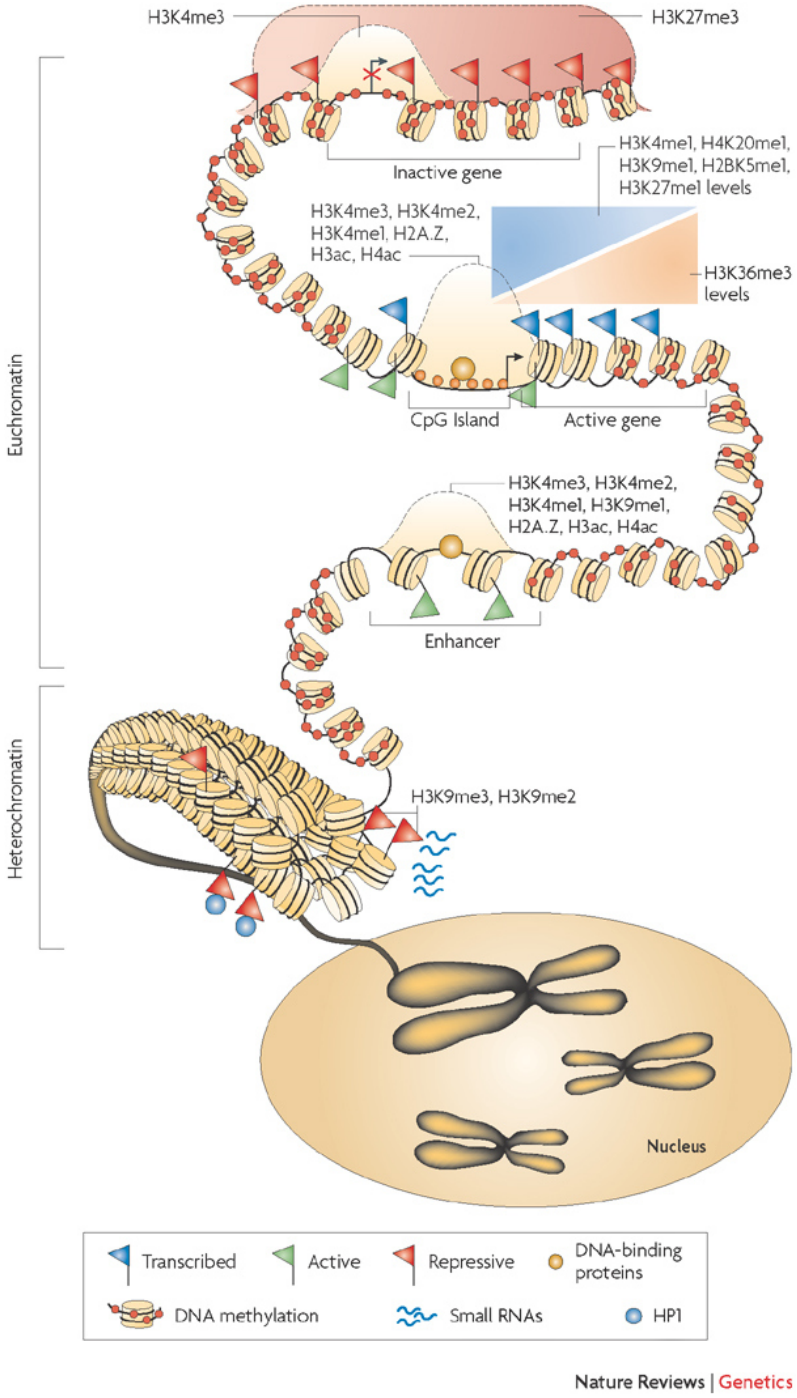


Figure 2.2.2. 2. Histone modifications showing both active and repressive marks and their mode of action (Schones and Zhao 2008)

In this study the focus was to re-establish and validate the main method to detect histone modifications which is chromatin immunoprecipitation (ChIP).

1.2.2.1. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation is an immunoprecipitation technique intended to investigate the interaction between DNA and protein. Its main aim is to explore the association between different protein like transcription factors and histone, and a specific region of the DNA. This method enables the researchers to look at both the exact enzymes and exact time window for this interaction to draw the map of signaling cascades for future understanding of the epigenetic mechanisms. There are different limitations of the ChIP method, starting with the high number of intact cells needed for this analysis. Furthermore, epigenetic marks can vary between cell populations which make the investigation using this method only valuable in pure cell populations to unmask any false positive or negative results. This method is not yet validated for cohort studies which are growing and the need of deep epigenetic analysis on the level of protein-DNA binding is needed. For that case, in this study the ChIP was re-established for a lower number of cells and validated to suit any future cohort studies.

1.2.3. Micro-RNAs and non-coding RNAs

MicroRNA (MiRNA) are short ~22 nucleotide long, non-coding, single-stranded RNAs that binds to complementary sequences in the target mRNA resulting in gene silencing (Pillai 2005) . They are usually encoded in the intron region of the DNA and their biogenesis is a complicated process. It begins with the transcription of pri-miRNA. Afterwards the pri-miRNA is cleaved by Drosha in the nucleus producing a ~70-90 nucleotide (nt) in length called precursor-miRNA (pre-miRNA) (Salam). This cleavage process is only possible with the help of DiGeorge syndrome critical region gene 8 (DGCR8) which is important for the maturation of miRNAs (Macias et al. 2012). The pre-miRNA is then exported to the cytoplasm by Exportin 5 where Dicer enzyme cleaves the pre-miRNA once again to generate a two stranded ~ 22 nt products. The first stand is recruited by Argonaut proteins to form RNA-induced silencing complex (RISC), which is the major driver for the other strand (miRNA) to target different mRNAs causing silencing of the genes (Mattick et al. 2009). MiRNAs interact with chromatin indirectly in different way causing different epigenetic changes by altering nuclear events in different cells (Li 2013).

1.3. Allergy

Allergy is a type of hypersensitivity of the immune system. It's manifested with different symptoms according to the organ that is hit by the hypersensitivity.

Allergies occur when the immune system reacts to a foreign substance — such as pollen, bee venom or pet dander — that doesn't cause a reaction in most people. Allergies can play a major role in conditions such as asthma. In some people, severe allergies to environmental or dietary allergens or to medication may result in life-threatening reactions called anaphylaxis. Food allergies and reactions to the venom of stinging insects such as wasps and bees are more often associated with these severe reactions (Nag et al. 2014).

1.3.1. Pathophysiology of allergy

All allergic diseases are characterized by a distinct pattern of inflammation that is largely driven via immunoglobulin E (IgE)-dependent mechanisms. There is different types of hypersensitivity ranging from Type I hypersensitivity, which is an allergic reaction provoked by re-exposure to a specific type of antigen referred to as an allergen (Bungum et al. 2014). Furthermore, Type II hypersensitivity is defined by the antibodies produced by the immune response bind to antigens on the patient's own cell surfaces. The antigens recognized in this way may either be intrinsic ("self" antigen, innately part of the patient's cells) or extrinsic (adsorbed onto the cells during exposure to some foreign antigen, possibly as part of infection with a pathogen) (Madden et al. 2013). These cells are recognized by macrophages or dendritic cells, which act as antigen-presenting cells. This causes a B cell response, wherein antibodies are produced against the foreign antigen. On the other hand Type III hypersensitivity is the hypersensitivity coming when antigen-antibody complexes that are not adequately cleared by innate immune cells accumulate, giving rise to an inflammatory response and attraction of leukocytes. It involves soluble antigens that are not bound to cell surfaces (Baldo 2013). In addition to these different types, the major type of hypersensitivity is Type IV hypersensitivity. It is usually called delayed type hypersensitivity as the reaction takes two to three days to develop. Unlike the other types, it is not antibody mediated but rather is a type of cell-mediated response (Smith and Miller 1979). In this type, $CD4^+$ helper T cells recognize antigen in a complex with Class II major histocompatibility complex (MHC II) (Miller 1981).

Genome-wide association studies (GWAS) have identified several genes that are associated with asthma and other allergic diseases showing the genetic background of this disease (AKHABIR and SANDFORD 2011). In many of these GWAS few novel genes have been identified that provided new insights of the pathophysiology of the allergic disease. In a GWAS of total serum IgE the most striking hits were functional variants in the genes encoding the α-chain of the high affinity receptor for IgE (FcεRα1), RAD50, located adjacent to the gene for interleukin-13 (IL-13), and signal transducer and activator of transcription 6 (STAT6), which is regulated by IL-4 and IL-13 (Weidinger et al. 2008). Environmental factors appear to be more important in determining whether an atopic individual develops a particular allergic disease, although genetic factors may exert an influence on how severely the disease is expressed and the extent of the allergic inflammatory response. The inflammatory process has several common characteristics shared between different allergic diseases, including asthma, allergic rhinitis/rhinosinusitis, and atopic dermatitis (eczema) (Broide 2010; Hamid and Tulic 2009; Sicherer and Leung 2010).

Allergic inflammation is characterized by IgE-dependent activation of mucosal mast cells and an infiltration of eosinophils that is orchestrated by increased numbers of activated $CD4^+$ Th2 lymphocytes (Sin and Togias 2011). However, in patients with more severe disease, other cells such as neutrophils, Th1, and $CD8^+$ (cytotoxic) lymphocytes may also be involved (Zhang et al. 2014b). The clinical differences between these diseases are largely determined by anatomical differences and the interaction between allergic inflammation and structural cells, such as airway smooth muscle cells in the lower airways, resulting in bronchoconstriction, vasodilatation in the upper airways leading to nasal blockage and rhinorrhea and activation of keratinocytes in the skin. The fact that there are common characteristics of allergic diseases suggests that it may be possible to treat these common diseases with single agents, particularly, as they often coexist (**Figure 2.3.1.1.**).

The major cell that orchestrates allergic inflammation is the Th2 cells by production of IL-4, IL-5, IL-9 and IL-13. Th2 cells are recruited at the site of allergic inflammation via antigen-presenting cells (APCs). Dendritic cells (DCs) are the main APCs that process allergens and present T-cell peptides to naive T cells, and DCs play a critical role in the

recruitment and activation of Th2 cells through the secretion of chemokines CCL17 and CCL22 (Lambrecht and Hammad 2010).

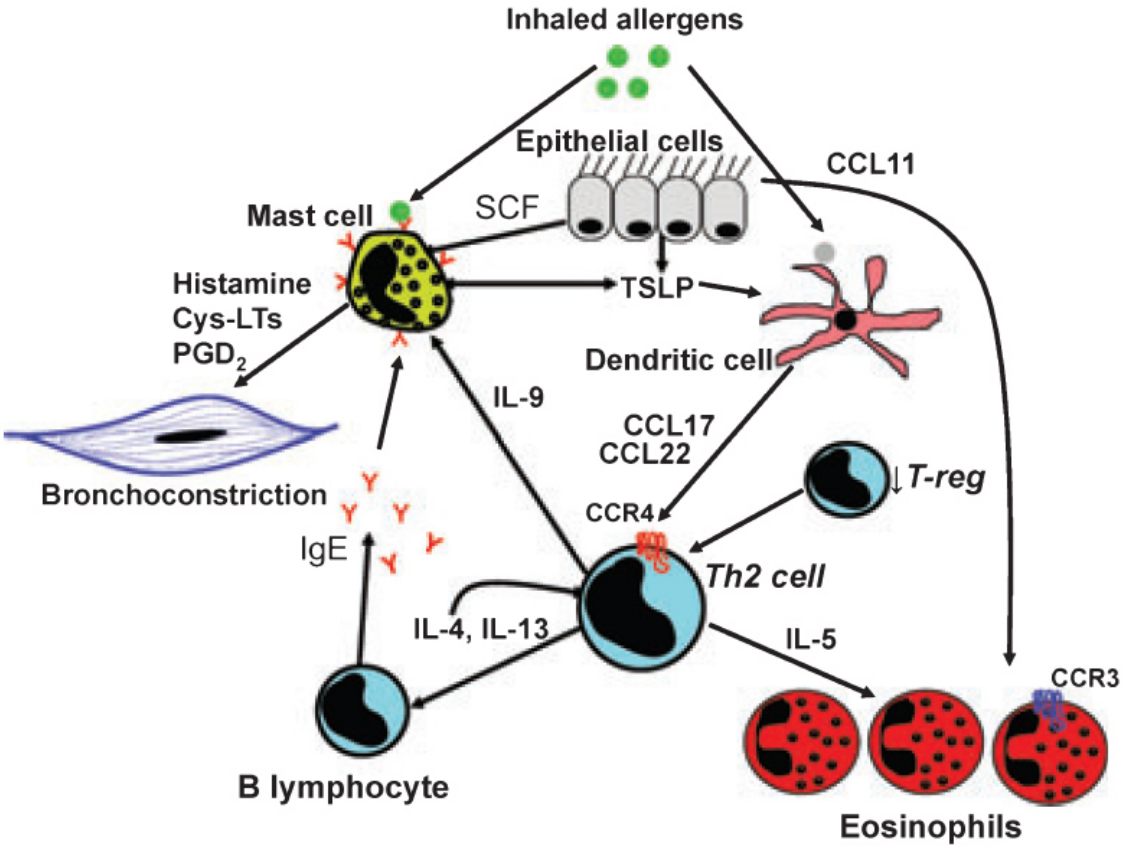


Figure 2.3.1. 1. Inflammation in allergy. A schematic draw by Barnes et.al showing the interaction between epithelial cells as the first line of defense against allergens and the different types of cells. Dendritic cells presenting the antigen to other types of cells like T-cells to prime them toward a more Th2 cells producing the main Th2 cytokines like IL4 and IL13 to activate B-cells to cause an IgG class switching toward IgE the main effector molecule in allergic inflammation(Barnes 2011).

1.4. Cell types involved in Allergy:

Immunologically, many cell types play a role in allergy pathogenesis. In particular, the contribution of dendritic cells, lymphocytes, mast cells, neutrophils and eosinophils will be discussed here.

1.4.1. Dendritic cells

Dendritic cells (DC) play a very important role in the development of allergy by capturing antigens, transporting them from the airway surface to regional lymph nodes and presenting them to T cells. In the lymph nodes, DC present processed antigen to T cells and stimulate the differentiation of naïve T cells into different T cell subtypes. Airway dendritic cells also play a crucial role in the local restimulation of circulating effector T cells upon allergen challenge (Pouliot et al.).

1.4.2. Mast cells

Mast cells are resident cells in many different tissues that play an important role in allergic disease. Activation of mast cells through the cross-linking of high-affinity IgE receptors causes the release of chemical mediators stored in granules, which contribute to the allergic reaction by production of different mediators like histamine the main chemical compound leading to the allergic reaction (Jensen et al.). Increased numbers of mast cells in airway smooth muscle may be linked to airway hyperresponsiveness due to the release of bronchoconstrictor mediators (histamine, cysteinyl-leukotrienes and prostaglandin D2) (Bradding and Brightling 2007; Galli et al. 2005; Prussin and Metcalfe 2003).

1.4.3. Eosinophils

Eosinophils are white blood cells responsible for combating multicellular parasites in the functioning immune system that, in a dysregulated immune response, contribute heavily to the allergic reaction (Chu and Martin 2001; Sampson 2000). These cells produce growth factors such as TGFβ, VEGF, and PDGF, where TGFβ is believed to be important in the regulation of the immune system by $CD25^+$ regulatory T cells and the development of both $CD25^+$ regulatory T cell and T_{h17} cells. Eosinophils also generate leukotrienes which affect allergy by increasing the vascular permeability and mucus production in bronchi, leading to the infiltration of inflammatory cells in the airway wall. In the same time eosinophils express a wide variety of pro-inflammatory cytokines such as IL-1, IL-2, IL-5, IL-6, IL-8, IL-13, and TNFα (Rothenberg and Hogan 2006). These cytokines contribute to the allergic reaction by activating IgE class switching in B cells and autocrine stimulation of eosinophils.

1.4.4. Neutrophils:

Neutrophils are the most abundant immune cells in the body; they are essential for innate immunity and are usually the first cells to migrate to an inflammation site (Witko-Sarsat et al. 2000). Like eosinophils, neutrophils are present in the airways of allergic patients, and in cases of corticosteroid resistant asthma, they are the most abundant cell type. Increasing evidence suggest that neutrophils may be central players with an important role in the pulmonary inflammatory processes present in airway allergic disease (Monteseirín 2009). Neutrophils contribute to the inflammatory process by secreting both lactoferrin and cathelicidin, which work as an antimicrobial compounds and act to attract other immune cell types, such as macrophages and lymphocytes, to the inflammation site. Neutrophil presence has been linked to severe allergic airway inflammation and the development of more chronic state of the disease (Monteseirín 2009).

1.4.5. Lymphocytes

Lymphocytes are considered the major cell type in the pathogenesis of allergy. Th2 cells play an important role in orchestrating allergic inflammation through the release of cytokines that include IL-4, IL-5, IL-9, and IL-13 (Barnes 2008) (**Figure 2.4.5.1.**). Th2 cells are recruited and activated at the sites of allergic inflammation, and a major focus of research has been to understand how topical allergens regulate Th2 cells via antigen-presenting cells (APCs). Although Th2 cells predominate in allergic disease and Th2 cytokines play a key role in the pathophysiology of these diseases, other types of T cells may also play a role, particularly in more severe disease (Lloyd and Hessel 2010). Th9 cells are $CD4^+$ T cells that produce IL-9, which are dependent on transforming growth factor-b (TGF-b) for their development through activation of the transcription factor PU.1, and are regulated by IL-25 (Soroosh and Doherty 2009). The Th9 cells play an important role in the pathogenesis of allergic disease. They are a key component in the memory of T-cell subsets in different allergies (Brough et al. 2014). Moreover, IL-9 contributes to disease by promoting mast cell expansion and production of IL-13 which in turn contributes to airway hyperresponsiveness.

Th17 cells may be associated with more severe asthma and may mediate a more neutrophilic pattern of inflammation. Although Th17 cells produce IL-22, a distinct set of $CD4^+$ IL-22 producing cells (Th22) has been described in allergic inflammatory diseases (Souwer et al. 2010). IL-17 in allergic asthma is most strongly expressed at the more severe stages, IL-17 in Atopic Dermatitis (AD) is absent in chronic lesions. The reason of this contrasting presence of IL-17 in these different allergic inflammations is enigmatic. Otherwise, both chronic allergic asthma and chronic AD inflammation are characterized by elevated amounts of IL-22 (Souwer et al. 2010).

Regulatory T cells (Tregs) may play an important role in suppressing allergic inflammation. Several types of Tregs are now recognized, including $CD4^+CD25^+$ innate Tregs that express the transcription factor Forkhead box protein 3 (FoxP3) and inducible Tregs. They may suppress inflammation through secretion of IL-10 or by inducing IL-10 secretion for bystander cells, as well as direct inhibition of cells, such as DCs, by cell–cell contact. There is some evidence that Treg function is impaired in patients with allergic diseases and that Treg function is enhanced by specific immunotherapy (Palomares et al. 2010).

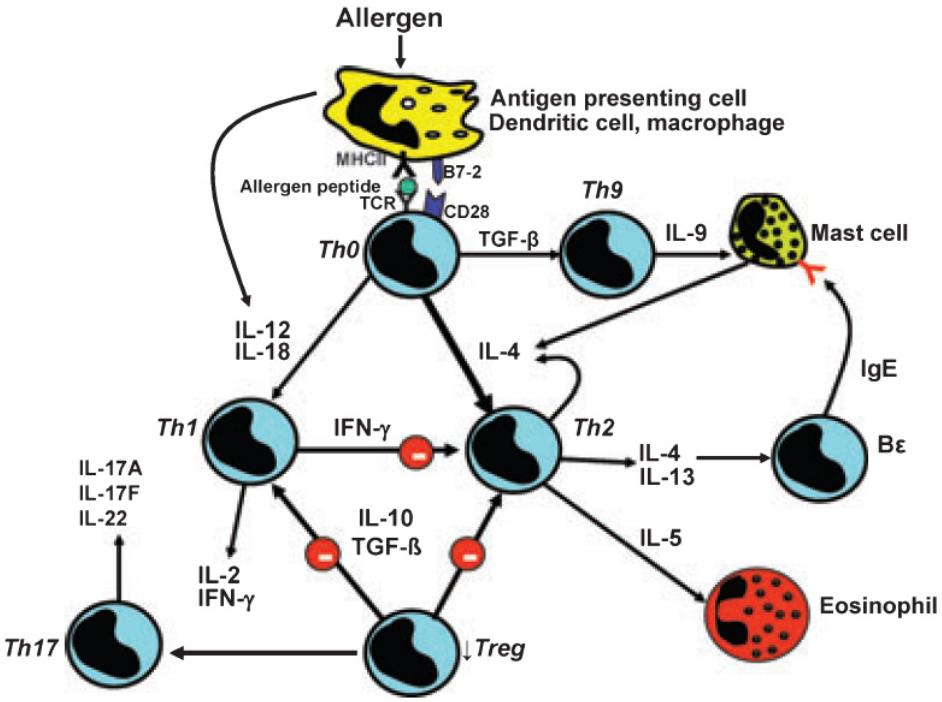


Figure 2.4.5. 1. Pathophysiology of allergic disease showing the role of different T-lymphocytes in the process (Barnes 2011).

1.4.5.1. T-cell epigenetics

Activation of naïve $CD4^+$ helper T cells via the T-cell receptor (TCR) and MHC II: peptide complex induces a rapid T-cell differentiation process. Differentiated helper T cells are classified according to the type of cytokines they produce into Th1, Th2, Th9, Th17 or Tregs. Both Th1 and Th2 have been extensively studied since the initial description of helper T-cell subsets (Mosmann et al. 2005). The epigenetic modifications of T-cells starts very early in the differentiation of T-cells toward Th1 or Th2 subsets. In naïve state histones in the IFNG and IL4 loci are hypoacetylated (Fields et al. 2002). Furthermore, DNA de-methylation at Th2 cytokine genes supports the development of Th2 cells from naïve T-cells (Makar and Wilson 2004b). Moreover, histone acetylation provides accessibility to both IFNG and IL4 loci for both Th1 and Th2 development. The induction of IFNG expression by H2.0-like homeobox protein (Hlx) depends on a permissive epigenetic state of the IFNG gene locus and/or the molecular context of the immature Th cells (Zheng et al. 2004). On the other hand DNA methylation has been shown to play a very important role in the regulation of IFNG expression. In naïve $CD4^+$ cells, IFNG locus is heavily methylated and no expression of IFNG possible. Upon differentiation toward a Th1 cells, the IFNG locus is hypomethylated and IFNG can be produced upon the right stimulus (Melvin et al. 1995). The development toward a Th2 cell on the other hand is connected with nearly complete methylation of IFNG locus while the IL4 locus is hypomethylated and IL4 can be expressed (Santangelo et al. 2002; Lee et al. 2002).

In addition to that, ChIP-seq analysis of genome wide histone modifications gave some insights how epigenetic modifications can regulate Th cell fate.

Upon differentiation from a naïve Th state into the various Th subsets, H3K4me3 deposition was observed at signature effector gene loci within distinct TH subsets (e.g., IFNG in TH1, IL4 in TH2, and IL17A in TH17). Moreover, H3K27me3 deposition was correlated with transcriptional shutdown of effector gene loci that are characteristic of other TH subsets (Wei et al. 2009). For example, the gene locus encoding the TH17 transcription factor retinoid-related orphan receptor-γ (RORC) was decorated with

H3K27me3 in the naïve state, and only acquired H3K4me3, and losing H3K27me3 after TH17 differentiation. In contrast, the repressive H3K27me3 signature was reinforced under TH1 and TH2 differentiation conditions (Araki et al. 2008). However, this was not always the case. The TBX21 (Th1) and GATA3 (Th2) gene loci in naïve TH cells were marked with both H3K4me3 and H3K27me3, and whilst these loci resolved to a permissive epigenetic signature (H3K4me3+/H3K27me3-) under Th1 and Th2 differentiation conditions, respectively, they did not acquire a repressive epigenetic signature when differentiated into opposing lineages, but rather maintained a bivalent state.

Similarly, the TBX21 locus within Th17 cells was also maintained in a bivalent state. In the case of Th17 cells, re-stimulation of Th17 cells in the presence of IL12 resulted in expression of IFNG and conversion to a Th1 phenotype. This was associated with acquisition of permissive epigenetic signatures (histone acetylation) at the IFNG locus and IL-12-dependent STAT4 and TBX21-dependent epigenetic silencing of the Th17 associated RORC locus (Mukasa et al. 2010). There are different enzymes affecting histone modifications processes and histone methyltransferases play a major role (Allan et al. 2012). SUV39H1 specifically trimethylates H3K9 – a PTM typically associated with transcriptional silencing of gene loci that is in turn recognized by heterochromatin protein 1α (HP1α) (Lachner et al. 2001)Docking of HP1α onto H3K9me3+ gene loci in turn recruits HDAC1 and 2, and the transcriptional repressor methyl-binding domain protein (MBD1) (Fujita et al. 2003). In this way, H3K9 acetylation, a PTM associated with transcriptional activation is limited. Thus, SUV39H1-mediated trimethylation of H3K9 is an initial step that triggers histone deacetylation and binding of transcriptional repressor protein complexes that stably silence targeted loci.

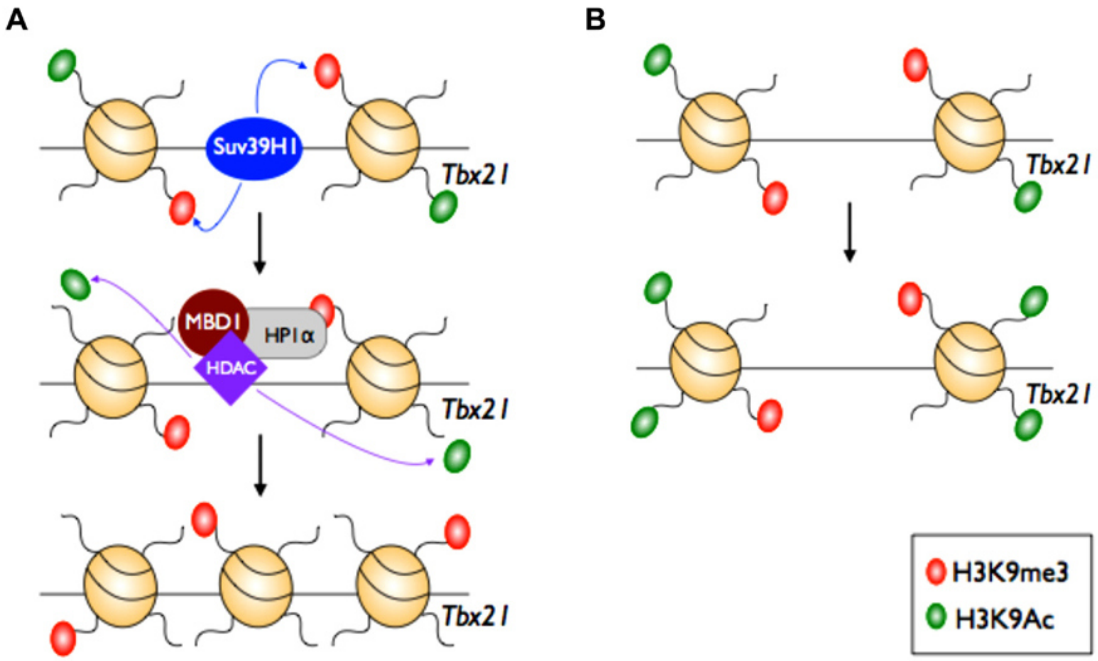


Figure 2.4.5.1. 1. Epigenetic maintenance of Th2 lineage commitment.

In the Th2 cell subset, the master regulator of Th1 cells (TBX21) is silenced. The histone methylase Suv39H1 adds the repressive H3K9me3 mark at the Tbx21 locus. This initiates recruitment and docking of heterochromatin protein 1 alpha (HP1α), histone deacetylase (HDAC1 and 2), and methyl-binding domain protein (MBD1). HDACs then remove the active H3K9ac mark to maintain silencing, mediated by H3K9me3, at the TbX21 locus (Russ et al. 2013). On the other hand, Inhibition of repressive histone methylation by the specific inhibitor 3-deazaneplanocin induced Th9-specific PU.1 expression, even in conditions that would normally yield only Th0 cytokines. Conversely, prevention of histone acetylation by the histone acetyltransferase inhibitor curcumin diminished PU.1 expression after IL-9-inducing stimulation. Our findings identify age- and differentiation-status-related epigenetic modifications of PU.1 as a unique regulator of Th9 memory acquisition and Th9 immunity (Ramming et al. 2012).

1.5. Environmental factors affecting epigenetic programming

Environmental factors play a very important role in changing different epigenetic marks in the mammalian cells. Starting with heavy metals like cadmium that can for example cross the placenta during pregnancy causing DNA methylation of different promoter regions of

different genes regulating apoptosis and transcriptional regulation (Sanders et al. 2013). Furthermore, folate supplementation during pregnancy can exert epigenetic modification on the offspring as well (Hollingsworth et al. 2008).

1.5.1. Tobacco smoke, Polycyclic aromatic hydrocarbon (PAH) and particulate matter (PM)

Airborne pollutions like tobacco smoke exert many harmful effects and an exposure to its fumes has been connected to hypermethylation of some genes (Bosetti et al. 2008). Furthermore, polycyclic aromatic hydrocarbon (PAH) is the most widespread pollutant on earth. Evidence is accumulating showing that PAH exposure can lead to many diseases, like lung cancer (Brodie et al. 2014), asthma (Tang et al. 2012), obstructive lung disease (Burstyn et al. 2003) and more. PAH exposure is associated with impaired systemic immunity and DNA methylation in a key locus involved in atopy: FOXP3, with a higher impact on atopic children (Hew et al. 2014). In addition to that, maternal exposure to PAHs was associated with hypermethylation of IFNγ in cord blood DNA from cohort children. These findings support a potential role of for PAH in reprogramming the fetal epigenome and thus leading to increased disease susceptibility (Tang et al. 2012). Another factor that can affect the epigenetic marks in the mammalian cells is particulate matter (PM) e.g. dust, diesel particulate matter. All these particles can cause different disease like asthma and cardiovascular diseases (Ji and Khurana Hershey 2012). In mice, the exposure to these PM particles caused an increase in DNMT1expression in lung epithelial cells and in the methylation of the P16 promoter that is linked to the development of cancers (Soberanes et al. 2012).

1.5.2. Bacterial exposure

According to the hygiene hypothesis proposed by Strachan et.al (Strachan 2000), different bacterial exposure during pregnancy and early life has an impact on the development of the immune system. One important environmental exposure during pregnancy and childhood influencing immune system development is bacterial exposure at the farm environment. Ege et.al showed that the bacterial exposure is directly proportional to the protection of asthma in children (Ege et al. 2011). This was done in two different European cohorts, the GABRIELLE study and the PASTURE study. Children who have been born and grown up

in farms were less susceptible to developing asthma and allergic disease when compared to children born and raised in the countryside but outside of a farm (Pfefferle et al. 2013). In a mouse model of asthma, offspring of mothers who were treated intranasally with *Acinetobacter lwoffii* were protected from developing asthma compared to sham treated mothers. This effect was mediated by histone acetylation of the IFNγ locus (Brand et al. 2011). Furthermore, commensal bacteria like *Lactobacillus rhamnosus GG* and *Bifidobacterium* caused a decrease in LPS-induced IL-17 and IL-23 production by suppressing histone acetylation in mice (Harb et al. 2013). This effect spreads beyond commensal bacteria to pathogens like *Helicobacter pylori* that causes DNA methylation (Ding et al. 2010), histone acetylation in gastric epithelial cells promoting the pathogenesis of the disease (Fehri et al. 2009) and even regulate different miRNAs like miR-222 and upregulation of miRNA-584 and miRNA-1290 by CagA of the bacteria in human epithelial cells(Li et al. 2012). These effects of bacteria that colonize the human gut suggests that these microorganisms play a very important role in the development of the human immune system, not only by shifting the immune reactions toward a Th1 rather a Th2 but also goes deep into the molecular level causing changes in our epigenome by different epigenetic mechanisms that can lead at the end to the shaping of our immune system.

1.5.3. Medication

In addition to the previously mentioned factors different pharmacological compounds can influence the epigenetic code. These compounds can be either of a nature origin or chemical compounds produced in the lab.

Mainly, pharmacological compounds cause an effect of the main metabolic enzymes cytochrome (CYP) p450. Many CYP genes do not have any apparent polymorphism or mutations that can cause the different effect of medication on the activity and sensitivity of these enzymes. In recent years, many studies have emerged showing the epigenetic effect of different medications and pharmacological compounds on certain genes.

DNA methylation contributes to the regulation of *CYP1A1* in prostate cancer cells. The lack of *CYP1A1* expression in the prostate cancer cell line LNCaP has been associated with methylation of the promoter region of the *CYP1A1* gene, which prevents the binding of the AhR complex to the dioxin response element (DRE) (Okino et al. 2006). Furthermore,

many pharmacological compounds were shown to have an effect on T-cell epigenetics. Allergen specific immunotherapy caused an increase in Treg numbers by the reduction of the DNA methylation on different CpG islands on the Foxp3 locus (Lal and Bromberg 2009). Additionally, demethylation agents like 5-azacytidine (5-azaC), procainamide and hydralazine could induce lupus-like autoimmunity in vitro and in vivo likely due to DNA demethylation in T cells (Zhou and Lu 2008; Richardson et al.).

1.5.4. Diet

There are many studies suggesting that the diet is affecting different epigenetic mechanisms both systematically and on specific targets. Here especially the effect of a methyl rich diet on DNA methylation was analyzed. . For instance, methyl donor rich diet of pregnant mice can cause a drastic change in the fur color of offspring due to changes in DNA methylation at the *agouti* locus(Shorter et al. 2014; Dolinoy 2008) (**Figure 1.5.4.1.**). Furthermore, maternal diet supplemented with methyl donors enhanced the severity of allergic airway disease that was inherited transgenerationally (Hollingsworth et al. 2008)

Figure 2.5.4. 1. Agouti mouse model, showing the effect on fur color through methyl donor high diet affecting the agouti gene responsible for the color of the mice. The blonde

mice represent high methyl diet and the methyl diet goes down as the color darkens (Dolinoy 2008).

One of the most prominent methyl donors is Folic acid and vitamin B12 that can affect the DNA methylation status universally (Zhang et al. 2014a). Furthermore, other nutrients like vitamin A and vitamin D3 can inhibit DNA methylation by regulating of different proteins like p21 and AP-1 involved in Dnmt1 activity through interaction with different receptors (Sundar and Rahman 2011). Nevertheless, also histone modifications can be modified by dietary compounds. Many vegetables like wasabi, horseradish, mustard, radish, Brussels sprouts, watercress, nasturtiums, and capers contains isothiocynate that can inhibit histone deacetylases (HDACs) causing a universal histone deacetylation and silencing of different genes (Meeran et al. 2012).

1.5.4.1. Folic Acid

Folic acid, also known as vitamin B9 is a water soluble vitamin found mostly in leafy vegetables. In figure 2, the chemical formula of folic acid is shown.

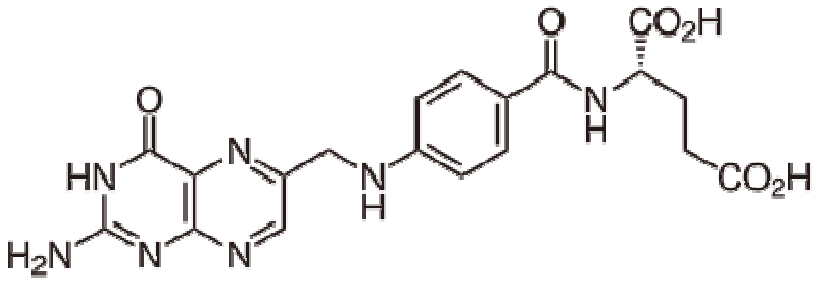


Figure 2.5.4.1. 1. The chemical formula of folic acid downloaded from http://en.wikipedia.org/wiki/File:Folic_acid.svg

Folic acid is considered one of the most important vitamins during pregnancy as it has a very important role in the development of neural tube and the development of the embryo (Jagerstad 2012). Moreover, folic acid supplementation during pregnancy was associated with less congenital heart diseases in newborns (Feng et al. 2015).

On the other hand, folic acid has been associated with the development of asthma and allergy later in life if supplemented in a high dose during pregnancy (Dunstan et al. 2012).

This effect is mainly elicited through different epigenetic mechanisms like DNA methylation (Ghoshal et al. 2006) and other mechanisms like decreasing different DNA methyltransferases and histone modifying enzymes like Dnmt1, Dnmt3, MBD2, HDAC1-9 and MBD4 mRNA and protein levels during early stages of hepatocarcinogenesis (James et al. 2003). On the level of T-cells folic acid supplementation can promote the survival of $Foxp3^+$ cells in the colon (Kinoshita et al. 2012).

1.5.4.2. Fish Oil

Fish oil is the product of oily fish like Salmon and Sardines. Fish oil contains the two major omega-3 (ω-3) fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), the precursors of different eicosanoids that play a very important role in the reduction of inflammation by the production of resolvins and defensins (Keelan et al. 2014). Fish oil supplementation in adults protects against heart diseases (Cleverley et al. 2013), brain diseases (Pascoe et al. 2015), insulin resistance (Bremer et al. 2013) and protection against some cancers (Runau et al. 2015). Furthermore, fish oil supplementation during pregnancy has great effect on the developing of the fetus. Omega–3 supplementation during pregnancy changes the whole inflammatory response of the fetus (Klemens et al. 2011). These findings were supported with what Miles et.al found that supplementation of ω-3 fatty acids reduces the risk of asthma and allergy development later in life (Miles and Calder 2013). This reduction was mediated by modulating the immune response by reduction of IL-13 and IL-5 levels in DHA supplemented infants compared to control (D'Vaz et al. 2012c).

1.6. Goal of the study

To study the role of epigenetic modifications in the development of chronic inflammatory disease DNA methylation has been analyzed in several cohort studies (Bose et al. 2014; Hagrass et al. 2014; Chang et al. 2014). Due to the challenging nature of the analysis of histone modifications these modifications have never been analyzed in the context of cohort studies until now.

The goal of the study was to establish a chromatin immunoprecipitation method suitable for cohort studies. As it is clear that the differentiation of T cells into effector T cells and the production of cytokines by effector cells plays a vital role in the development of chronic inflammatory disease this study focuses on histone modifications of Th1, Th2, Th9, Th17 and Treg gene loci in $CD4^+$ T cells from peripheral blood. The study concentrate on histone H3 and H4 acetylation as once the analysis of these markers has been established it should be relative easy to analyze also other modifications just by using other antibodies against e.g. methylated histones. The method should be validated according to validation protocols to ensure the redundancy and repeatability of the results. The reason for the validation is the following:

- Humans are unique individuals and usually display a relatively high variance between individuals so that is important to analyze the normal range of H3 and H4 modifications at a given locus.
- To determine the best working conditions for this method on the long run for cohort studies, including storage conditions effects, temperature effect and the effect of repeated freezing and thawing of the samples.

Until now, all published methods needs a high number of cells up to 5 million cells. As chronic inflammatory disease often have an origin in early childhood so that it is especially interesting to analyze epigenetic modifications in children. Here it is hard to get these amounts of pure $CD4^+$ T cells so that it the number of cells needed to produce reliable and redundant results for such cohort samples should be reduced.
To prove that it is possible to use the method to show differences between cohort group's samples from different cohorts should be analyzed. These cohorts include:

- A cohort from Australia was the folate level of pregnant mother was analyzed. The goal of this study is to show if high maternal folate levels influence epigenetic modifications including histone acetylation in $CD4^+$ T cells of the neonates.
- An additional Australian cohort were the effect of fish oil supplementation during pregnancy vs. placebo supplementation on histone acetylation and their correlation with disease development later in life should be analyzed.

- A comparison between healthy children vs. allergic asthmatic children on the level of histone acetylation to analyze H3 and H4 acetylation in this cohort for future analysis and planning of new cohorts focusing on the origins of allergic disease.

2. Materials and Methods

2.1. Cells

Human $CD4^+$ cells.

2.2. Chemicals and reagents

Sodium Butyrate	Sigma – Aldrich, Germany
Glycine	Sigma – Aldrich, Germany
Lithium Chloride (LiCl)	Jena Bioscience, Germany
Sodium Chloride (NaCl)	Roth, Germany
Tris – HCl	Roth, Germany
Tween 20	Roth, Germany
Sodiumdodecylsulfate (SDS)	Roth, Germany
Igepal CA-630	Sigma – Aldrich, Germany
Sodium carbonate (NaHCO3)	Sigma – Aldrich, Germany
Sodium Hydroxide (NaOH)	Merk, Germany
Triton – X100	Sigma – Aldrich, Germany
Sodium Deoxycholclate (DOC)	Sigma – Aldrich, Germany
Potassium Chloride (KCl)	Sigma – Aldrich, Germany
Ethylenediaminetetraacetic acid	Sigma – Aldrich, Germany
Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES)	Sigma – Aldrich, Germany

2.3. Kits

Full blood $CD4^+$ isolation kit	Miltenyi, Germany
PCR purification kit (250)	Qiagen, Germany

2.4. Deoxyribonucleic acid

Different Th1, Th2, Th17, Th9 and Treg gene promoter primers were established and validated in both normal and quantitative PCR.

2.4.1. Oligonucleotides

Table 3.4.1. Promoter primers used for both validation and measurement of different enrichment values on both H3 and H4 histone acetylation.

Locus	Forward	Reverse
RPL32	5'-GGAAGTGCTTGCCTTTTTCC-3'	5'-GGATTGCCACGGATTAACAC-3'
IL-1β	5'-CGTGGGAAAATCCAGTATTTTAATG-3'	5'-CAAATGTATCACCATGCAAATATGC-3'
IL-4	5'-TGGGTAAGGACCTTATGGACC-3'	5'-GGTGGCATCTTGGAAACTGTC-3'
IL-5	5'-AGGAGATCTTTTTAGTCACTGGCAACA-3'	5'-CGTCTCGAGGGCAAAGAAAGTGCATAG-3'
IL-9	5'-CGTTAGAACACCCATGAC-3'	5'-TTCTGGTTGTGAGAGTTAG-3'
IL-10	5'-GACAACACTACTAAGGCTCCTTTGGGA-3'	5'-GTGAGCAAACTGAGGCACAGAAAT-3'
IL-13	5'-TGTGGGAGATGCCGTGGG-3'	5'-TCTGACTCCCAGAAGTCTGC-3'
IL-17A	5'-AATTTCTGCCCTTCCCATTT-3'	5'-CCCAGGAGTCATCGTTGTTT-3'
GATA-3	5'-CACATTTAAAGGGCCAGAGC-3'	5'-AAGGAAACTGCAACCCAAAC-3'
T-bet	5'-TGGCCAAGAGCGTAGAATTT-3'	5'-GCTTTGCTGTGGCTTTATGA-3'
RORc	5'-TCTCCCCTATGCCTGTCACCTG-3'	5'-TGATTTTGCCCAAGGACTCACAC-3'
Foxp3	5'-ATCGTGAGGATGGATGCATTAATA-3'	5'-CCACTGGGAAGGTCCCTAGC-3'
IRF-1	5'-GTACTTCCCCTTCGCCG-3'	5'-GCGTACTCACCTCTGCTGC-3'
TNFα	5'-AGAAGACCCCCCTCGGAACC-3'	5'-ATCTGGAGGAAGCGGTAGTG-3'
IFNγ	5'-AATCCCACCAGAATGGCACAGGTG-3'	5'-GAACAATGTGCTGCACCTCCTCTGG-3'
FcεRIβ	5'-CTTAGGGGTTAGATTTTATGTGTTTGAACCCCAA-3'	5'-CCATCTTCTTCATGGACTCCTGGTGCTTAC-3'

2.5. Antibodies

IgG antibody	Abcam, Cambridge, UK
Anti- H3 acetylation Rat anti Human	Millipore, Germany
Anti-H4 acetylation Rat anti Human	Millipore, Germany
Anti -CD4 FITC labelled Rat anti Human	BD, Germany
Anti-CD45R PE labelled Rat anti Human	BD, Germany

2.6. Reagents and Buffers

Ready to ChIP chromatin	Diagenode, Germany
Protease Inhibitor cocktail	Roche, Germany
RNase A	Invitrogen, Germany
Proteinase K from Tritrichium Album	Sigma – Aldrich, Germany
nProtein A Sepharose Beads	GE Healthcare, USA

nProtein G Sepharose Beads	GE Healthcare, USA
Sybr Green	Qiagen, Germany
PBS	PAA, USA
Cellfix	BD, Germany
Human Serum	Linco, Germany
Fetal Calb Serum	PAA, USA
$CD4^+$ dynalbeads	Invitrogen, Germany

Lysis Buffer I	for 50 ml
5 mM PIPES pH 8	0,5 ml 0,5M PIPES pH 8
85 mM KCl	1,4 ml 3M KCl
0,5% NP40 (Igepal-CA630)	0,25 ml Igepal (100%)
Protease Inhibitor cocktail tablets	One Tablet

Lysis Buffer II	for 50 ml
10 mM Tris-HCl pH 7,5	0,5 ml 1M Tris-HCl pH 7,5
150 mM NaCl	1,5 ml 5M NaCl
1% NP40 (Igepal-CA630)	0,5 ml Igepal (100%)
1% DOC (Natriumdeoxycholat)	0,5 g
0,1% SDS	0,25 ml 20% SDS
1 mM EDTA	0,1 ml 0,5M EDTA pH 8
Protease Inhibitor cocktail tablets	One Tablet

Wash Buffer I	for 50 ml
20 mM Tris-HCl pH 8	1 ml Tris-HCl pH 8
150 mM NaCl	1,5 ml 5M NaCl
2 mM EDTA	0,2 ml 0,5M EDTA pH8
0,1% SDS	0,25 ml 20% SDS
1% Triton X100	0,5 ml Triton X100

Wash Buffer II	for 50 ml
20 mM Tris-HCl pH 8	1 ml Tris-HCl pH 8
500 mM NaCl	5 ml 5M NaCl
2 mM EDTA	0,2 ml 0,5M EDTA pH8
0,1% SDS	0,25 ml 20% SDS
1% Triton X100	0,5 ml Triton X100

Wash Buffer III	for 50 ml
10 mM Tris-HCl pH 8	0,5 ml Tris-HCl pH 8
1% NP40 (Igepal-CA630)	0,5 ml Igepal (100%)
1% DOC	0,5 g
1 mM EDTA	0,1 ml 0,5M EDTA pH 8
0,25 M LiCl	1,25 ml 10MLiCl

1xTE	for 50 ml
10 mM Tris-HCl pH 8	0,5 ml Tris-HCl pH 8
1 mM EDTA	0,1 ml 0,5M EDTA pH 8

Elution Buffer	for 25 ml
1% SDS	1,25 ml 20% SDS
0,1 M $NaHCO_3$	2,5 ml 1M $NaHCO_3$

2.7. Consumables

Reaction tubes (1.5 ml) Eppendorf, Germany

Reaction tubes (0.1 ml) LTF, Germany

FACS tubes BD Falcon, Germany

2.8. Machines

Tip-Sonicator (Heilscher, Germany)

Bioruptor Power up	(Diagenode, Belgium)
Flow Cytometry Canto	(BD, Germany)

2.9. Isolation of $CD4^+$ cells from human full blood.

2.9.1. Ethical Approval

10 healthy subjects were recruited for ChIP analysis and method validation. The ethical approval was obtained from the ethic committee of the Philipps University Marburg.

2.9.2. Isolation of $CD4^+$ cells from human full blood

2 ml full blood was withdrawn in EDTA tubes and a direct isolation of $CD4^+$ cells was run as follows:

$CD4^+$ magnetic beads were added to the blood directly in a ratio of 50 µl for each 1 ml blood. The beads and blood were incubated for 15 min in 4°C. Afterwards, 20 ml MACS buffer was added and blood was centrifuged at 800 g for 10 min without brakes. The supernatant was discarded and the rest were suspended in 1 ml MACS buffer. The suspension mixture was added on the LS/MS columns (Meltinye, Germany) attached to the magnet. The columns were washed 3 times with 3 ml MACS buffer to ensure that no red blood cells were left in the column. Then the columns were removed from the magnet and the cells were washed out using 5 ml elution buffer containing 0.009 % sodiumazide. The cells were centrifuged at 600 g for 10 min and the pellet was resuspended in 1 ml MACS buffer for further analysis.

2.9.3. $CD4^+$ cells purification test through flow cytometry

To measure the purity of the isolated cells, the human $CD4^+$ cells were resuspended in 1 ml FACS buffer in FACS tubes. The cells were then stained with anti-CD45R and anti-CD4 antibodies to measure the purity of our population according to the following scheme

- Tube	- FITC	- PE
- 1	- CD 4	- CD 3
- 2	-	- CD45R
- 3	- CD4	- CD 45R

The FACS tubes were centrifuged at 350 g for 10 min. afterwards, the supernatant was discarded and the pellet was suspended in 50 µl human serum + 9 µl FACS buffer. 1 µl of the antibodies were added as shown in the scheme. The cells were incubated for 15 min with the antibodies at RT in a dark place. Once the incubation was completed 1 ml FACS buffer was added and the tubes were centrifuged once again with 350 g for 10 min. the supernatant was discarded and the pellet was resuspended in 300 µl in Cell fix. The cells were measured using FACS Canto II.

2.10. Chromatin immunoprecipitation (ChIP).

2.10.1. Preparation of the $CD4^+$ cells chromatin for chromatin immunoprecipitation.

$CD4^+$ cells were isolated using either $CD4^+$ beads (Miltenyi, Germany) or $CD4^+$ dynalbeads (Invitrogen GmbH, Darmstadt, Germany). For the crosslinking of the histones with the DNA, cells were fixated for 8 min on RT using 1% formaldehyde. The reaction was stopped using 55 µl 2.5 M glycine. The chromatin was treated for 3 min with 5 M sodium butyrate to enhance the binding of the acetyl group to the histone and then centrifuged for 5 min at 8000 rcf (relative centrifugal force) at room temperature (RT). Moreover, the chromatin was incubated with lysis buffer I for 20 min on RT. After spinning down the chromatin for 5 min at 8000 rcf on RT, the chromatin was incubated with lysis buffer II containing 0.1 – 1.5% sodium dodecyl sulfate (SDS) for 5 min on RT and 3 min on ice then put directly in either the tip sonicator or the power-up bioruptor (Diagenode, Belgium) for 30 cycles, where each cycle was 30` on and 30` off (high power). Afterwards, the chromatin was centrifuged for 15 min at 28000 rcf to

remove any debris that may interfere in the process. The supernatant was transferred to a new reaction tube and diluted to an SDS concentration of 0.1%.

2.10.2. Chromatin Immunoprecipitation (ChIP)

1. For the Chromatin immunoprecipitation, Sepharose beads (GE Healthcare Bio-sciences AB, Uppsala, Sweden) were washed once with lysis buffer II then centrifuged at 1800 rcf for 2 min. After removal of the supernatant the beads were blocked with 1 mg/ml BSA and 400 µg salmon sperm DNA overnight at 4°C. The beads were then centrifuged at 1800 rcf for 5 min and washed once with 5 ml lysis buffer II. 30 µl of the beads per IP per number of samples were transferred to a new reaction tube and stored to be used for step 6.
2. 20 µl beads per antibody per sample were added to the chromatin and incubated for 2 hours at 4°C for removal of chromatin which is binding unspecific to the Sepharose beads (preclearing 1). After the incubation time the supernatant with the precleared chromatin was transferred to a new reaction cup (1.5 ml).
3. In the meantime the rest of the beads were filled with 500 µl lysis buffer II per sample and 1 µg of an unspecific IgG antibody (Abcam, Cambridge, UK) was added per sample. The beads with the unspecific antibody were incubated for 1 hour at 4°C. Afterwards the beads coupled with IgG antibody were centrifuged for 5 min with 1800 rcf and washed 3 times each with 5 ml lysis buffer II and centrifuged for 5 min with 1800 rcf.
4. 20 µl of the IgG coupled beads were added to the precleared chromatin from step 2 and incubated for another 2 hours at 4°C for removal of chromatin which is binding unspecific to IgG antibodies (preclearing 2). At the end of the incubation time, the precleared chromatin was centrifuged at 8000 rcf for 5 min at 4°C.
5. The supernatant containing the chromatin was transferred to a new reaction cup. 10% volume of the chromatin was set aside for the input control. The H3 or H4 acetylation antibodies (4 µg) (Millipore, Darmstadt, Germany) were added to the chromatin and incubated overnight at 4°C. A negative control IgG was also prepared (0.5 µg) (Abcam, USA).

6. 30 µl bead that are been saved from step 1 were added to each IP and then incubated for 2 hours at 4°C. Once the incubation was completed the beads were centrifuged at 8000 rpm for 2 min and washed two times with wash buffer I, two times with wash buffer II, 3 times with wash buffer III, and finally two times with 1X TE buffer. After discarding the supernatant, 500 µl of the elution buffer were added to the Sepharose beads, vortexed and incubated for 30 min on the fast rotor at RT. Afterwards, the mixture is being centrifuged at 8000 rpm for 2 min. The supernatant contains the precipitated chromatin and was transferred to a new reaction tube.

7. For crosslinking reversal following mixture is being added to the IP product as well as to in input controls saved from day two with 500 µl elution buffer to the input controls.

20µL	5M	NaCl	
10µL	0,5M	EDTA	pH8
20µL	1M	Tris	pH7.2
1µL		ProteinaseK	(20mg/ml)
1µL		RNaseA	(10mg/ml)

to make a 52µL Master mix for each sample.

The mixture will ensure the detachment of the histones from the DNA and denaturation of the rest of proteins and any RNA found. The samples were incubated for 3 hours at 55°C then 65°C overnight. On the fourth day the DNA will be purified using QIAquick kit according to the manufacturer's manual protocol.

All incubation steps were performed on an overhead rotator.

The purified DNA was used in real time PCR to measure the enrichment of different promoter regions of different genes.

2.10.3. Enrichment measurement

To measure the effect of the acetylation on different gene promoter per cent enrichment was measured to a control that represents 100 per cent.

The enrichment is calculated using the following formula:

$$\% \text{ Enrichment} = 100 * 2^{[(CT\text{input} - 3.3) - CT\text{sample}]}$$

The adjusted CT value used comes from a 10% input from the starting materials if the starting input fraction is 10%, then a dilution factor (DF) of 10 or 3.3 cycles (i.e., log2 of 10) is subtracted from the Ct value of diluted input.
The final enrichment percentage was calculated by subtracting the enrichment of our negative IgG-Mock control from the enrichment of our wanted antibody.
The per cent enrichment was then normalized to the RPL32 house keeping gene as: % Enrichment to desired gene/ % Enrichment to positive control.

2.11. Validation of the ChIP method.

After establishing the method, different validation techniques and protocols were implemented for the following reasons:

- Humans are unique individuals. To consider if a measured value in any individual is "normal" it is necessary to know which values can be measured in healthy subjects. This is the so-called reference range
- To determine the lowest values which could be measured it is necessary to determine the lower limit of the blank. To determine the lowest number of cells needed for an acceptable measurement the lower limit of quantification must be analyzed
- It is common to freeze samples during collection within cohort studies so that it is necessary to analyze if freezing and storage could affect the analysis.
- As histone modifications could be temperature sensitive it is also necessary to analyze if e.g. if exposure to higher temperatures (room temperatures) is affecting the outcome of the ChIP.
- An internal standard is needed to ensure that a measurement is valid.

2.11.1. Reference range, inter- and intra-assay coefficient of variance

To analyze the normal variation of H3 and H4 histone acetylation at a certain locus in healthy individuals, a reference range for each locus measured was set for both H3 and

H4 histones. To do so histone acetylation at certain loci was measured in 10 healthy subjects and taking the upper and lower 95 % confidence interval for the measurement. These values represent the normal values that a healthy person should have for both H3 and H4 acetylation for different enrichment values for different gene promoters. Furthermore, both inter- and intra-assay coefficient of variance were measured to test both the specificity and sensitivity of the ChIP respectively. The inter-assay of variance is the variance when measuring the same sample many times in different runs, whereas the intra-assay coefficient of variance is variance when measuring the same sample many times in the same run.

2.11.2. Lower limit of the blank (LOB).

Lower limit of the blank (LOB) is the measurement of the background of our test. It's measured by measuring 5 blank samples in one run and taking the mean value of the enrichment + 3 standard deviations (Armbruster and Pry 2008).

2.11.3. Lower limit of quantification (LOQ).

Lower limit of quantification (LOQ) is the lowest number of cells needed for a repeatable and redundant enrichment measurement (Armbruster and Pry 2008). $CD4^+$ cells were titrated from $1X10^6$, $5X10^5$, $1X10^5$, $5X10^4$ and $1X10^4$ cells. *RPL32* and *IL10* were picked for the testing of the lower limit of quantification. *RPL32* was picked as the positive gene for this validation. Moreover, *IL10* was used as an examplatory gene as a representative of the asthma related genes.

2.11.4. Freeze – thawing effect.

It is well known that most of the cells that are used for any type of analysis are usually stored and thawed throughout this collection and analysis process. To ensure that the chromatin do not undergo any changes or destruction through freezing and thawing procedures, chromatin was frozen and thawed two times and a comparison between freshly isolated chromatin and frozen – thawed chromatin was done looking at the enrichment values for both histone 3 (H3) and histone 4 (H4) acetylation.

2.11.5. Temperature effect.

Furthermore, it is known that DNA – Protein binding is relatively weak binding and it is temperature sensitive (Bi 2014; Ausió 2015). To test the effect of normal temperatures, chromatin isolated from human $CD4^+$ cells was left on room temperature (RT) for 24 hours and enrichment values for acetylated histones H3 and H4 at different gene loci were measured and compared to enrichment values from freshly isolated and ChIPed chromatin. .

2.11.6. Long time stability test.

It is well known, that for cohort studies samples will be harvested, prepared and frozen for longer time periods until the required number is reached for the measurement of different parameters. To look if longer storage time has an effect on the histone modifications and the enrichment values of different asthma/allergy associated genes. For this reason, $CD4^+$ cells were stored for 7 and 30 days in -80°C then chromatin immunoprecipitation protocol was run and a comparison between freshly isolated and ChIPed chromatin and the chromatin isolated from 7 and 30 days frozen cells was done. Enrichment values for both histone 3 (H3) and histone 4 (H4) acetylation were measured for different asthma/allergy associated genes.

2.11.7. Setting an internal standard.

To set an internal standard that shows us that our work is standardized and any changes in the measurements seen is due to a mistake in the protocol or a real difference in the samples. As our method is a house established and validated method, the best standard to be used was a chromatin with a well-known enrichment values for different genes for both H3 and H4 acetylation. This standardization can be achieved by one of two ways; establishing our own pool of chromatin, measuring its enrichment values and uses its aliquots for our measurement. The second way was to use a ready to ChIP chromatin from Diagenode. The chromatin was taken from leukemia cell line, which was the

closest found to human $CD4^+$ cell. Chromatin from one million cells were measured each second ChIP run throughout the measurement of the different cohorts.

2.12. Selection of different study groups.

2.12.1. Validation study group.

For the validation of the ChIP 10 healthy adults were recruited. Persons with allergies and other chronic diseases were excluded from the study population. This study was approved by the ethics committee in the medical faculty in Philipps-University Marburg.

2.12.2. Folic acid study group.

The study population included 23 neonates selected from a larger prospective birth cohort comprising 628 mother-infant pairs recruited through the allergy research clinic in the Princess Margaret Hospital for Children (Dunstan et al. 2012). Mothers were recruited during the last trimester (>=28 weeks) of pregnancy at which time maternal blood samples were collected, and cord blood samples were subsequently collected at the time of birth. Peripheral blood mononuclear cells were harvested from blood samples within 12 hours of collection according to standard protocols (Martino et al. 2011). Serum folate measurements were available for maternal (n=435) and cord (n=285) blood. Extensive clinical and dietary data collected through the semi-quantitative food frequency questionnaire and information on maternal socio-demographic factors were also available.

The sample population was selected based on the following criteria: For the purposes of this study we excluded any infants that developed subsequent allergic disease, in order to reduce potential biases in gene expression that may be associated with the development of later disease. Infants were defined as ‘non-atopic’ based on clinical assessment and skin prick allergy test (SPT) to a range of inhalant and dietary allergens, availability of matched maternal and cord blood samples. Infants who developed positive SPT or clinical disease in the first 5 years of age were excluded. The high (HF)

and low folate (LF) groups were defined according to the first and third quartiles from the distribution of maternal serum folate levels in conventional extremes of exposure design. All study procedures were carried out in accordance with full institutional ethics.

2.12.3. Fish oil study group.

Ninety-eight pregnant, atopic women booked for delivery at St John of God Hospital, Subiaco, Western Australia, between January 1999 and September 2001 were recruited into the study because their offspring were considered to be at high risk of allergic disease. All women had a history of physician-diagnosed allergic rhinitis and/or asthma and 1 or more positive skin prick tests to common allergens (house dust mite; grass pollens; molds; and cat, dog, and cockroach extracts; Hollister-Stier Laboratories, Spokane, Wash). Women were ineligible for the study if they smoked; if they had other medical problems, complicated pregnancies, or seafood allergy; or if their normal dietary intake exceeded 2 meals of fish per week. At 20 and 30 weeks' gestation, the women completed a validated, semi quantitative food frequency questionnaire, which was used to identify any background dietary change in fish consumption in each group. Pregnancy outcomes were assessed using data from all participants. The trial protocol was approved by the Ethics Committees at St John of God Hospital and Princess Margaret Hospital, and all women gave informed consent. The groups were block-randomized according to parity (no previous term childbirth versus 1 or more), pre pregnancy body mass index (BMI), age, and maternal allergy (allergic rhinitis or asthma). Women in the fish oil group received 4 (1-g) fish oil capsules per day (Ocean Nutrition, Halifax, Nova Scotia, Canada) comprising a total of 3.7 g of n-3 PUFAs with 56.0% as docosahexaenoic acid (DHA) and 27.7% as eicosapentaenoic acid (EPA) (confirmed by gas chromatography). The control group received 4 (1-g) capsules of olive oil per day (containing 66.6% n-9 oleic acid and <1% n-3 PUFAs; Pan Laboratories, Moorebank, NSW, Australia). From those who completed the study, 70 neonates were picked, 34 in the placebo group and 36 in the fish oil group (D'Vaz et al. 2012c).

2.12.4. Children asthma study group.

This study was carried on a sub-population from the CLARA study. From that study, peripheral blood (n= 230) was sampled from children age 4 to 15 years in the CLARA study, Munich, Germany. Enrollment occurred from January 2009 and is still ongoing. Parents were approached for consent and completed a detailed questionnaire that assessed infant data.

Upon enrollment, all asthmatic children underwent full clinical examination, pulmonary function test, chest radiograph and blood count. In addition, total and specific IgE (RAST) was measured. A positive specific IgE was defined as one or more positive reactions [≥0.35 IU/ml] to a panel of 20 common allergens (Mediwiss Analytic, Moers, Germany; for allergen details see 2.2.1.1). Reversible airway obstruction was defined as significant difference of the forced expiratory volume in one second before and after inhalation of the short-acting β2-adrenergic receptor agonist salbutamol (ΔFEV1), analogous to ATS/ERS criteria(Beydon et al. 2007; Bisgaard and Nielsen 2005; Crapo et al. 2000)

Inclusion criteria for asthmatic children comprised at least 3 attacks of obstructive bronchitis and/or a doctor´s diagnosis of recurrent episodes of obstructive bronchitis and/or a history of asthma medication and a typical lung function showing reversible pulmonary obstruction. Children were defined as asthmatics if they met the inclusion criteria for asthmatics and had a ΔFEV1 of higher than 10%. Allergic asthmatics (AA) were defined based on the criteria above and a positive (>0.35 IU/ml) specific allergic sensitization assessed by RAST test. The definition for healthy children (HC) was based on having no allergies and any chronic diseases. Children with other pulmonary, chronic or autoimmune diseases were excluded, similarly children with immunodeficiency and subjects taking steroids, antibiotics, probiotics or suffering from an infection within 14 days before blood withdrawal. From this cohort 28 sub-sample collective was collected to look at the epigenetic profile in a pure $CD4^+$ cells and compare between healthy control children and allergic asthmatics.

2.13. Statistical Analysis

If not otherwise stated, qualitative variables were compared by Pearson's $\chi 2$ test. Partial pairwise relationships between two continuous parameters were analyzed using Spearman's rank correlation coefficient. Direct comparisons of qualitative parameters between two groups were always conducted by multiple linear regression models including adjustment for potential confounders specific for the purposes of the relevant sub-studies belonging to this work, i.e. binarized folic acid or continuous vitamin D (folic acid/vitamin D part) or fish oil supplementation (fish oil part). Before entering any regression model, variables of distribution other than normal as estimated by Shapiro-Wilk test, which was characterized in all cases by a positive skewness, had it approximated to normality by square-root transformation.

In the fish oil study, the potential predictive value of a certain locus histone acetylation for binary traits, such as the development of atopy or subsequent allergic diseases, was also tested but only if a significant association in initial analysis by multiple linear regression was observed (see respective parts of Results section). First, logistic regression models (with adjustment for fish oil supplementation) were developed providing odds ratio [95% confidence interval] as an output. Second, receiver operating characteristic (ROC) curves were created, in which the true positive rate (sensitivity) is plotted in function of the false positive rate (100-specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity). Therefore the closer the ROC curve is to the upper left corner, the higher the overall accuracy of the test (Zweig and Campbell 1993). This analysis was accompanied by plotting the corresponding dot diagrams, in which the data of the groups negative and positive for the best cut-off point are displayed as dots on two vertical axes, and a horizontal line indicates the best cut-off point established based on ROC curve analysis. Such cut-off point corresponds to the best combination of sensitivity and specificity in ROC curve, i.e. the best separation (minimal collective

false negative and false positive results) between the two groups with respect to the binary parameter.

A P-value of less than 0.05 was considered statistically significant. Analyses were performed using STATISTICA Version 10.0 software (StatSoft, Inc., Tulsa, OK, USA) or MedCalc Version 13 program (MedCalc Software bvba, Ostend, Belgium).

3. Results

3.1. Establishment and validation of a chromatin immunoprecipitation (ChIP) method for cohort studies.

Chromatin immunoprecipitation (ChIP) is a method where an immunoprecipitation is done pulling down a specific protein that is linked to DNA in order to study DNA – Protein interactions. This method requires usually a high number of cells. Additionally, the method is relative sensible and results might vary depending on factors like buffer composition, shearing method, temperature and the analyzed cell subset.. The goal of this part of the study is to build a redundant method able to deliver repeatable and acceptable data/results in large number of samples (Human cohorts) using a relatively low number of cells. This part of the study was carried on using $CD4^+$ cells as they are considered the primary vessel for the changes in allergic and immune diseases.

3.2. Establishment of the chromatin immunoprecipitation protocol for human $CD4^+$ cells.

The method was established according to the protocol described previously. There were different critical points that were to be validated throughout the protocol. The validation was done to ensure a reliable measurement of the different histone modifications in cohort studies. The first important step is the crosslinking of the DNA and the histone. It is very important to set both the concentration of the fixative and the timing very accurately, as any changes in one or both of them may lead to over crosslinking rendering the de-crosslinking step impossible. Formaldehyde is considered one of the most stable corss-linkers with a short arm that binds the any protein directly adjacent to the DNA. Nevertheless, this binding if left unstopped can be irreversible. The optimal length for the chromatin is between 500 – 1000 bp for normal ChIP. Furthermore, sonication of chromatin to its proper size is critically important and sonication efficiency is influenced by several parameters like fixation of cells, SDS concentration

in the buffer and the sonication device. Additionally, sonication efficiency depends on the chromatin structure and it is known that the compact chromatin of the human $CD4^+$ cells is relative sonication resistant.

3.2.1. Shearing of chromatin from human $CD4^+$ needs high SDS concentrations in lysis buffer II and long sonication times

Before sonication $CD4^+$ cells were fixed with 1% paraformaldehyde for 8 min at room temperature and lysed with two different lysis buffers before put in the sonicator. The first lysis buffer has the ability to destroy the cell membrane and keeps the nucleus intact. The second lysis buffer had the ability to lysate the nucleus to reveal chromatin for sonication. The tip sonicator was used as the mainly used sonicator in different protocols.

In figure 4.2.1.1, it shows how problematic the shearing can be without the proper conditions. Using low number of cycles (10 min) and low SDS concentration, resulted in no shearing at all using the tip sonicator (**Figure 4.2.1.1.A**) After changing the conditions using a tip sonicator the shearing efficiency improved but the nevertheless, it was not optimum (**Figure 4.2.1.1.B**). Changing the SDS concentration produced a huge change in the shearing efficiency. In figure 3.2.1.C, moving up from 0.1% SDS concentration in lane 3 toward 1% and 1.5% SDS concentration in lanes 2 and 1 respectively, produced a fully functional shearing and an optimum shearing efficiency between 500 – 1000 bp for ChIP.

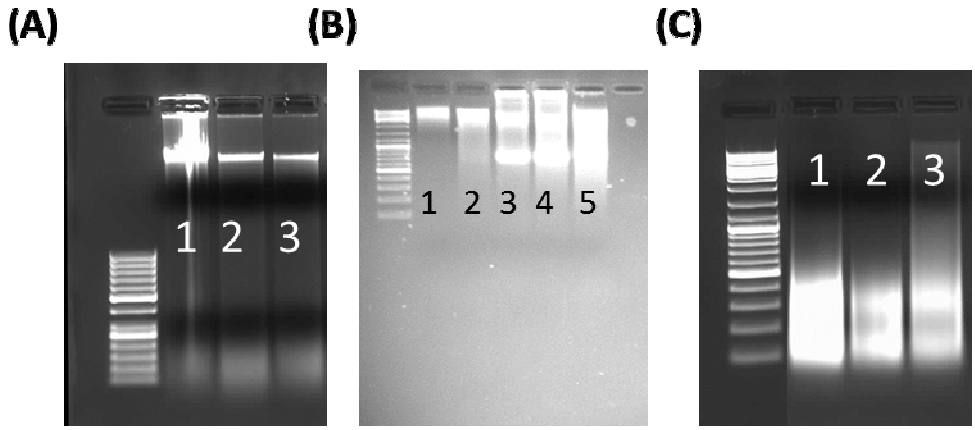
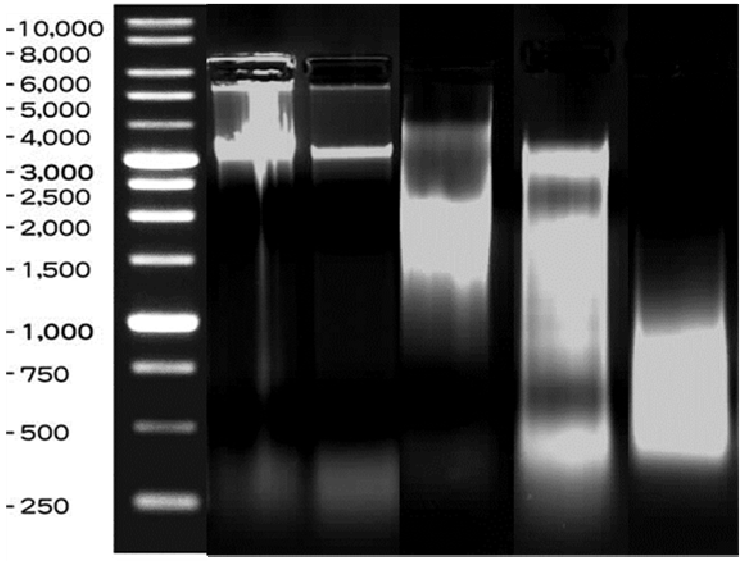


Figure 4.2.1. 1. Shearing Efficiency of human CD4$^+$ cells. (**A**) sonication for 15 minutes using the tip sonicator without protein digestion in lanes 1,2 and 3 with 0.1% SDS (**B**) sonication for different time points using tip sonicator in 4°C without protein digestion lane 1: 10 min, lane 2: 15 min, lane 3: 20 min, lane 4: 25 min, and lane 5: 30 min with 0.1% SDS (**C**) Sonication with 30 min in tip sonicator using different SDS concentrations ranging from 0.1 – 1.5% SDS. Lane 1: 1.5% SDS, lane 2: 1% SDS and lane 3: 0.1% SDS.

3.2.2. Chromatin from CD4$^+$ T cells can reliable be sonicated with the Bioruptor Power up

With the tip sonicator only one sample can be sonicated at the same time. Additionally, the samples have to be cooled during sonication to avoid overheating of the chromatin and protein denaturation. The tip sonicator is working without an integrated cooling device and the samples have to be cooled manually with an ice-bath. With the Bioruptor Power up device six samples can be processed at the same time and the samples are cooled down and maintained at 4°C during the sonication process with in an integrated water bath. A concentration of either 0.1% or 1% SDS was used with different cycle number ranging from 10 to 30 cycles. The optimal shearing conditions lays with 30 cycles (30 on/ 30 off seconds) with the Bioruptor-Power up machine from Diagenode with a final concentration of 1 % SDS in the lysis buffer solution (**Figure 4.2.2.1.A**).



Cycles	0	10	20	30	30
SDS	0	1%	1%	0.1%	1%

Figure 4.2.2.1. Shearing Efficiency of human CD4$^+$ cells. sonication for 30 cycles (30 sec on and 30 sec off for each cycle) using the bioruptor with protein digestion. All lanes represent a repeatable measurement to prove the repeatability of the machine. In lane one, Chromatin is shown without any shearing. Lane two, the chromatin was sonicated for 10 cycles with 1% SDS concentration in Lysis buffer II. Lane three, the chromatin the chromatin was sonicated for 20 cycles with 1% SDS concentration in Lysis buffer II. In lane four, the chromatin was sonicated for 30 cycles with 0.1% SDS concentration in Lysis buffer II. In lane five, the chromatin was sonicated for 30 cycles with 1% SDS concentration in Lysis buffer II

3.3. Validation of the ChIP protocol.

The validation of the method for cohort studies and large number of samples would follow. For the validation of the ChIP first the reference ranges for different gene loci were determined. Additionally, the specificity and sensitivity were measured by determining both - the inter- and intra-assay coefficient of variance.

3.3.1. Measurement of the Reference Range, inter and intra-assay coefficient of variance

The reference range for each locus measured was set for both H3 and H4 histones. The reference range was set after measuring 10 healthy controls and taking the upper and lower 95 % confidence interval for the measurement. These values represent the normal values that a healthy person should have for both H3 and H4 acetylation for different enrichment values for different gene promoters. Furthermore, both inter- and intra-assay coefficient of variance were measured to test both the specificity and sensitivity of the ChIP respectively. The inter-assay of variance is the variance when measuring the same sample many times in different runs, whereas the intra-assay coefficient of variance is variance when measuring the same sample many times in the same run.

RPL32 was used as our positive control gene as it is a house keeping gene and should be expressed in all cells and in all conditions, thus the chromatin should be always open.

On the other hand *MS4A2* gene that encodes the FcεRIβ, which is not found on $CD4^+$ cells, was used as our negative control. In this case the immunoprecipitation result should be negative for each sample measured.

For each histone a reference range was set and measured for each gene as seen in (**Table 4.3.1.1.**). Moreover both inter- and intra – assay coefficient of variance were measured for both H3 and H4 histones which laid mostly below 10 % for most of the genes measured which is acceptable for genetic tests. For the positive control *RPL32* the histone H3 acetylation reference range was 0.5 – 3 % and 0.12 – 3.70 % for acetylation at histone H4. The inter- and intra-assay coefficient of variance was 6.67% and 5.36% for histone H3 and 1.76% and 2.80% for histone H4. For *IL4* the histone H3 acetylation reference range was 0.5 – 1.4 % and 0.92 – 4.7 % for acetylation at histone H4. The inter- and intra-assay coefficient of variance was 15.23% and 9.36% for histone H3 and 0.001% and 7.36% for histone H4. For *IL5* the histone H3 acetylation reference range was 0.001 – 0.93 % and 0.12 – 1.32 % for acetylation at histone H4. The inter- and intra-assay coefficient of variance was 0.001% and 0.001% for histone H3 and 0.001% and 0.001% for histone H4. Another Th2 gene was measured and validated, *IL13* as a main modifier of the Th2 response. For *IL13* the histone H3 acetylation reference range

was 0.2 – 0.84 % and 0.14 – 0.53 % for acetylation at histone H4. The inter- and intra-assay coefficient of variance was 0.001% and 3.36% for histone H3 and 5.36% and 5.89% for histone H4. *GATA3* the main transcription factor for Th2 cells had a reference range of 0.001 – 1.9 % for H3 acetylation and 0.001 – 2.3 % for acetylation at histone H4. The inter- and intra-assay coefficient of variance was 2.65% and 4.65% for histone H3 and 4.78% and 5.78% for histone H4. On the other side *TBX21,* the main transcription factor for Th1 cells had a reference range of 0.001 – 2.36 % for H3 acetylation and 0.001 – 1.98 % for acetylation at histone H4. The inter- and intra-assay coefficient of variance was 4.65% and 8.65% for histone H3 and 5.47% and 7.65% for histone H4. For the negative control gene *MS4A2* there is no values shown as no enrichment at all at that gene is possible as it is not transcribed of even found in $CD4^+$ cells.

The only outlier was the intra – assay coefficient of variance for H3 histone acetylation for *IL4* which lay by 15.23% which needed to be fine-tuned. The inter and intra-coeffiecnt of variance should not exceed 10% in order to accept the measurement of each gene.

Table 4.3.1. 1. Validation of ChIP method for human cohorts. In this table the reference ranges for both H3 and H4 acetylation for Th1, Th2, TH17 and Treg genes and positive and negative controls are shown as well as both inter- and intra-assay coefficient of variance concluding both specificity and accuracy of the measurement. n=10 healthy subjects.

	Locus	H3acet	Intra-assay coefficecent of variance H3	Inter-assay coefficecent of variance H3	H4acet	Intra-assay coefficecent of variance H4	Inter-assay coefficecent of variance H4
Reference Range	RPL32	0.5 – 3.00	6.67%	5.36%	0.12 – 3.70	1.76%	2.80%
	IL4	0.5 – 1.4	15.23%	9.36%	0.92 – 4.7	0.001%	7.36%
	IL5	0.001 – 0.93	0.001%	0.001%	0.12 – 1.32	0.001%	0.001%
	IL9	0.59 – 2.07	0.001%	0.001%	0 – 2.1	0.001%	0.001%
	IL10	0,001 – 4.6	3.53%	9.85%	0.66 – 4.6	7.9%	9.81%
	IL13	0.2 – 0.84	0.001%	3.36%	0.14 – 0.53	5.36%	5.89%
	IL-17A	0.12 – 1.54	0.001%	2.65%	0.74 – 0.9	5.77%	6.12%
	IFNG	0.001 – 2.12	0.001%	4.23%	0.12 – 1.42	1.25%	9.36%
	TNF	0.001 – 2.1	8.18%	9.78%	0.23 – 0.6	3.36%	10.47%
	GATA3	0.001 – 1.9	2.65%	4.65%	0.001 – 2.3	4.78%	5.78%
	TBX21	0.001 – 2.36	4.65%	8.65%	0.001 – 1.98	5.47%	7.65%
	RORC	0.11 – 1.11	7.98%	9.65%	0.1 – 1.41	7.54%	1.23%
	FOXP3	0.06 – 1.06	9.2%	9.54%	0.001 – 0.35	9.6%	5.22%
	MS4A2	0	-	-	0	-	-

3.3.2. Determination of the Lower Limit of the blank (LOB)

Lower limit of the blank is the lowest measurable value when a sample without analyte (blank sample) is measured and it reflects the background. The LOB was measured using 5 blank samples and the enrichment for each gene was measured + 3 standard deviations. The LOB for most of the samples was below 0.001 % to the input control for both H3 and H4 acetylation. Some genes showed a stronger background with higher LOB e.g. *IL13* with a 0.012 LOB for H3 histone and 0.014 LOB for H4 histone and *FOXP3* with a 0.06 LOB for H3 histone (**Table 4.3.2.1.**).

Table 4.3.2. 1. validation of ChIP method for human cohorts. In this table the lower limit of the blank for both H3 and H4 acetylation for all asthma related genes are shown. n=10 healthy subjects.

Locus	LOB H3	LOB H4
RPL32	0.001	0.001
IL4	0.001	0.001
IL5	0.001	0.001
IL9	0.001	0.001
IL10	0.001	0.1
IL13	0,012	0,014
IL-17A	0,001	0,001
IFNG	0.001	0.001
TNF	0.001	0.001
GATA3	0.001	0.001
TBX21	0.01	0.1
RORC	0.001	0.001
FOXP3	0.06	0.001
MS4A2	0.001	0

3.3.3. Determination of Lower Limit of Quantification (LOQ)

The lower limit of quantification was set to be measured. Lower limit of quantification (LOQ) is the lowest number of cells needed in the ChIP protocol to deliver an acceptable reliable data. The normal starting number of cells used in any published protocol is about $5X10^6$ cells. To validate the lowest number of cells was needed to deliver a reliable enrichment values. A dilution set starting with one million cells going to half million, one hundred thousand, 50 thousand down till ten thousand cells were prepared. The ChIP protocol was conducted as described earlier with one million, half a million, one hundred thousand, fifty thousand and ten thousand cells. Looking at examplatory genes like *RPL32* and *IL10*, it is clear that going below $1X10^5$ cells the

ChIP loses its sensitivity and no enrichment can be detected (**Figure 4.3.3.1.**). This result was repeated with all other loci measured losing enrichment measurement going below $1X10^5$ cells.

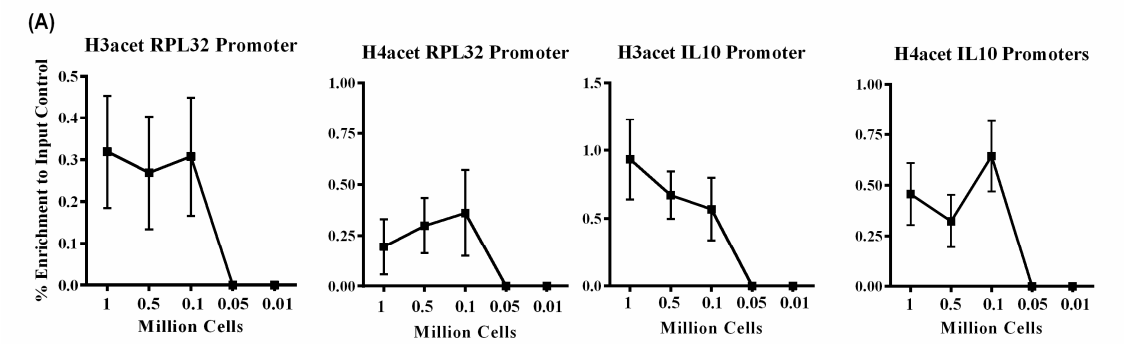


Figure 4.3.3. 1. validation of ChIP method for human cohorts. Lower Limit of Quantification (LOQ). Lower limit of quantification for both *RPL32* as a positive control gene and *IL10* as an examplatory gene showing that below $1X10^5$ cells. n=10 healthy subjects.

3.3.4. Repeated freezing and thawing has no significant effect upon enrichment values during ChIP.

Repeatable freezing and thawing is known to have a destructive effect on proteins and thus might also have negative effects histone proteins linked to DNA. As during epidemiological trial samples are routinely frozen the Freeze-Thawing effect was tested. In practice, the chromatin was frozen to -20°C and thawed twice. Afterwards, a comparison of the enrichment values for H3 and H4 histones at the *RPL32* locus (positive control), the *MS4A2* locus (negative control), *IFNG* locus (Th1), the *IL4* locus (Th2) and *IL10* locus (Treg) between freshly isolated and ChIPed chromatin and frozen and thawed chromatin was conducted. Enrichment values were slightly reduced at all tested loci. This reduction was not significant. (**Figure 4.3.4.1.**).

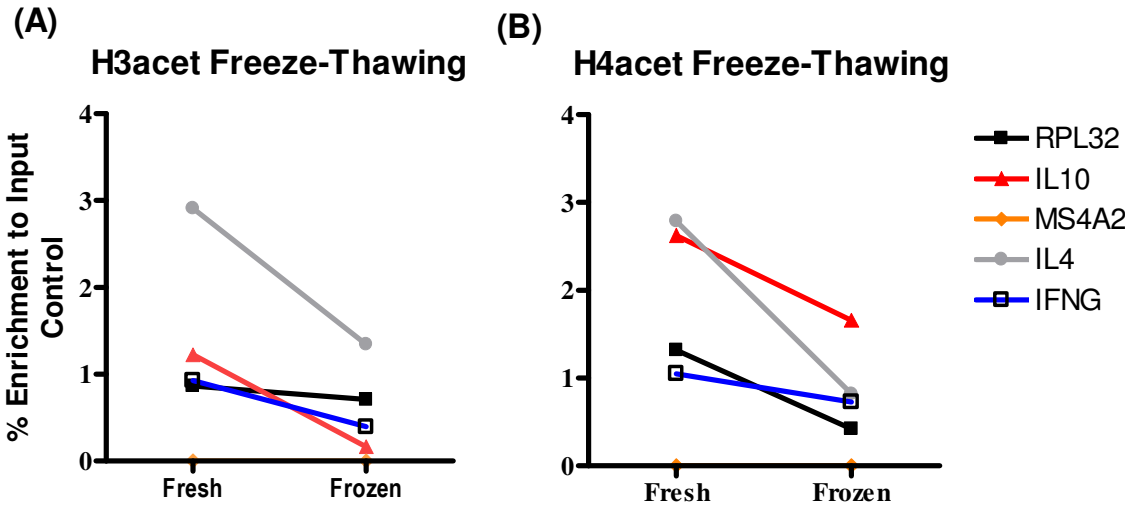


Figure 4.3.4. 1. validation of ChIP method for human cohorts. Effect of freezing and thawing. The chromatin was frozen and thawed twice and a comparison between freshly ChIPed and frozen-thawed ChIPed chromatin was done. (**A**) Histone H3 acetylation at the *RPL32*, *MS4A2*, *IFNG*, *IL4* and *IL10* gene loci. (**B**) Histone H4 acetylation at the *RPL32*, *MS4A2*, *IFNG*, *IL4* and *IL10* gene loci. n=10 healthy subjects.

3.3.5. Room temperature can lead to decreased enrichment values during ChIP.

In this validation step, the effect of room temperature was tested. The chromatin was left for 24 hours on room temperature to see if this may cause a degradation of the chromatin stability. A comparison between freshly isolated and ChIPed chromatin versus 24 hours room temperature left chromatin was done. There is a significant reduction in the enrichment percentage between the fresh chromatin and 24 h RT chromatin in both RPL32 and IL4 loci. This reduction was not seen in IL10 and other measured loci (**Figure 4.3.5.1.**). Nevertheless, a significant reduction in one of the loci is enough to prohibit working in RT for longer time intervals.

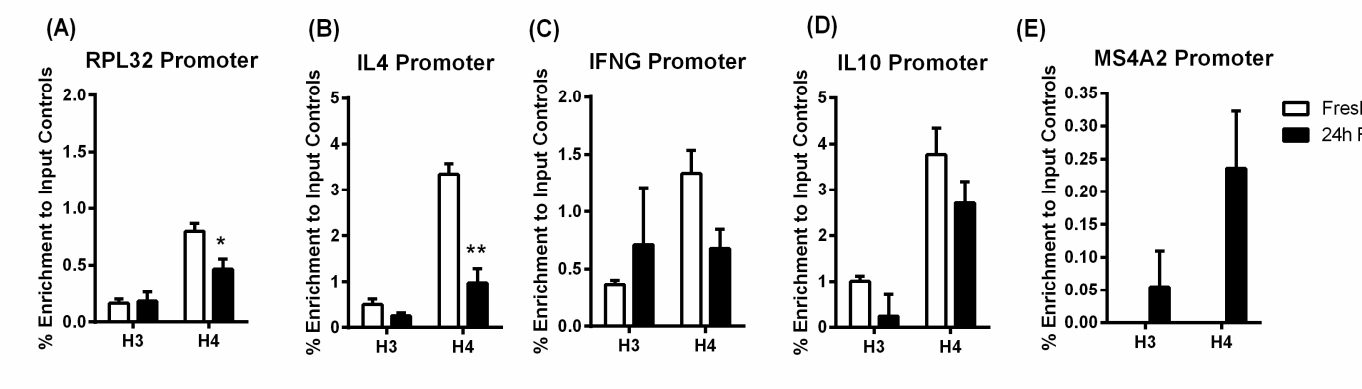


Figure 4.3.5. 1. validation of ChIP method for human cohorts. Temperature effect. The chromatin was left on room temperature (RT) for 24 hours and a comparison between fresh and 24 h left chromatin was done. H3: Histone H3, H4: Histone H4. ** P<0.01. (**A**) *RPL32* promoter (positive control) for both acetylation on H3 and H4 histones. (**B**) *IL4* promoter for both acetylation on H3 and H4 histones (**C**) *IFNG* promoter for both acetylation on H3 and H4 histones (**D**) *IL10* promoter for both acetylation on H3 and H4 histones (**E**) *MS4A2* promoter (negative control) for both acetylation on H3 and H4 histones. n=10 healthy subjects.

3.3.6. Sample for ChIP can be stored for at least 30 days at -80°C without significant changes of the measured enrichment values.

Long time storage stability test was conducted by storing freshly isolated chromatin for either 7 days or 30 days by -80°C. Afterwards, the chromatin was ChIPed and a comparison was conducted with freshly isolated and ChIPed chromatin. RPL32, IL4, IFNG, IL10 and the negative control MS4A2 gene loci were analyzed. There was no difference in the enrichment percentage between the three time points in all the genes measured and tested (**Figure 4.3.6.1**).

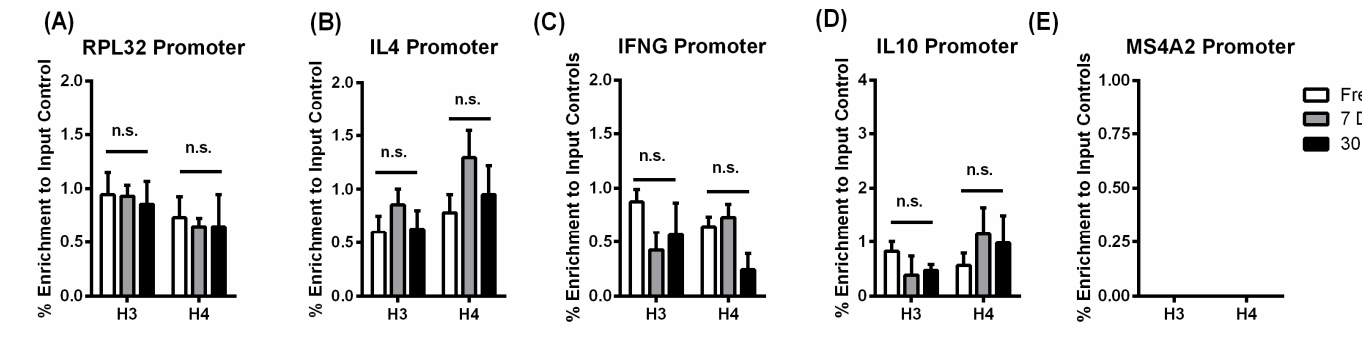


Figure 4.3.6. 1. Validation of ChIP method for human cohorts. Long Storage stability test. Cells were ChIPed either freshly after isolation or after 7 days storage in -80°C or 30 days storage in -80°C. (**A**) *RPL32* promoter (positive control) for both acetylation on H3 and H4 histones. (**B**) IL4 promoter for both acetylation on H3 and H4 histones (**C**) *IFNG* promoter for both acetylation on H3 and H4 histones (**D**) *IL10* promoter for both acetylation on H3 and H4 histones (**F**) *MS4A2* promoter (negative control) for both acetylation on H3 and H4 histones. n=10 healthy subjects.

3.4. The effect of folic acid levels during pregnancy on epigenetic modifications in cord blood CD4$^+$ cells.

Folate is an important methyl donor for the organism and folate levels can influence DNA methylation as well as histone methylation (Dobosy et al., 2008). Beside this also effects upon Histone acetylation have been described (reference, I think we have one in the manuscript). Folate is regularly taken by pregnant women to avoid neural tube defects in the unborn child. Nevertheless, it is discussed an excess of folate during pregnancy also increase the incidence of allergic disease in the offspring. To analyze the effect of high maternal folate levels upon allergic disease relevant epigenetic modifications like histone acetylation in CD4$^+$ T cells in newborns a subgroup from an Australian cohort study was analyzed. Here, the folic acid status was measured in pregnant mothers and two groups were established using the first and third quartiles of the folic status levels. The sample population was selected based on the following criteria: For the purposes of this study we excluded any infants that developed subsequent allergic disease, in order to reduce potential biases in gene expression that

may be associated with the development of later disease. Infants were defined as 'non-atopic' based on clinical assessment and skin prick allergy test (SPT) to a range of inhalant and dietary allergens, availability of matched maternal and cord blood samples. Infants who developed positive SPT or clinical disease in the first 5 years of age were excluded. The high (HF) and low folate (LF) groups were defined according to the first and third quartiles from the distribution of maternal serum folate levels in conventional extremes of exposure design. All study procedures were carried out in accordance with full institutional ethics.

3.4.1. Folic acid exposure during pregnancy increases histone acetylation at GATA3 and IL9 locus

A set of Th1/Th2/Th17/Treg loci were analyzed on the level of both histone 3 and histone 4 acetylation. GATA3, IL4, IL5, IL9, IL13 and IRF1 were analyzed as Th2 genes. IFNG, IL1B, TBX21 and TNF were analyzed as Th1 genes. IL10, FOXP3 were analyzed as the main Treg genes. Furthermore, IL17A and RORC were analyzed as Th17 genes. For the Th2 genes, there was a significant increase in the acetylation levels of *GATA3* locus in both H3 (**Figure 4.4.1.1.A**) and H4 histone (**Figure 4.4.1.2.A**) in the high folate group in comparison to the low folate group. For the rest of the Th2 genes there was no differences between low and high folate group for both H3 (**Figure 4.4.1.1.B, C, E and F**) and H4 acetylation (**Figure 4.4.1.2.B, C, E and F**). Furthermore, there was a significant increase in the histone acetylation at histone H4 at the *IL9* locus (**Figure 4.4.1.2.D)** paralleled with an increase at the same locus at histone H3 (P=0.074) in the high folate group (**Figure 4.4.1.1.D**). On the other hand, there was a decrease in the histone acetylation at the histone H4 for the *IFNG* locus (P=0.086) in the high folate group in comparison with the low folate group (**Figure 4.4.1.4.A**). Moreover, there were no differences between low vs. high folate group for the Th1 genes measured at histone H3 acetylation (**Figure 4.4.1.3.**). In addition to the Th1 and Th2 loci measured, Treg and Th17 major gene were analyzed. There was no significant difference between low vs. high folate group at histone H3 neither for IL10 (**Figure 4.4.1.5.A**), FOXP3 (**Figure 4.4.1.5.B**) as the major Treg genes nor IL17A (**Figure**

4.4.1.5.C) and RORC (**Figure 4.4.1.5.D**). Concomitant with these results there was no significant difference between low vs. high folate group at histone H3 neither for IL10 (**Figure 4.4.1.6.A**), FOXP3 (**Figure 4.4.1.6.B**) as the major Treg genes nor IL17A (**Figure 4.4.1.6.C**) and RORC (**Figure 4.4.1.6.D**). All the data is adjusted for vitamin D levels as it was the only confounder that was significantly different between the two groups (This study was conducted in collaboration with Susan L. Prescott and Manori Amarasekera).

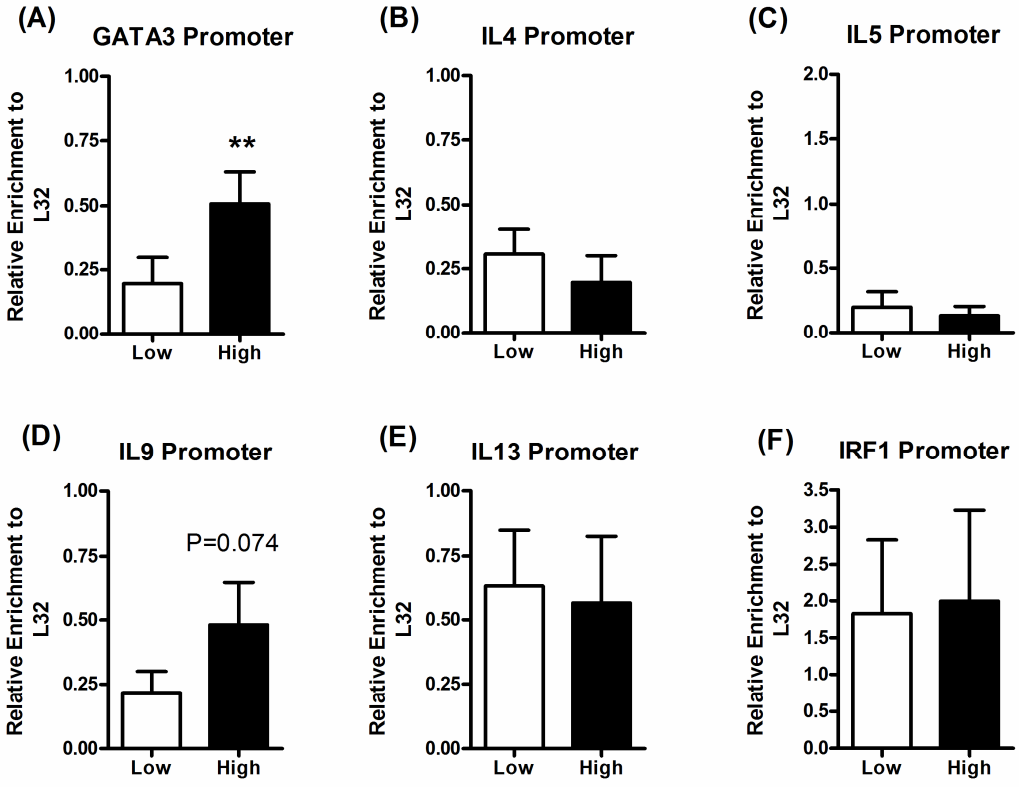


Figure 4.4.1. 1. Effect of folic acid status during pregnancy on histone H3 acetylation at Th2 gene loci in CD4$^+$ T cells from the newborns. (**A**) *GATA3* the major transcription factor for Th2 genes showing a significant increase in histone acetylation at histone H3 in the high folate compared to the low folate. (**B**) *IL4* gene showing no difference between the two groups. (**C**) *IL5* gene showing no difference between the two groups. (**D**) *IL9* gene showing a slight increase in the high group

compared to the low group (P=0.074). (**E**) *IL13* locus showing no difference between the two groups. (**F**) *IRF1* locus showing no difference between the two groups. * P<0.05, ** P<0.01

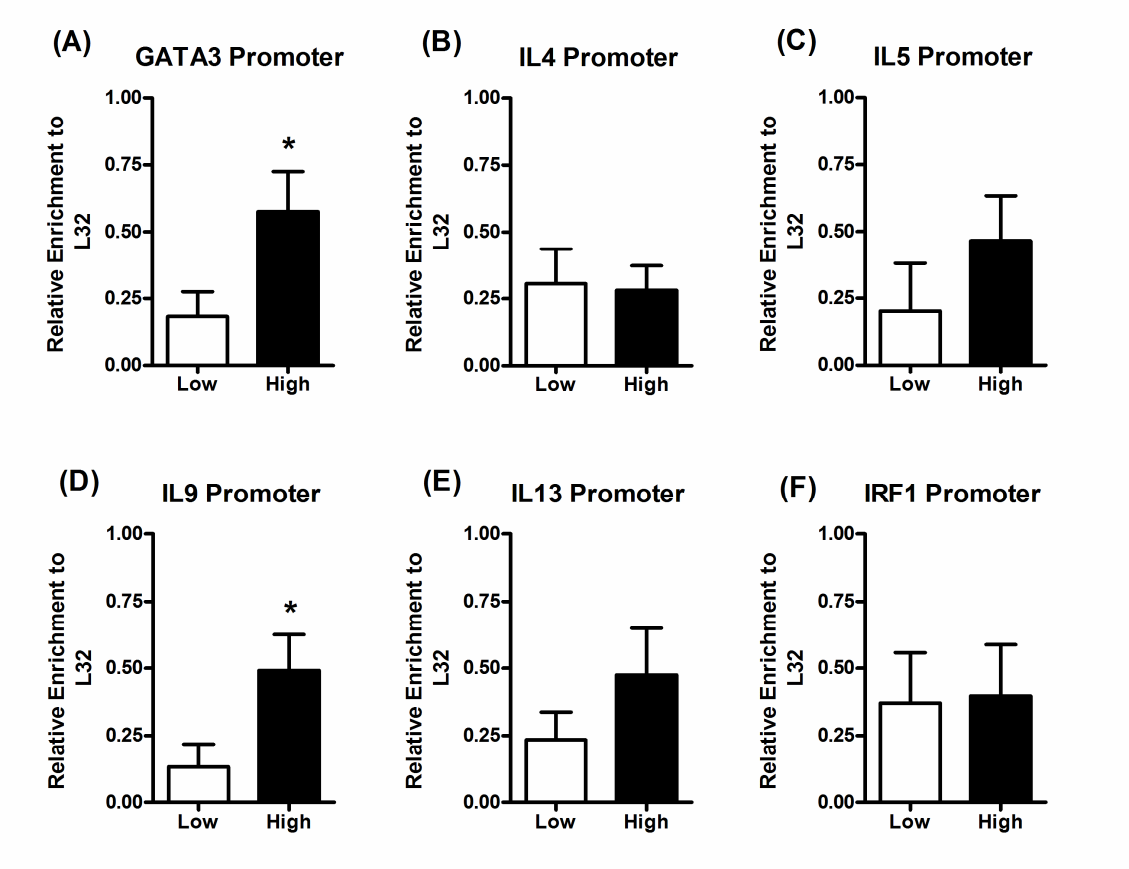


Figure 4.4.1. 2. Effect of folic acid status during pregnancy on histone H4 acetylation at Th2 gene loci in CD4$^+$ T cells from the newborns. Different loci were analyzed comprising Th2 genes. (**A**) *GATA3* the major transcription factor for Th2 genes showing a significant increase in histone acetylation at histone H3 in the high folate compared to the low folate. (**B**) *IL4* gene showing no difference between the two groups. (**C**) *IL5* gene showing no difference between the two groups. (**D**) *IL9* gene showing a significant increase in histone acetylation at histone H3 in the high folate compared to the low folate. (**E**) *IL13* locus showing no difference between the two groups. (**F**) *IRF1* locus showing no difference between the two groups. * P<0.05, ** P<0.01

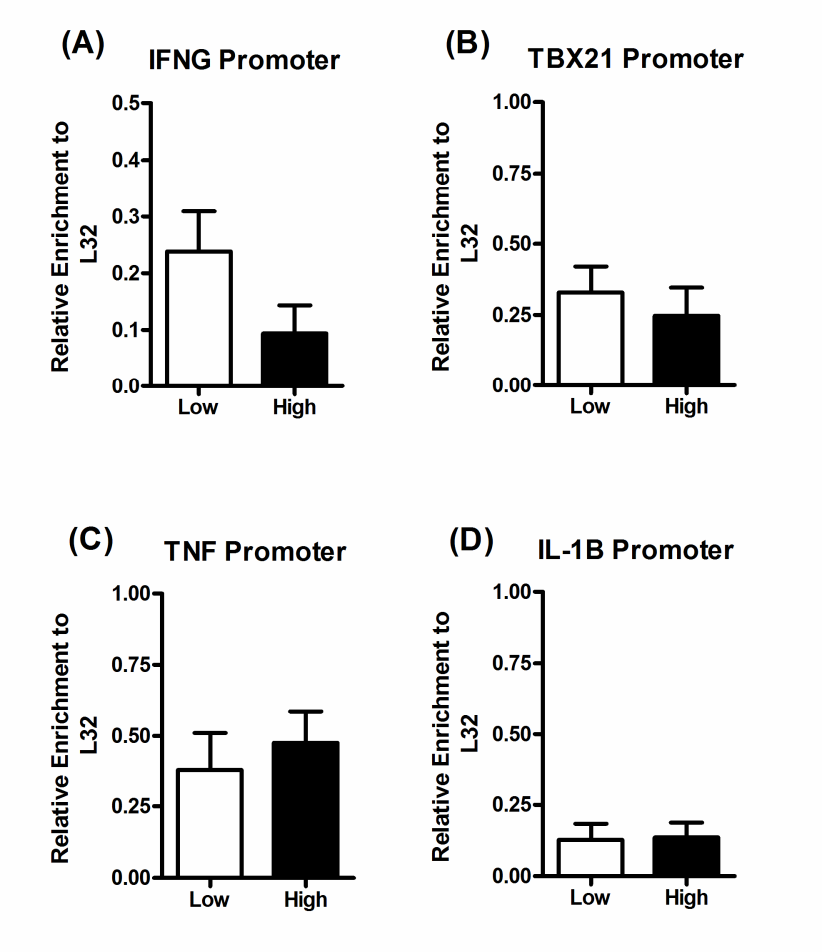


Figure 4.4.1. 3. Effect of folic acid status during pregnancy on histone H3 acetylation at Th1 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IFNG* the major Th1 cytokine gene showing no difference between low vs. high folate group. (**B**) *TBX21* the major transcription factor for Th1 cells showing no difference between low vs. high folate groups. (**C**) *TNF* gene showing no difference between the two groups. (**D**) *IL1B* gene showing no difference between the two groups.

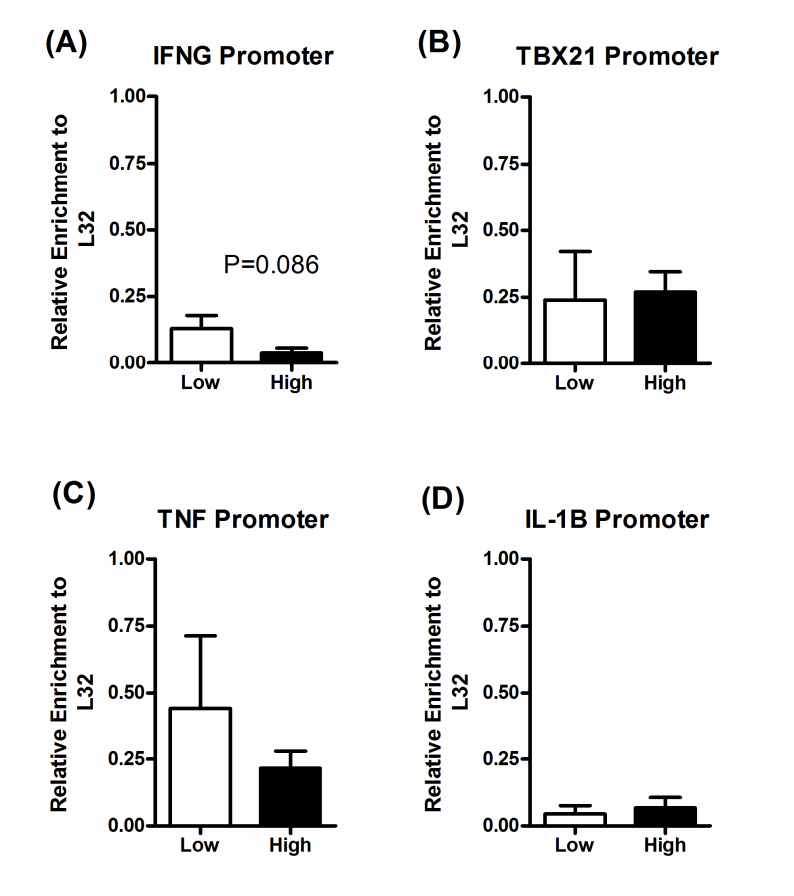


Figure 4.4.1. 4. Effect of folic acid status during pregnancy on histone H4 acetylation at Th1 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IFNG* the major Th1 cytokine gene showing a reduction in histone acetylation in the high folate group in comparison to the low folate group (P=0.086). (**B**) *TBX21* the major transcription factor for Th1 cells showing no difference between low vs. high folate groups. (**C**) *TNF* gene showing no difference between the two groups. (**D**) *IL1B* gene showing no difference between the two groups.

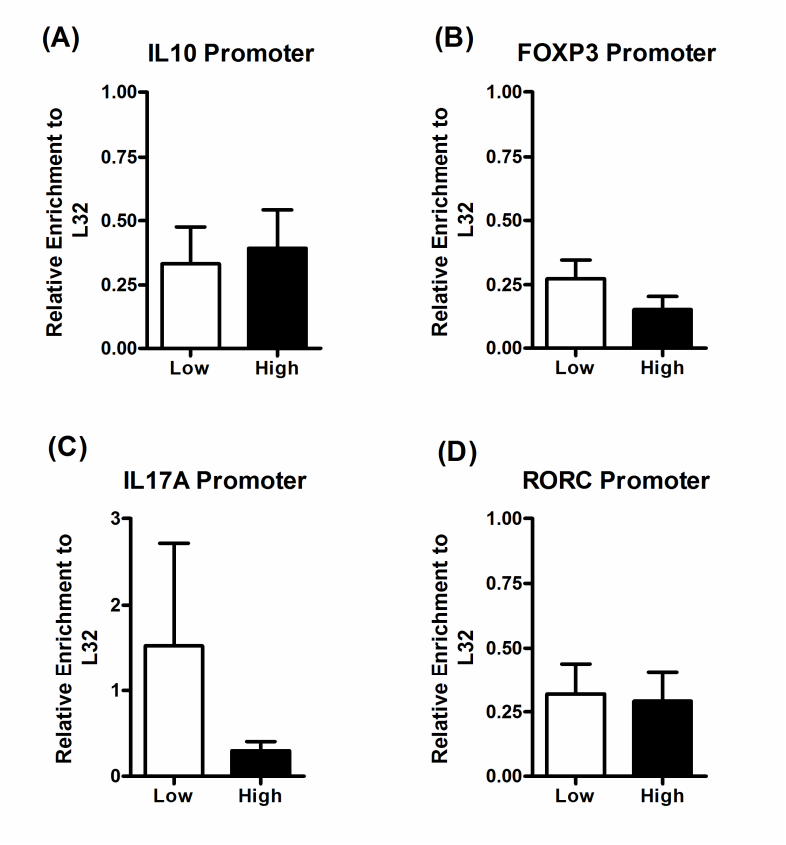


Figure 4.4.1. 5. Effect of folic acid status during pregnancy on histone H3 acetylation at Treg/Th17 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IL10* locus showing no difference between the low vs. high folate groups. (**B**) *FOXP3* the major transcription factor for T-regulatory cells showing no difference between the two groups. (**C**) *IL17A* the major cytokine for Th17 cells showing no difference in histone acetylation between low vs. high folate group. (**D**) *RORC* the major transcription factor for Th17 cells showing no difference between the two groups.

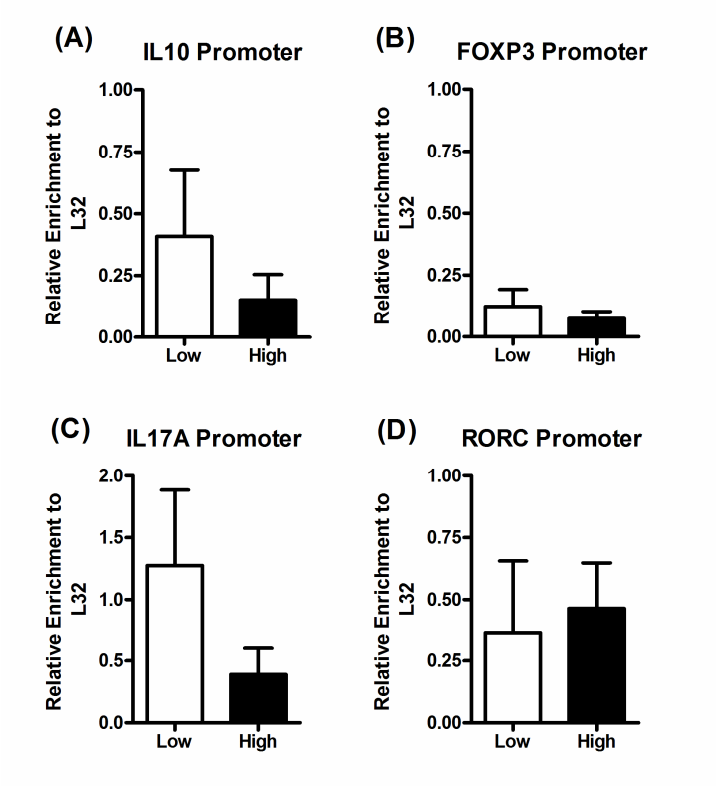


Figure 4.4.1. 6. Effect of folic acid status during pregnancy on the effect of histone H4 acetylation at Treg/Th17 gene loci in CD4$^+$ T cells from the newborns.. (**A**) *IL10* locus showing no difference between the low vs. high folate groups. (**B**) *FOXP3* the major transcription factor for T-regulatory cells showing no difference between the two group. (**C**) *IL17A* the major cytokine for Th17 cells showing no difference in histone acetylation between low vs. high folate group. (**D**) *RORC* the major transcription factor for Th17 cells showing no difference between the two groups.

3.4.2. Vitamin D exposure during pregnancy causes an increase in histone acetylation at GATA3 and IL9 loci in neonatal CD4$^+$ cells

Interestingly, it seems that folate and vitamin D levels are correlated with each other and women with a high folate level exhibit also a high vitamin D level (results are the courtesy of Manori Amarasekera). It is well know that the vitamin D receptor can interact with several chromatin modifying enzymes like histone acetyltransferases and histone deacetylases thus vitamin D levels might modulate histone acetylation (Fetahu

et al., 2014). For this reason the effects of vitamin D levels upon histone acetylation were analyzed. The groups were divided according to their vitamin D level in mother serum and the results were adjusted for folic acid status in mothers as well (Results are courtesy of Manori Amarasekera).

A set of Th1/Th2/Th17/Treg loci were analyzed on the level of both histone 3 and histone 4 acetylation. GATA3, IL4, IL5, IL9, IL13 and IRF1 were analyzed as Th2 genes. IFNG, IL1B, TBX21 and TNF were analyzed as Th1 genes. IL10, FOXP3 were analyzed as the main Treg genes. Furthermore, IL17A and RORC were analyzed as Th17 genes. For the Th2 genes, there was a significant increase in the acetylation levels of *GATA3* locus in both H3 (**Figure 4.4.2.1.A**) in the high vitamin D group in comparison to the low Vitamin D group. For the rest of the Th2 genes there was no differences between low and high folate group for both H3 (**Figure 4.4.2.1.B, C, E and F**) and H4 acetylation (**Figure 4.4.2.2.B, C, E and F**). Furthermore, there was a significant increase in the histone acetylation at histone H3 at the *IL9* locus (**Figure 4.4.2.1.D)** paralleled with a significant increase at the same locus at histone H4 in the high Vitamin D group (**Figure 4.4.2.2.D**). Moreover, there were no differences between low vs. high Vitamin D group for the Th1 genes measured at histone H3 acetylation (**Figure 4.4.2.3.**) and Histone H4 (**Figure 4.4.2.4.**). In addition to the Th1 and Th2 loci measured, Treg and Th17 major gene were analyzed. There was no significant difference between low vs. high folate group at histone H3 neither for IL10 (**Figure 4.4.2.5.A**), FOXP3 (**Figure 4.4.2.5.B**) as the major Treg genes nor IL17A (**Figure 4.4.2.5.C**) and RORC (**Figure 4.4.2.5.D**). Concomitant with these results there was no significant difference between low vs. high folate group at histone H3 neither for IL10 (**Figure 4.4.2.6.A**), FOXP3 (**Figure 4.4.2.6.B**) as the major Treg genes nor IL17A (**Figure 4.4.2.6.C**) and RORC (**Figure 4.4.2.6.D**).

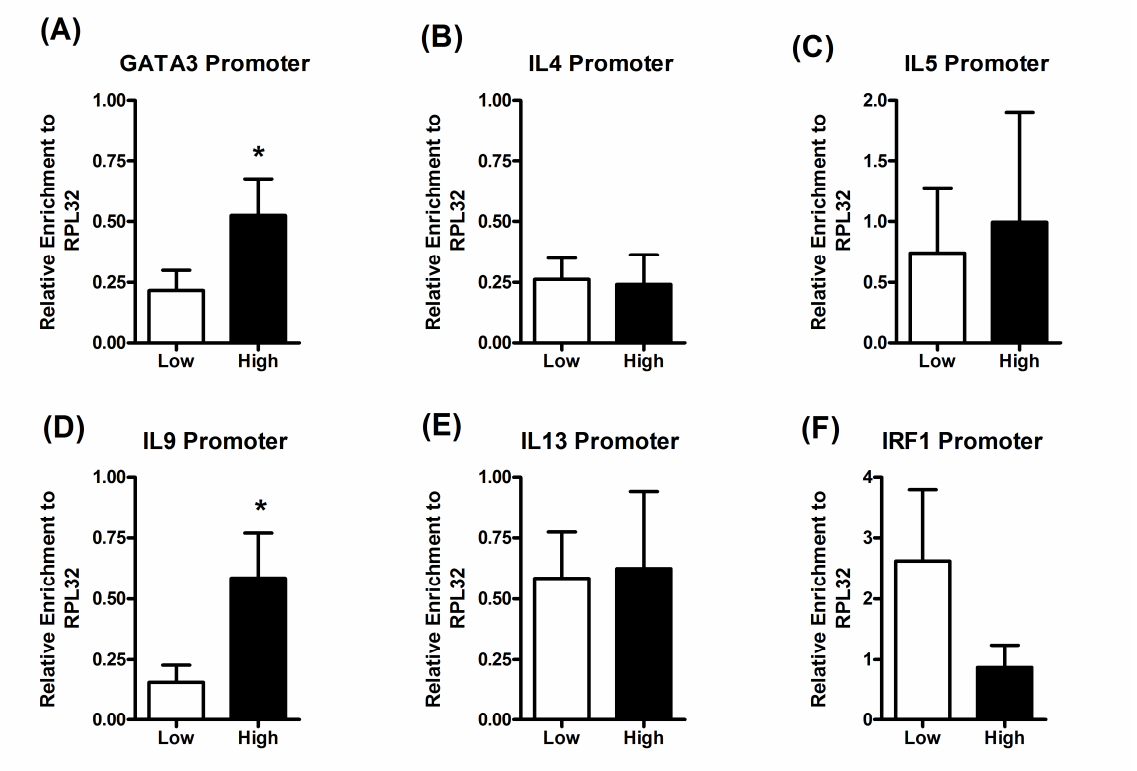


Figure 4.4.2. 1. Effect of Vitamin D status during pregnancy on histone H3 acetylation at Th2 gene loci in CD4$^+$ T cells from the newborns. (**A**) *GATA3* the major transcription factor for Th2 genes showing a significant increase in histone acetylation at histone H3 in the high Vitamin D group compared to the low vitamin D group. (**B**) *IL4* gene showing no difference between the two groups. (**C**) *IL5* gene showing no difference between the two groups. (**D**) *IL9* gene showing significant increase in histone acetylation at histone H3 in the high Vitamin D group compared to the low vitamin D group. (**E**) *IL13* locus showing no difference between the two groups. (**F**) *IRF1* locus showing no difference between the two groups. * P<0.05.

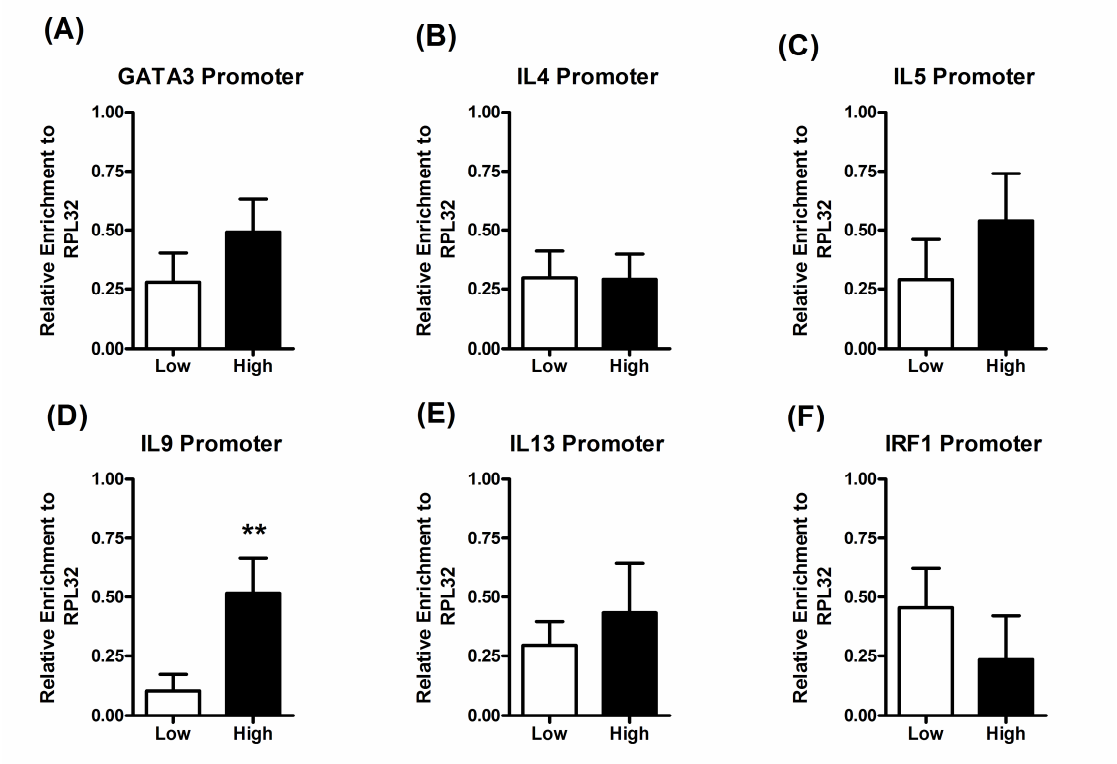


Figure 4.4.2. 2. Effect of Vitamin D status during pregnancy on histone H4 acetylation at Th2 gene loci in CD4$^+$ T cells from the newborns. (**A**) *GATA3* the major transcription factor for Th2 genes showing no difference in histone acetylation at histone H3 in the high Vitamin D group compared to the low Vitamin D group. (**B**) *IL4* gene showing no difference between the two groups. (**C**) *IL5* gene showing no difference between the two groups. (**D**) *IL9* gene showing a significant increase in histone acetylation at histone H3 in the high Vitamin D group compared to the low Vitamin D group. (**E**) *IL13* locus showing no difference between the two groups. (**F**) *IRF1* locus showing no difference between the two groups. ** P<0.01.

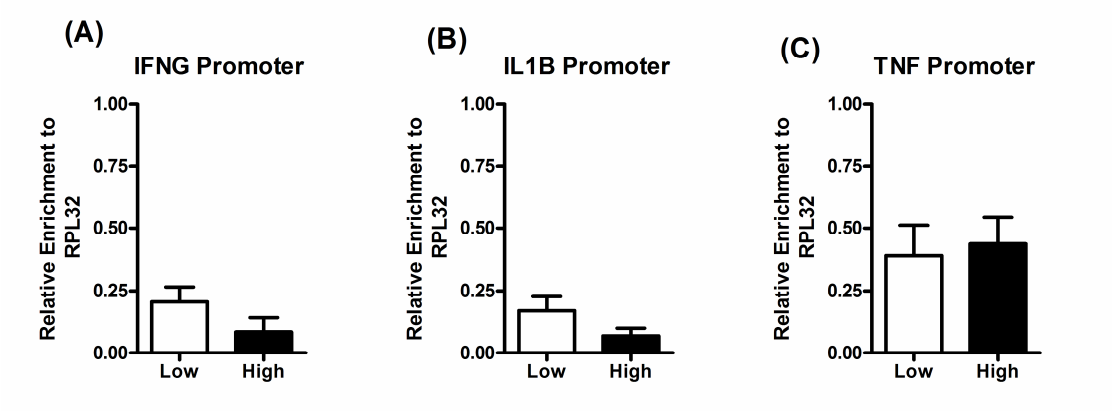


Figure 4.4.2. 3. Effect of Vitamin D status during pregnancy on histone H3 acetylation at Th1 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IFNG* the major Th1 cytokine gene showing no difference between low vs. high vitamin D group. (**B**) *IL1B* gene showing no difference between the two groups. (**C**) *TNF* gene showing no difference between the two groups.

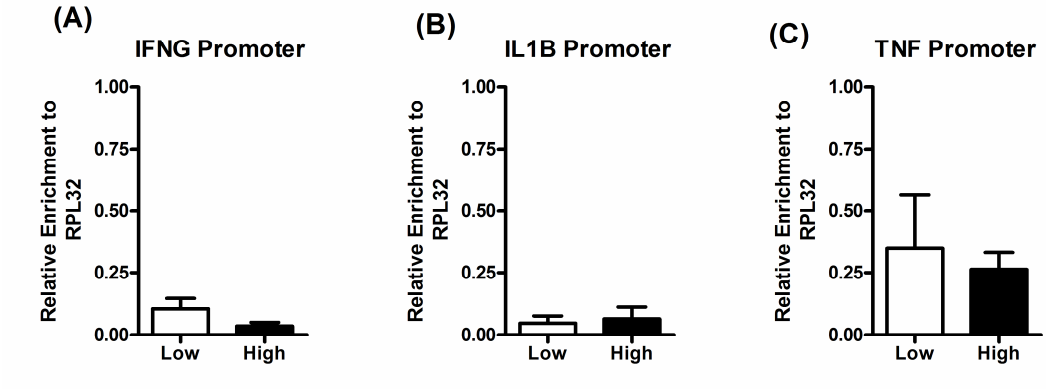


Figure 4.4.2. 4. Effect of Vitamin D status during pregnancy on histone H4 acetylation at Th1 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IFNG* the major Th1 cytokine gene showing no difference between low vs. high Vitamin D group. (**B**) *IL1B* gene showing no difference between the two groups. (**C**) *TNF* gene showing no difference between the two groups.

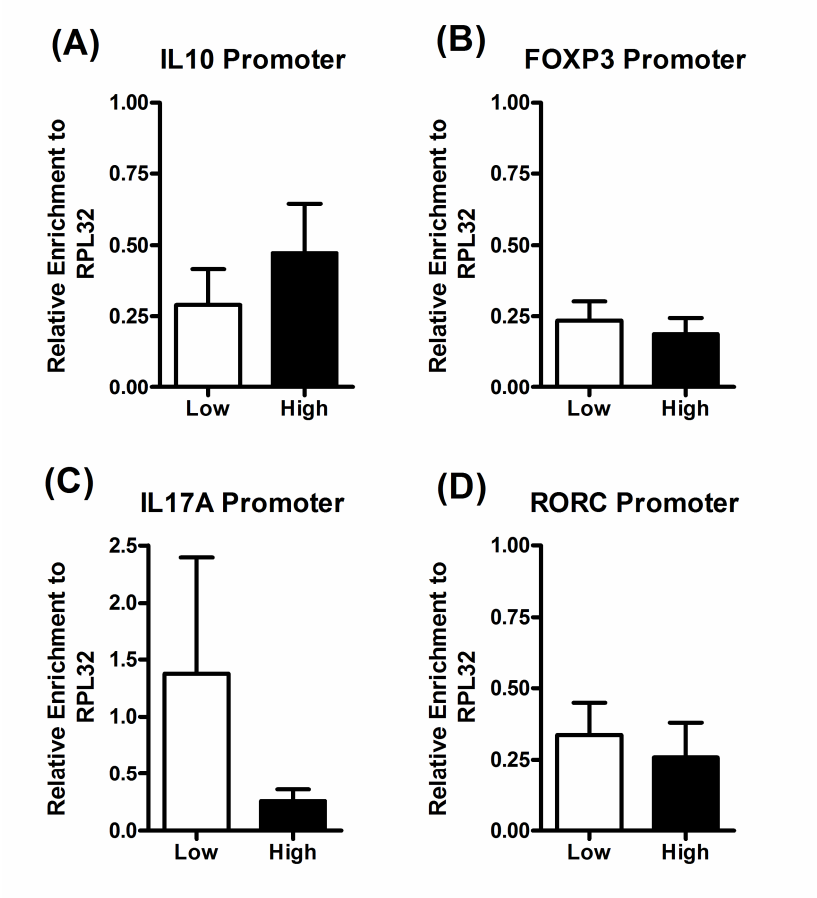


Figure 4.4.2. 5. Effect of Vitamin D status during pregnancy on histone H3 acetylation at Treg/Th17 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IL10* locus showing no difference between the low vs. high Vitamin D groups. (**B**) *FOXP3* the major transcription factor for T-regulatory cells showing no difference between the two groups. (**C**) *IL17A* the major cytokine for Th17 cells showing no difference in histone acetylation between low vs. high Vitamin D group. (**D**) *RORC* the major transcription factor for Th17 cells showing no difference between the two groups.

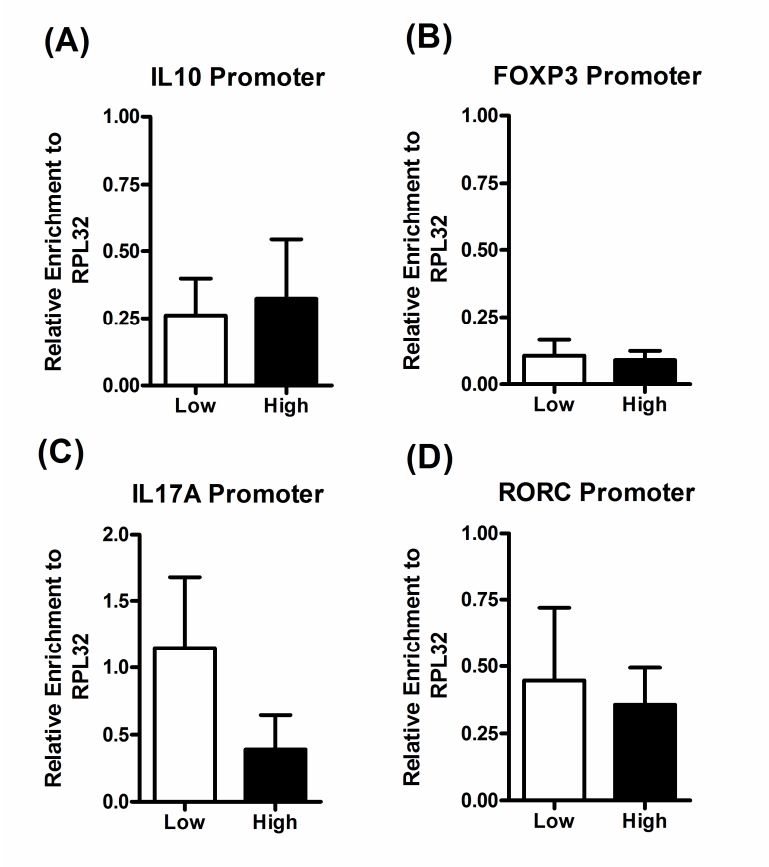


Figure 4.4.2. 6. Effect of Vitamin D status during pregnancy on histone H4 acetylation at Treg/Th17 gene loci in CD4$^+$ T cells from the newborns. (**A**) *IL10* locus showing no difference between the low vs. high Vitamin D groups. (**B**) *FOXP3* the major transcription factor for T-regulatory cells showing no difference between the two groups. (**C**) *IL17A* the major cytokine for Th17 cells showing no difference in histone acetylation between low vs. high Vitamin D group. (**D**) *RORC* the major transcription factor for Th17 cells showing no difference between the two groups.

3.5. The effect of fish oil supplementation during pregnancy on the histone acetylation in cord blood CD4$^+$ cells.

In this cohort the effect of fish oil supplementation during pregnancy was studied. Fish oil is considered one of the first supplements prescribed for pregnant mothers during the whole period of pregnancy. Throughout a clinical study looking at the effect of fish oil

supplementation on asthma and allergy development later in life in the children, Prescott and colleagues showed that fish oil supplementation was able to reduce the risk of allergy and atopy (D'Vaz et al. 2012c; D'Vaz et al. 2012d). To illustrate the effect of fish oil supplementation, different epigenetic analyses were carried out and histone modifications/acetylation was carried out here in Marburg. For our analysis $CD4^+$ cells were isolated from cord blood mononuclear cell (CBMCs) from newborns born to mothers either supplemented with fish oil or the olive oil as a placebo. $CD4^+$ cells were selected as the major vehicle for any changes that is happened in the immune system related to allergic disease. Different analyses were conducted for this study.

In short, the groups were block-randomized according to parity (no previous term childbirth versus 1 or more), pre pregnancy body mass index (BMI), age, and maternal allergy (allergic rhinitis or asthma). Women in the fish oil group received 4 (1-g) fish oil capsules per day (Ocean Nutrition, Halifax, Nova Scotia, Canada) comprising a total of 3.7 g of n-3 PUFAs with 56.0% as docosahexaenoic acid (DHA) and 27.7% as eicosapentaenoic acid (EPA) (confirmed by gas chromatography). The control group received 4 (1-g) capsules of olive oil per day (containing 66.6% n-9 oleic acid and <1% n-3 PUFAs; Pan Laboratories, Moorebank, NSW, Australia). From those who completed the study, 70 neonates were picked, 34 in the placebo group and 36 in the fish oil group (D'Vaz et al. 2012c).

3.5.1. Fish oil supplementation causes a decrease in histone acetylation on both histones H3 and H4 at IL13 and TBX21 loci

For this analysis we looked at the histone acetylation at both histones H3 and H4. We looked at different Th1/Th2/Treg genes as well as the protein kinase zeta genes described in D'Vaz publication as a predictor of the development and severity of infant allergic disease (D'Vaz et al. 2012a). *GATA3*, *IL4*, *IL5*, *IL9*, *IL13* and *IRF1* were analyzed as Th2 genes. *IFNG*, *IL1B*, *TBX21* and *TNF* were analyzed as Th1 genes. *IL10*, *FOXP3* were analyzed as the main Treg genes.

The analysis of different Th2 loci showed no significant difference between the placebo and the fish oil supplemented group in different genes at both histone H3 (**Figure 4.5.1.1 A-C**) and histone H4 (**Figure 4.5.1.1. D-F**)

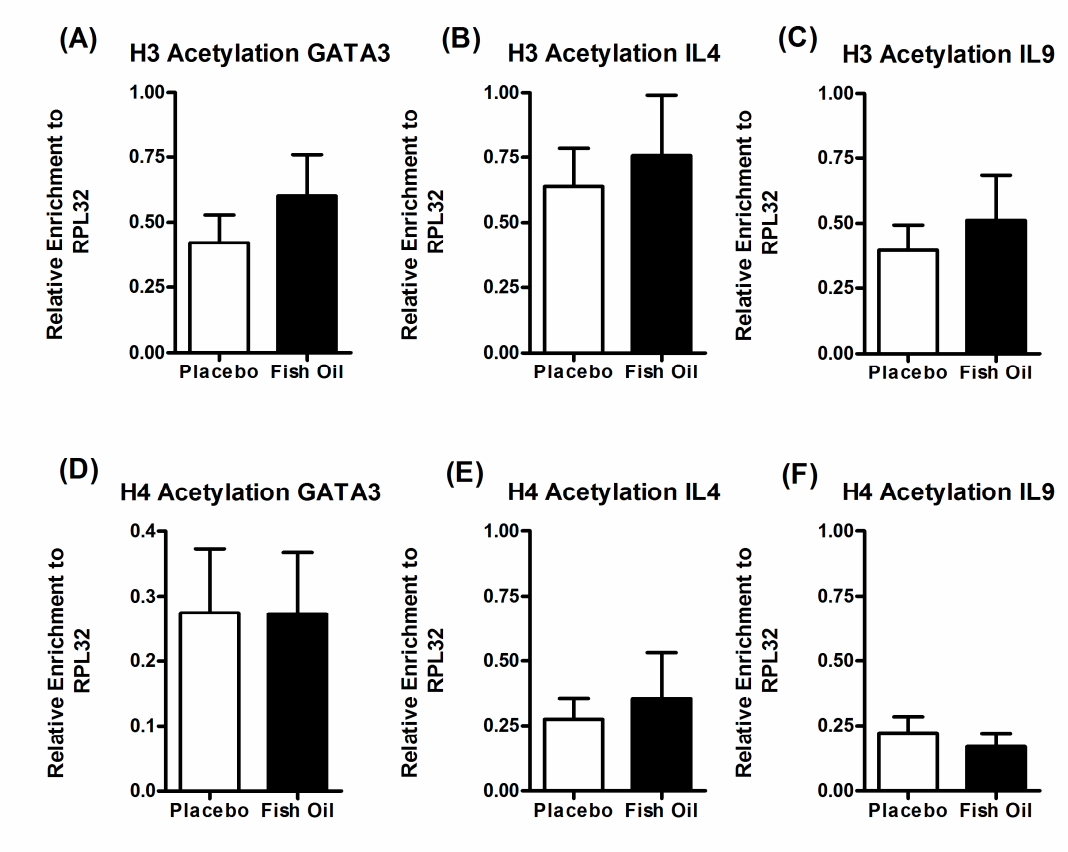


Figure 4.5.1. 1. Effect of fish oil supplementation on the histone acetylation in different Th2 genes at both H3 and H4 histones (**A**) *GATA3* locus showed no difference in histone acetylation at histone H3 between placebo and fish oil groups. (**B**) *IL4* locus showed no difference in histone acetylation at histone H3 between placebo and fish oil groups. (**C**) *IL9* locus showed no difference in histone acetylation at histone H3 between placebo and fish oil groups. (**D**) *GATA3* locus showed no difference in histone acetylation at histone H4 between placebo and fish oil groups. (**E**) *IL4* locus showed no difference in histone acetylation at histone H4 between placebo and fish oil groups. (**F**) *IL9* locus showed no difference in histone acetylation at histone H4 between placebo and fish oil groups.

Furthermore, the percent enrichment for Th2 gene loci there was a significant decrease in the histone acetylation at the *IL13* locus on both H3 and H4 histones in the fish oil group compared to the placebo group (**Figure 4.5.1.2.A, B**). Furthermore, there was a significant decrease in *TBX21* acetylation at histone H3 in fish oil group in comparison with the placebo group (**Figure 4.5.1.2.A**).

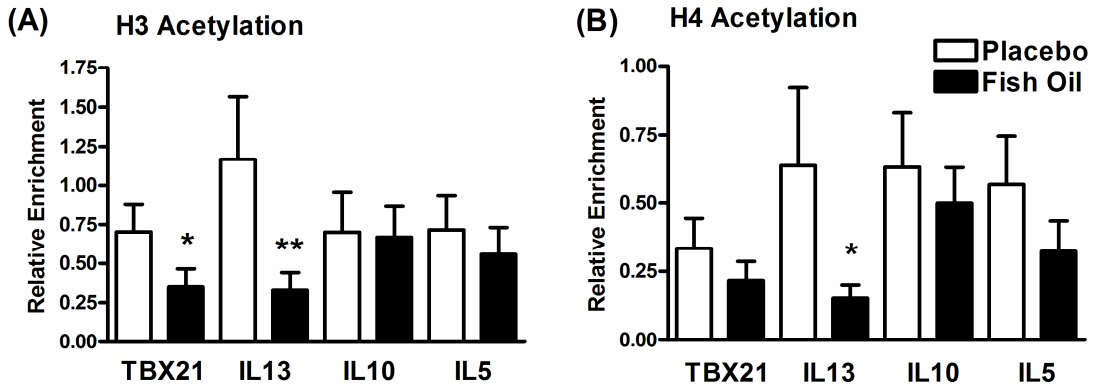


Figure 4.5.1. 2. Effect of fish oil supplementation during pregnancy on histone acetylation at both histones (**A**) Histone acetylation at histone H3 for the following genes, *TBX21*, *IL13*, *IL10* and *IL5*, showing a significant reduction at both *TBX21* and *IL13* acetylation in the fish group compared to placebo group. (**B**) Histone acetylation at histone H3 for the following genes, *TBX21*, *IL13*, *IL10* and *IL5*, showing a significant reduction at *IL13* acetylation in the fish group compared to placebo group. * $P<0.05$, ** $P<0.01$.

In addition to the Th2 loci, Th1 genes were as well analyzed. Histone acetylation at both histone H3 and H4 showed no difference between placebo and fish oil supplemented group for *IFNG* (**Figure 4.5.1.3.A, C**) and *IL10* genes (**Figure 4.5.1.3.B, D**)

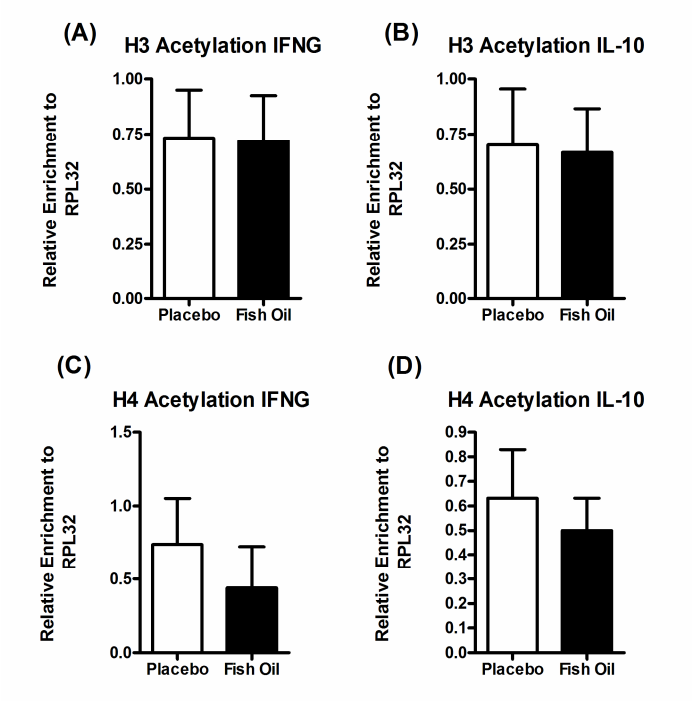


Figure 4.5.1. 3. Effect of fish oil supplementation on the histone acetylation in different Th2 genes at both H3 and H4 histones (**A**) *IFNG* locus showed no difference in histone acetylation at histone H3 between placebo and fish oil groups. (**B**) *IL10* locus showed no difference in histone acetylation at histone H3 between placebo and fish oil groups. (**C**) *IFNG* locus showed no difference in histone acetylation at histone H4 between placebo and fish oil groups. (**D**) *IL10* locus showed no difference in histone acetylation at histone H4 between placebo and fish oil groups.

Finally, FOXP3 the major transcription factor for T-regulatory cells was analyzed and there was no significant difference between placebo and fish oil group at both H3 and H4 histones.

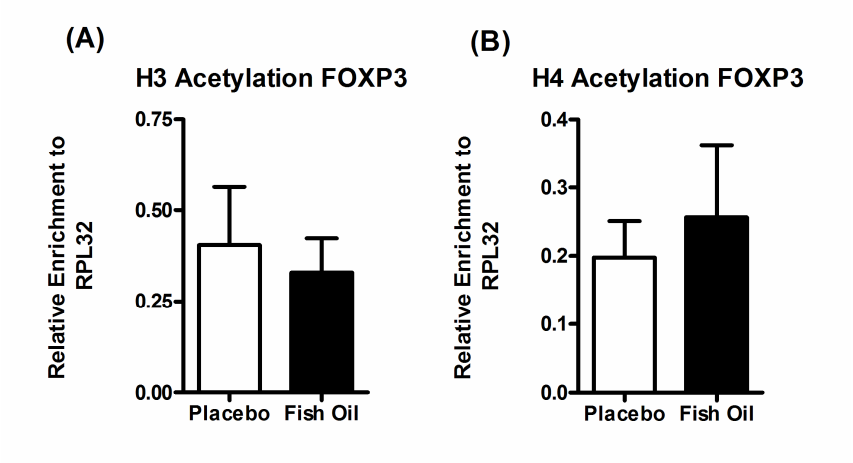


Figure 4.5.1. 4. Effect of fish oil supplementation on the histone acetylation in different Th2 genes at both H3 and H4 histones (**A**) *FOXP3* locus showed no difference in histone acetylation at histone H3 between placebo and fish oil groups. (**B**) *FOXP3* locus showed no difference in histone acetylation at histone H4 between placebo and fish oil groups.

Moreover, I looked at protein kinase zeta (*PRKCz*) to assess the effect and what D'Vaz saw in the same cohort earlier, where neonatal PRKCz expression determines the neonatal T-Cell cytokine phenotype and predicts the development and severity of infant allergic disease (D'Vaz et al. 2012b). PRKCz has 17 different transcripts encoding proteins. Among them many which are tissue specific like liver (Farese et al. 2014) or nervous system (Kwapis and Helmstetter 2014). These transcripts have distinct transcription starting sites. In $CD4^+$ cells

I looked at three different promoter regions of *PRKCz*. *PRKCz* has 27 different transcripts, which are differentially expressed in different tissues. At the PRKCz gene there is different transcription starting sites. It is not clear which transcript(s) is/are mainly expressed in $CD4^+$ T cells thus three different regions covering the putative promoter regions of transcripts *PRKCZ*-001, 009 and 015, to see which are expressed in $CD4^+$ cells (**Figure 4.5.1.5.**).

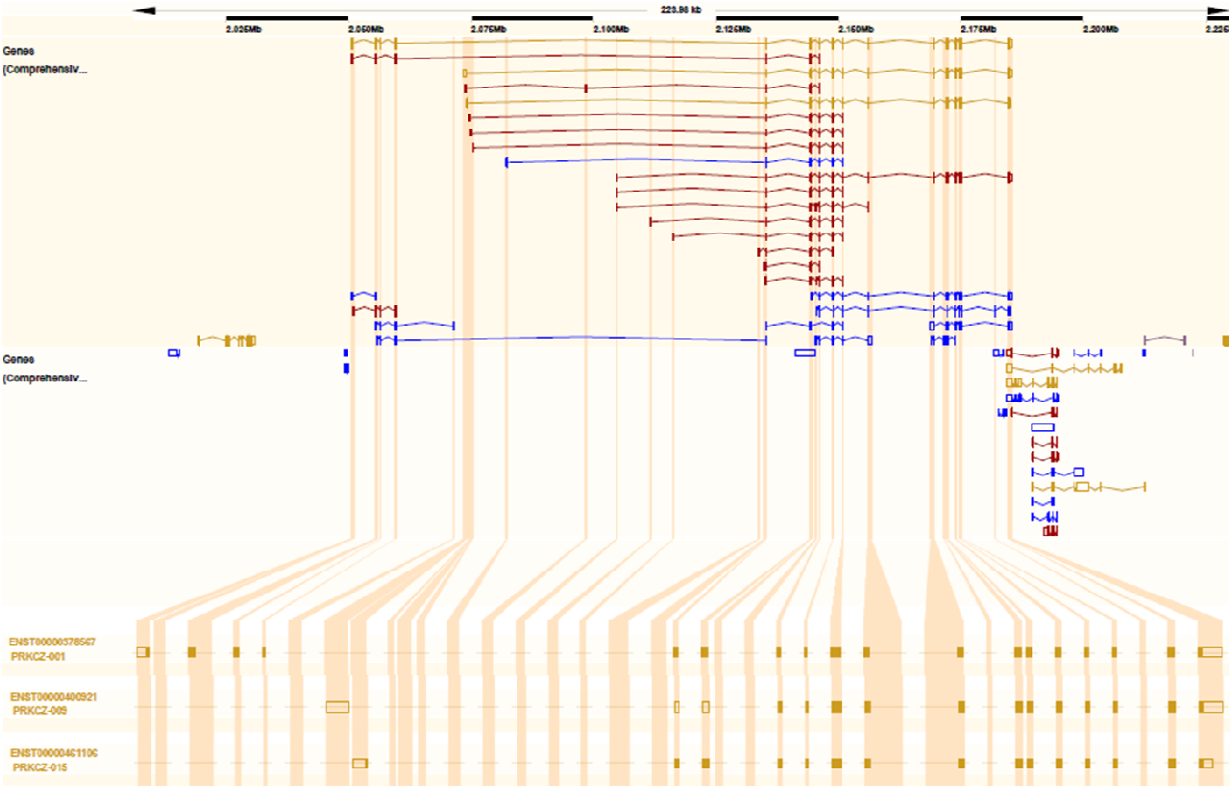


Figure 4.5.1. 5. Transcripts of Protein Kinase Zeta. Three different major transcription starting sites have been used in order to look at the right promoter region at the *PRKCz* expressed in $CD4^+$ cells. PRKCz001 (PRKCz_1), PRKCz009 (PRKCz_2) and PRKCz015 (PRKCz_3) have been analyzed.

In two out of three promoter regions there was a significant increase in the histone acetylation at histone H3 in the fish oil group compared to the placebo group.

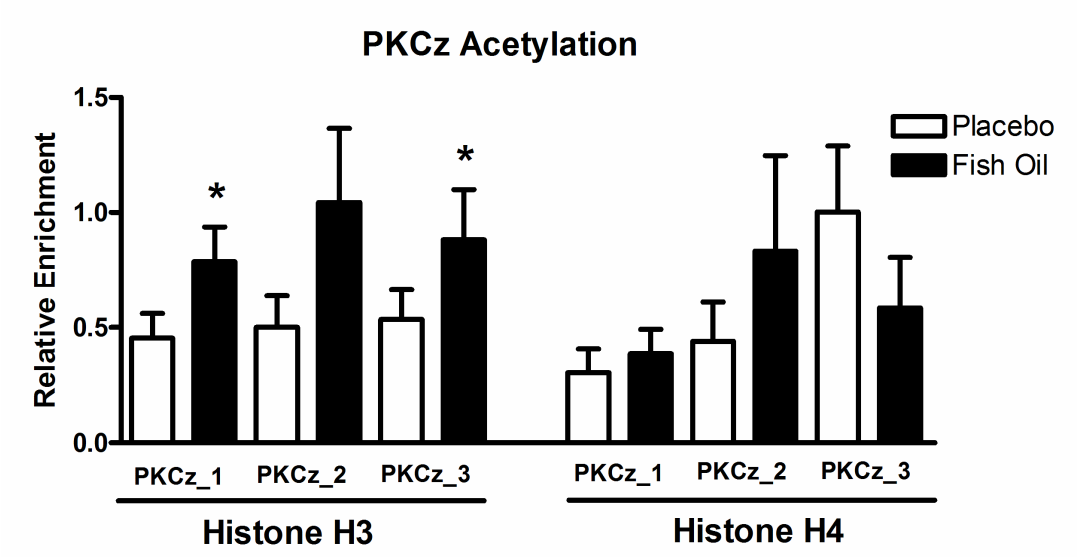


Figure 4.5.1. 5. Histone acetylation at both histones H3 and H4 for different promoter regions at the protein kinase zeta (PRKCz) gene. There is a significant increase in the histone acetylation at both PRKCz_1 and _3 promoter in the fish oil group compared to the placebo group. * P<0.05

3.5.2. PRKCz and TBX21 are correlated with disease development later in life

An additional goal of this analysis was to see if there is a good predictor for allergic disease in later life using histone acetylation analysis at different allergy related loci in $CD4^+$ T cells from cord blood of newborn children. For that I looked at different allergic parameters starting from skin prick test (SPT), wheezing, eczema until reaching asthma or even a more universal approach looking at any allergic disease at the age of 1 year old. First I looked if there was any correlation between SPT and histone acetylation. For this analysis I used both multiple-linear regression and logistic regression to look how the acetylation affects the disease and vice versa. In the linear regression there is a dependent variable (acetylation), independent variable, i.e. predictor (SPT) and, eventually, adjusting variable (fish oil). On the other hand logistic regression looks at the dependent variable as a binary (here SPT) and the predictor can be of any kind (here in my case acetylation). In my case, the interpretation is that the level of acetylation tells

us about the chance of having positive SPT at certain age. If OR is > 1.0, the increase of acetylation increases the chance for positive SPT. If OR is < 1, increasing acetylation decreases the chance of positive SPT. Moreover, if the 95% CI crosses 1.0, the result is not significant (it corresponds to the p-value). Furthermore the analysis looked at two different conditions. The crude condition without any adjustment to different variables and the adjusted condition using fish oil consumption during pregnancy as a confounding variable for the analysis.

Looking the histone H3, there was a positive correlation between SPT and acetylation at the *FOXP3* locus (**Table 4.5.2.1.**).

Table 4.5.2. 1. Correlation analysis between skin prick test (SPT) and histone acetylation at histone H3. Histone H3 acetylation at the FOXP3-locus is positively correlated with SPT.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H3	0.320148	0.270804						
IL9-H3	0.760880	0.806012						
IL13-H3	0.200690	0.321456						
IFNG-H3	0.324724	0.278909						
GATA3-H3	0.907531	0.808011						
TBX21-H3	0.294834	0.409761						
FOXP3-H3	0.025629	0.015441	3.7914	1.0702 to 13.4316	0.0389	4.6987	1.1842 to 18.6436	0.0278
PKCz-H3	0.861364	0.627206						
IL-10-H3	0.896949	0.828359						
PKCz_2-H3	0.351341	0.366067						
PKCz_3-H3	0.258474	0.357684						
IL-5-H3	0.321476	0.296306						

If we looked at the histone H4 we can see a positive correlation as well between SPT and histone acetylation at the *TBX21* and *PRKCz*_2 loci.

Table 4.5.2. 2. Correlation analysis between skin prick test (SPT) and histone acetylation at histone H4. Histone H4 acetylation at the TBX21- and PRKCz_2-locus are positively correlated with SPT.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H4	0.316356	0.302505						
IL9-H4	0.588494	0.621487						
IL13-H4	0.334480	0.460246						
IFNG-H4	0.425737	0.366781						
GATA3-H4	0.213379	0.237905						
TBX21-H4	0.016839	0.021134	4.8521	1.2062 to 19.5188	0.0262	4.7584	1.1554 to 19.5963	0.0308
FOXP3-H4	0.602004	0.558184						
PKCz-H4	0.781025	0.665771						
IL-10-H4	0.113611	0.145995						
PKCz_2-H4	0.023333	0.022637	2.9133	1.0899 to 7.7873	0.033	2.9991	1.0870 to 8.2750	0.0339
PKCz_3-H4	0.295273	0.395255						
IL-5-H4	0.849028	0.989088						

Furthermore, it was looked at the prognostic marks both the sensitivity and specificity of the histone acetylation and the prediction of the SPT in the significantly changed genes at both H3 and H4 histones. Generally, the receiver operatic characteristic curve ROC shows the possible combinations of sensitivity and specificity (actually 100-specificity), i.e. certain value of the continuous parameter to become predictor (in this case acetylation). There is always one possible combination.

The higher are both specificity and sensitivity at once for a certain cut-off, the better possible marker the analyzed continuous variable can be. Moreover, at the ROC: the further it is from the line dividing the square into two triangles (the closer to the shape of the upper-left triangle it is), the better. The p-value given is for the chance that the curve is different from the line dividing the square (i.e. that it is really moved toward upper left corner). The best cut-off criterion (the best predictive value, i.e. the best

combination of specificity and sensitivity is the one closest to the left upper corner of the square in ROC diagram.

On the other hand the dot-plot shows how the exact values of our continuous parameter (acetylation) are distributed between those positive and negative for the binary trait to be predicted (SPT, asthma, etc.). The horizontal line shows the best criterion (cut-off for the continuous parameter) estimated by ROC, i.e. the one closest to the left upper corner. *FOXP3* acetylation at histone H3 could predict positive SPT at children by age 6 with a specificity of 60.5% and sensitivity of 70.6% (**Figure 4.5.2.1.A**). *PRKCz* acetylation at histone H4 could predict positive SPT at children by age 6 with a specificity of 53.5% and sensitivity of 82.4% (**Figure 4.5.2.1.B**). Moreover, *TBX21* acetylation at histone H4 could predict positive SPT at children by age 6 with a specificity of 83.7% and sensitivity of 47.1% (**Figure 4.5.2.1.C**).

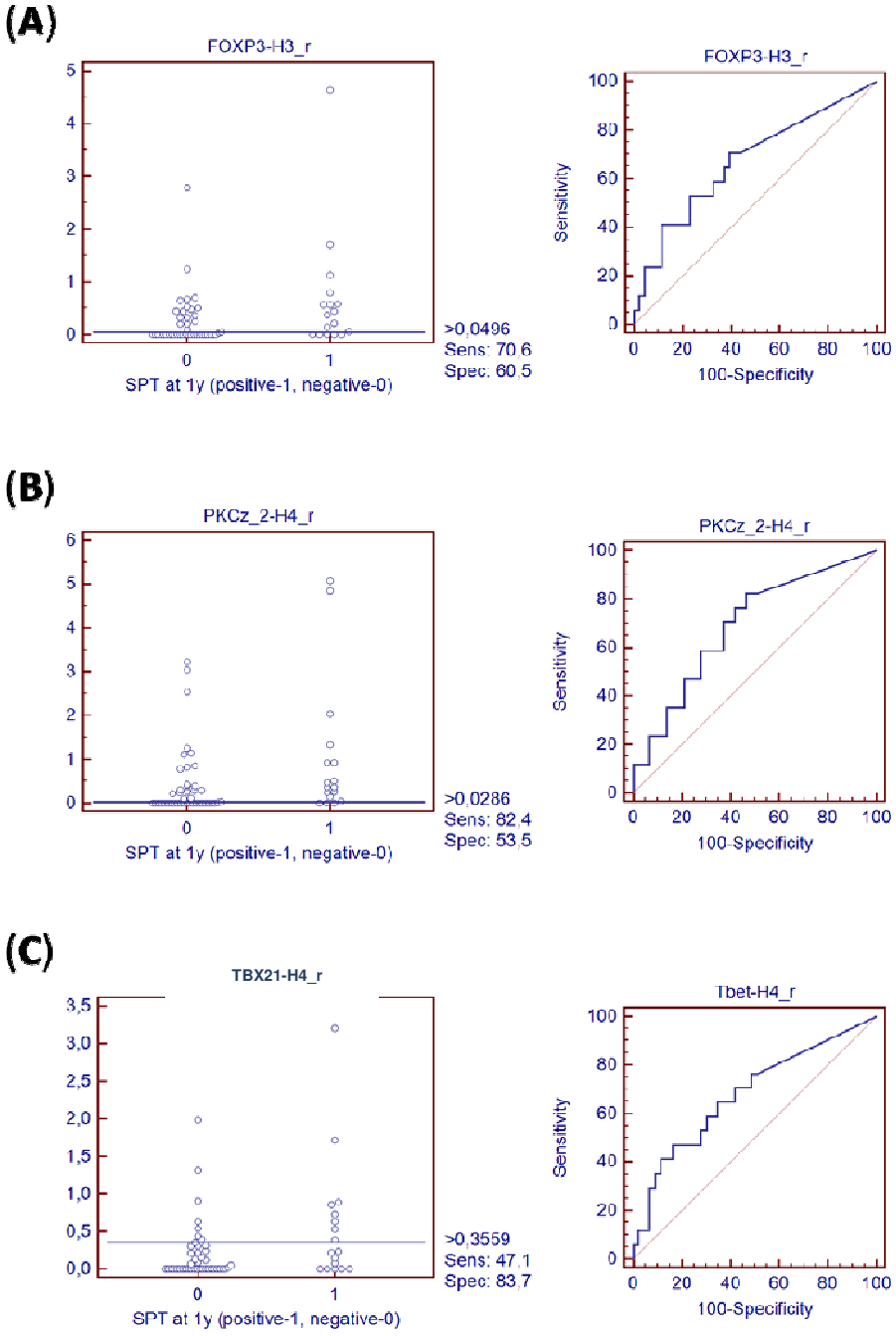


Figure 4.5.2. 1. Dot plot and receiver operatic characteristic curve (ROC curve) looking at the sensitivity and specificity of the prognosis of skin prick test (SPT) using acetylation status for different allergy related genes. (**A**) Dot plot and ROC showing H3 acetylation at *FOXP3* locus is correlated with SPT with a sensitivity of

70.6% and specificity of 60.5% (**B**) Dot plot and ROC showing histone H4 acetylation at *PRKCz*_2 locus is correlated with SPT with a sensitivity of 82.4% and specificity of 53.5% (**C**) Dot plot and ROC showing histone H4 acetylation at *TBX21* locus is correlated with SPT with a sensitivity of 47.1% and specificity of 83.7%.

Another disease status was investigated and the correlation between wheezing and histone acetylation was analyzed. At the level of acetylation at histone H3, a significant positive correlation between histone acetylation at the *IL5* locus and wheezing paralleled with a trend of a positive correlation of *TBX21* and *PRKCz_3* and wheezing. Nevertheless, this was not significant (**Table 4.5.2.3.**).

Table 4.5.2. 3. Correlation analysis between wheezing and histone acetylation at histone H3. There is a positive trend for a correlation between histone H3 acetylation at the *TBX21*- and *PKC*_2-locus with wheezing later in life. Nevertheless, this was not significant. On the other hand, histone H3 acetylation at the *IL5*-locus is highly positively correlated with wheezing development later in life.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H3	0.849028	0.950513						
IL9-H3	0.750024	0.631661						
IL13-H3	0.587796	0.482704						
IFNG-H3	0.235663	0.223300						
GATA3-H3	0.393323	0.377087						
TBX21-H3	0.075886	0.054347	0.3143	0.0852 to 1.1599	0.0823	0.2718	0.0698 to 1.0585	0.0604
FOXP3-H3	0.825910	0.791004						
PKCz-H3	0.851194	0.930278						
IL-10-H3	0.474368	0.491355						
PKCz_2-H3	0.983671	0.972370						
PKCz_3-H3	0.072609	0.082482	2.0516	0.9185 to 4.5827	0.0797	2.0602	0.8970 to 4.7317	0.0884
IL-5-H3	0.038448	0.035508	2.0648	1.0017 to 4.2560	0.0495	2.1318	1.0264 to 4.4274	0.0424

At histone H4, the correlation between wheezing and acetylation status was positively significant at the *IFNG* locus and there was a trend toward a positive correlation at the *PRKCz* locus.

Table 4.5.2. 4. Correlation analysis between wheezing and histone acetylation at histone H4.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H4	0.569664	0.574528						
IL9-H4	0.884467	0.872298						
IL13-H4	0.231771	0.262465						
IFNG-H4	0.024919	0.028468	2.6536	1.0645 to 6.6147	0.0362	2.6332	1.0464 to 6.6263	0.0398
GATA3-H4	0.431394	0.452069						
TBX21-H4	0.360109	0.386458						
FOXP3-H4	0.781080	0.775728						
PKCz-H4	0.119188	0.108198						
IL-10-H4	0.627537	0.655403						
PKCz_2-H4	0.062901	0.064300	2.3871	0.9217 to 6.1822	0.0731	2.3849	0.9174 to 6.2000	0.0746
PKCz_3-H4	0.274549	0.306671						
IL-5-H4	0.345011	0.302781						

Furthermore, the sensitivity and specificity of the prediction of wheezing by histone H3 and H4 acetylation for the significantly changed loci was analyzed. *IL5* acetylation at histone H3 could predict positive wheezing at children by age 6 with a specificity of 67.9% and sensitivity of 64.7% (**Figure 3.5.2. 2. A**). PRKCz acetylation at histone H4 could predict positive wheezing at children by age 6 with a specificity of 90.6% and sensitivity of 35.3% (**Figure 3.5.2. 3. B**).

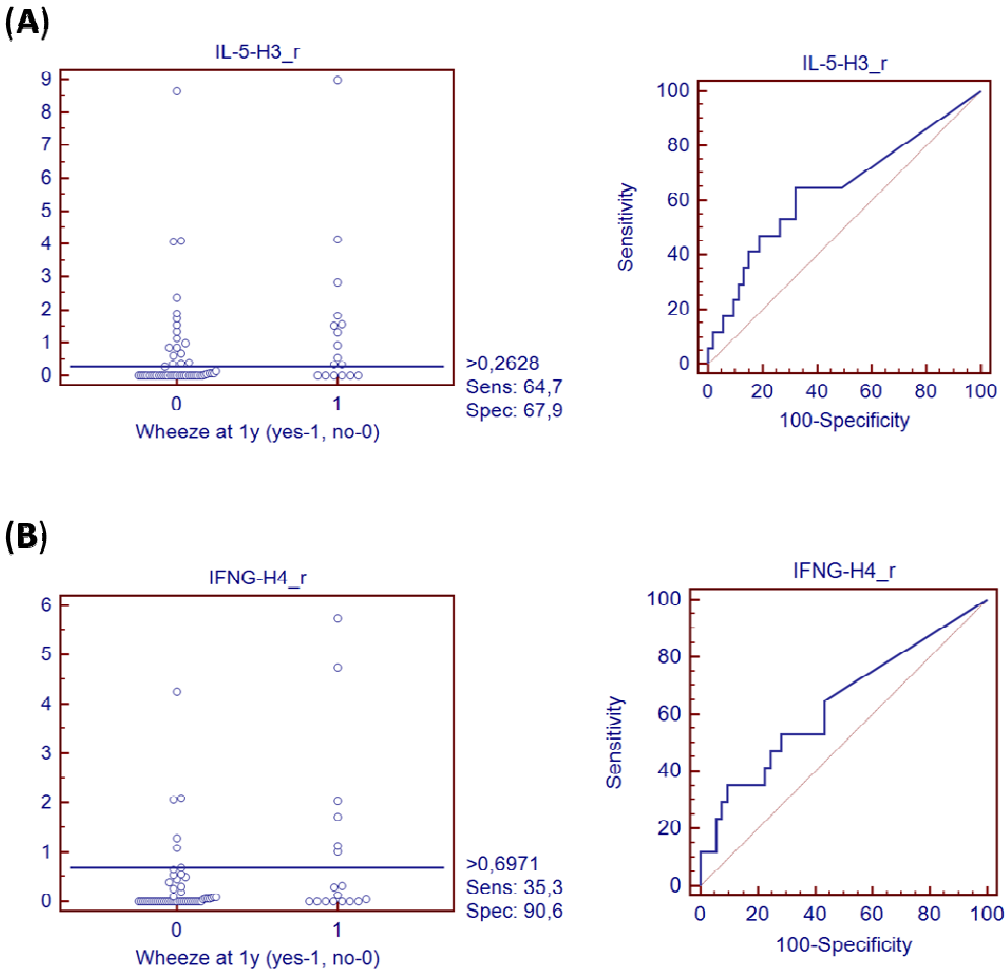


Figure 3.5.2. 4. Dot plot and receiver operatic characteristic curve (ROC curve) looking at the sensitivity and specificity of the prognosis of wheezing using acetylation status for different allergy related genes. (**A**) Dot plot and ROC showing that H3 histone acetylation at the *IL5* locus is correlated with wheezing with a sensitivity of 64.7% and specificity of 67.9% (**B**) Dot plot and ROC showing that histone H4 acetylation at the *IFNG* locus is correlated with wheezing with a sensitivity of 35.3% and specificity of 90.6%.

Afterwards, another allergic disease which is eczema was explored. This was done to widen the spectrum of the analysis and to cover all different aspects and parameters of allergic disease. The correlation between eczema at the age of 1 year and histone

acetylation of H3 and H4 histones was analyzed. There was no correlation between the analyzed loci and eczema neither at histone H3 (**Table 4.5.2.5.**) nor at histone H4 (**Table 4.5.2.6.**).

Table 4.5.2. 5. Correlation analysis between eczema and histone acetylation at histone H3.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H3	0.417985	0.432204						
IL9-H3	0.561603	0.583800						
IL13-H3	0.209085	0.134115						
IFNG-H3	0.908597	0.872795						
GATA3-H3	0.070200	0.077903	2.2863	0.9223 to 5.6680	0.0742	2.258	0.9069 to 5.6225	0.0801
TBX21-H3	0.648386	0.547802						
FOXP3-H3	0.461610	0.498444						
PKCz-H3	0.140977	0.170191						
IL-10-H3	0.145377	0.137981						
PKCz_2-H3	0.909141	0.924558						
PKCz_3-H3	0.475253	0.554592						
IL-5-H3	0.421550	0.449979						

Table 4.5.2. 6. Correlation analysis between eczema and histone acetylation at histone H4.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H4	0.105400	0.108312						
IL9-H4	0.896662	0.880178						
IL13-H4	0.626692	0.721361						
IFNG-H4	0.836797	0.774579						
GATA3-H4	0.249035	0.268277						
TBX21-H4	0.741459	0.791334						
FOXP3-H4	0.739652	0.732303						
PKCz-H4	0.496139	0.457564						
IL-10-H4	0.265468	0.288357						
PKCz_2-H4	0.279857	0.283378						
PKCz_3-H4	0.466798	0.532941						
IL-5-H4	0.349561	0.397373						

Next the correlation between any kind of food allergy at one year of age and histone acetylation in the $CD4^+$ cells from cord blood was analyzed. There was no correlation between the children having food allergy at 1 year old and histone acetylation status at birth, neither on histone H3 (**Table 4.5.2.7.**) nor histone H4 (**Table 4.5.2.8.**).

Table 4.5.2. 7. Correlation analysis between food allergy and histone acetylation at histone H3.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H3	0.868684	0.896504						
IL9-H3	0.378343	0.406992						
IL13-H3	0.305331	0.438455						
IFNG-H3	0.708066	0.650651						
GATA3-H3	0.735905	0.790407						
TBX21-H3	0.228167	0.312007						
FOXP3-H3	0.956433	0.962961						
PKCz-H3	0.421660	0.554180						
IL-10-H3	0.838254	0.880435						
PKCz_2-H3	0.463033	0.483254						
PKCz_3-H3	0.403157	0.265947						
IL-5-H3	0.408712	0.454358						

Table 4.5.2. 8. Correlation analysis between food allergy and histone acetylation at histone H4.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H4	0.729499	0.723683						
IL9-H4	0.924753	0.895864						
IL13-H4	0.511814	0.354590						
IFNG-H4	0.994538	0.887359						
GATA3-H4	0.468617	0.424223						
TBX21-H4	0.799712	0.713058						
FOXP3-H4	0.909539	0.925054						
PKCz-H4	0.660745	0.736086						
IL-10-H4	0.897653	0.965181						
PKCz_2-H4	0.347119	0.345819						
PKCz_3-H4	0.394089	0.283590						
IL-5-H4	0.502091	0.608440						

Furthermore, the correlation between histone H3 and H4 acetylation and the development of asthma was investigated. First the correlation between having asthma later at life and histone acetylation at histone H3 in $CD4^+$ cells from cord blood was explored. There was a tendency towards a positive correlation between *IL13* and *FOXP3* acetylation at birth with developing asthma later in life, nevertheless, this correlation remains insignificant (**Table 4.5.2.9**). On the other hand there was a high correlation between asthma status in children and histone acetylation at histone H4 at different genes. There was a correlation at *IFNG* locus (*P= 0.066*), *PRKCz* locus (*P=0.0471*) (**Table 4.5.2.10**).

In addition to that the prognostic marks both the sensitivity and specificity of the histone acetylation and the prediction of the asthma in the significantly changed genes at both H3 and H4 histones were measured. Histone acetylation on histone H4 at *IFNG* locus could predict asthma incidence at children at the age of 1 year old with a specificity of 57.1% and sensitivity of 85.7% (**Figure 4.5.2. 3. A**). *PRKCz_2* acetylation

at histone H4 could predict asthma incidence at children by age 6 with a specificity of 87.3% and sensitivity of 42.9% (**Figure 4.5.2. 3. B**). The other promoter regions of the *PRKCz (1 and 3)* showed similar results with a specificity of 79.4% and 68.3% and a sensitivity of 57.1% and 71.4% respectively.

Table 4.5.2. 9. Correlation analysis between asthma and histone acetylation at histone H3. Histone H3 acetylation at the *IL13* and *FOXP3*- locus is positively correlated with asthma development but nonetheless, not significant.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H3	0.463487	0.431989						
IL9-H3	0.352601	0.313770						
IL13-H3	0.097168	0.183719	2.1946	0.8042 to 5.9887	0.1249	1.8982	0.6590 to 5.4674	0.235
IFNG-H3	0.217481	0.176275						
GATA3-H3	0.959565	0.961271						
TBX21-H3	0.760506	0.998299						
FOXP3-H3	0.083719	0.058238	3.2796	0.7912 to 13.5939	0.1016	3.5001	0.8248 to 14.8523	0.0893
PKCz-H3	0.529987	0.326441						
IL-10-H3	0.993047	0.947688						
PKCz_2-H3	0.806854	0.771688						
PKCz_3-H3	0.216637	0.346040						
IL-5-H3	0.325312	0.269973						

Table 4.5.2. 10. Correlation analysis between asthma and histone acetylation at histone H4. H4 histone acetylation at the *IFNG* locus and at the *PKC-z*, and *PRKCz*_2-promoter regions are positively correlated with the development of asthma at 1 year of age.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H4	0.784176	0.774593						
IL9-H4	0.517607	0.548704						
IL13-H4	0.067690	0.120665	2.6454	0.8163 to 8.5732	0.1049	2.2361	0.6635 to 7.5363	0.1942
IFNG-H4	0.027555	0.041065	3.0683	1.0292 to 9.1476	0.0443	2.9045	0.9521 to 8.8611	0.061
GATA3-H4	0.081242	0.098215	2.355	0.8059 to 6.8813	0.1174	2.1839	0.7312 to 6.5228	0.1618
TBX21-H4	0.079385	0.106189	4.053	0.7837 to 20.9591	0.0951	3.6706	0.6826 to 19.7392	0.1298
FOXP3-H4	0.221855	0.209606						
PKCz-H4	0.051820	0.034220	4.2453	0.9086 to 19.8364	0.066	5.0067	1.0210 to 24.5521	0.0471
IL-10-H4	0.079388	0.099311	2.1757	0.8581 to 5.5164	0.1015	1.9873	0.7842 to 5.0361	0.1477
PKCz_2-H4	0.052129	0.050420	3.0331	0.9226 to 9.9718	0.0677	2.9216	0.8870 to 9.6235	0.0779
PKCz_3-H4	0.036516	0.062244	2.0356	0.9642 to 4.2975	0.0623	1.8777	0.8689 to 4.0576	0.109
IL-5-H4	0.517416	0.370029						

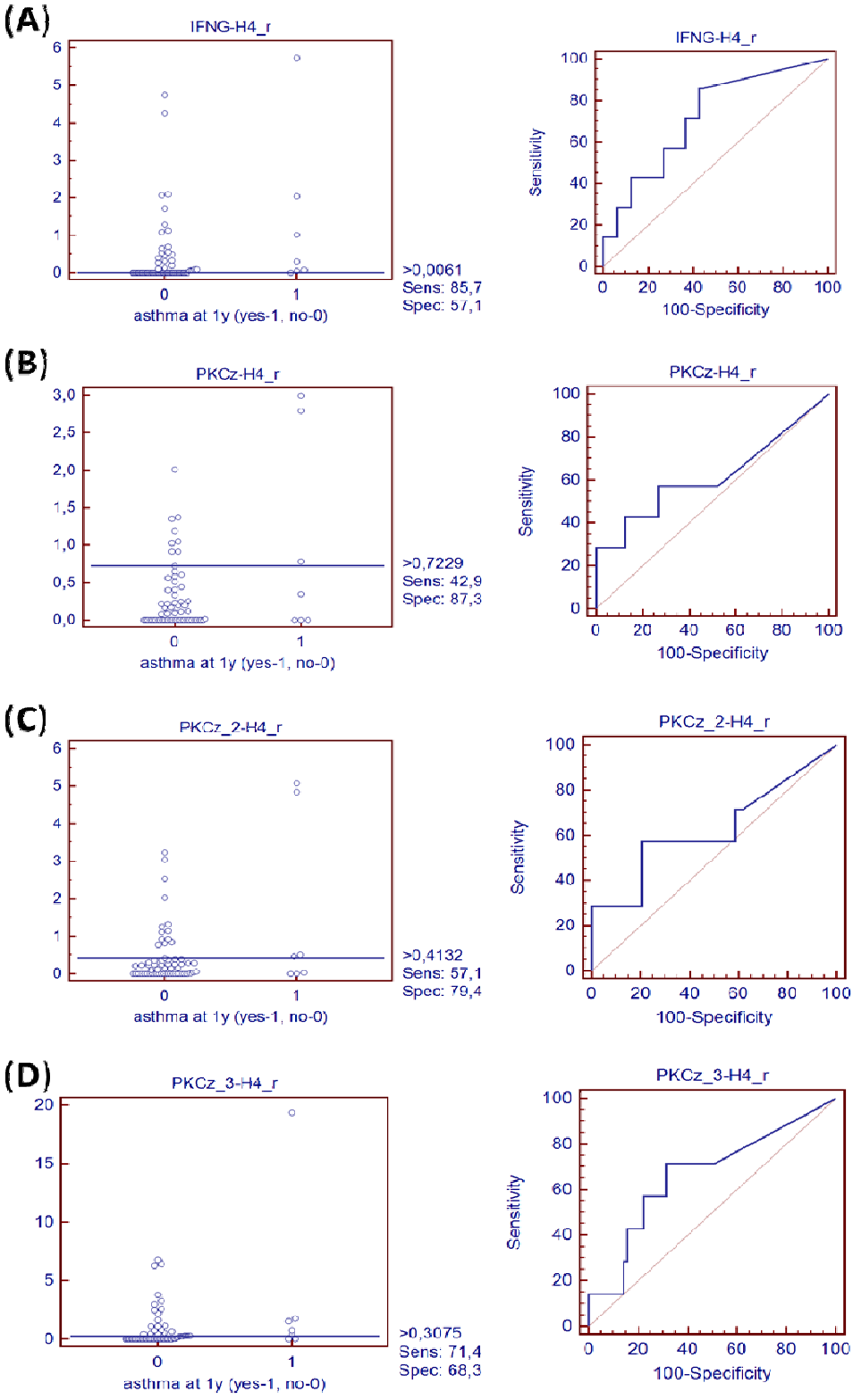

(A)
IFNG-H4_r
asthma at 1y (yes-1, no-0)
>0,0061
Sens: 85,7
Spec: 57,1
IFNG-H4_r
Sensitivity
100-Specificity
(B)
PKCz-H4_r
asthma at 1y (yes-1, no-0)
>0,7229
Sens: 42,9
Spec: 87,3
PKCz-H4_r
Sensitivity
100-Specificity
(C)
PKCz_2-H4_r
asthma at 1y (yes-1, no-0)
>0,4132
Sens: 57,1
Spec: 79,4
PKCz_2-H4_r
Sensitivity
100-Specificity
(D)
PKCz_3-H4_r
asthma at 1y (yes-1, no-0)
>0,3075
Sens: 71,4
Spec: 68,3
PKCz_3-H4_r
Sensitivity
100-Specificity

Figure 4.5.2. 5. Dot plot and receiver operatic characteristic curve (ROC curve) looking at the sensitivity and specificity of the prognosis of asthma, using histone acetylation status for different allergy related genes. (**A**) Dot plot and ROC showing histone H4 acetylation at the *IFNG* and its correlation with asthma with a sensitivity of 85.7% and specificity of 57.1% (**B**) Dot plot and ROC showing *PRKCz*_1 locus at histone H4 correlation with asthma with a sensitivity of 42.9% and specificity of 87.3% (**C**) Dot plot and ROC showing *PRKCz*_2 locus at histone H4 correlation with asthma with a sensitivity of 57.1% and specificity of 79.4% (**D**) Dot plot and ROC showing *PRKCz*_3 locus at histone H4 positive correlation with asthma with a sensitivity of 71.4% and specificity of 68.3%.

Afterwards, the combination of any allergic disease and its correlation with histone acetylation was analyzed.. There was no correlation at all between having an allergic disease later in lifer and histone acetylation neither at histone H3 (**Table 4.5.2.11**), nor at histone H4 (**Table 4.5.2.12**).

Table 4.5.2. 11. Correlation analysis between having any allergic disease and histone acetylation at histone H3.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H3	0.534299	0.525159						
IL9-H3	0.525159	0.586800						
IL13-H3	0.127941	0.148474						
IFNG-H3	0.702742	0.675600						
GATA3-H3	0.189598	0.178980						
TBX21-H3	0.617267	0.688167						
FOXP3-H3	0.498983	0.467921						
PKCz-H3	0.337326	0.270331						
IL-10-H3	0.180466	0.191217						
PKCz_2-H3	0.789878	0.778932						
PKCz_3-H3	0.915220	0.818939						
IL-5-H3	0.385229	0.365177						

Table 4.5.2. 12. Correlation analysis between having any allergic disease and histone acetylation at histone H3.

Linear Regression			Logistic Regression					
Locus	Crude	Adj. Fish Oil	Crude			Adjusted for Fish oil		
	P	P	Odds ratio	95% CI	P	Odds ratio	95% CI	P
IL4-H4	0.361459	0.362190						
IL9-H4	0.926394	0.912666						
IL13-H4	0.710494	0.792892						
IFNG-H4	0.326192	0.357373						
GATA3-H4	0.970068	0.942013						
TBX21-H4	0.672473	0.712409						
FOXP3-H4	0.738296	0.746910						
PKCz-H4	0.779757	0.818794						
IL-10-H4	0.527398	0.555713						
PKCz_2-H4	0.127640	0.129454						
PKCz_3-H4	0.840904	0.914479						
IL-5-H4	0.873320	0.807621						

3.6. Histone acetylation is changed at several loci in healthy children compared to asthmatic children.

To investigate if allergic disease like asthma correlates with differences in histone acetylation at allergy-relevant gene loci histone acetylation was analyzed in $CD4^+$ T cells from healthy children and allergic asthmatic children. For that purpose, 28 subjects were picked (a subpopulation from The CLARA cohort) , 15 healthy and 13 allergic asthmatics.

In short, upon enrollment, all asthmatic children underwent full clinical examination, pulmonary function test, chest radiograph and blood count. In addition, total and specific IgE (RAST) was measured. A positive specific IgE was defined as one or more positive reactions [≥0.35 IU/ml] to a panel of 20 common allergens (Mediwiss Analytic, Moers, Germany; for allergen details see 2.2.1.1). Inclusion criteria for asthmatic

children comprised at least 3 attacks of obstructive bronchitis and/or a doctor´s diagnosis of recurrent episodes of obstructive bronchitis and/or a history of asthma medication and a typical lung function showing reversible pulmonary obstruction. Children were defined as asthmatics if they met the inclusion criteria for asthmatics and had a ΔFEV1 of higher than 10%. Allergic asthmatics (AA) were defined based on the criteria above and a positive (>0.35 IU/ml) specific allergic sensitization assessed by RAST test. The definition for healthy children (HC) was based on having no allergies and any chronic diseases. Children with other pulmonary, chronic or autoimmune diseases were excluded, similarly children with immunodeficiency and subjects taking steroids, antibiotics, probiotics or suffering from an infection within 14 days before blood withdrawal (Raedler et al. 2015). From this cohort 28 sub-sample collective was collected to look at the epigenetic profile in a pure $CD4^+$ cells and compare between healthy control children and allergic asthmatics.

Histone acetylation at histones H3 and H4 was analyzed. Different Th1 (*TBX21, IFNG, TNF* and *IL10*) and Th2 (*GATA3, IL4, IL5, IL9* and *IL13*) loci were measured. There was a significant increase in the acetylation status of the *IL13* at both histones H3 (**Figure 4.6.1.A**) and H4 (**Figure 4.6.1.C**) in the asthmatic children compared to the healthy controls. Acetylation of histone H3 at GATA3, IL4, IL5 and IL9 showed no differences between the healthy control (C) group and allergic asthmatic group (A) (**Figure 4.6.1.A**). Furthermore, none of the Th1 genes analyzed (IFNG, IL10, TNF, and TBX21) showed any difference between the healthy control group vs. the allergic asthmatic group (**Figure 4.6.1.B**). Investigating the acetylation status between the two analyzed group on histone H4, there was no changes in the acetylation status at all different Th2 loci (GATA3, IL4, IL5 and IL9) (**Figure 4.6.1.C**) as well as Th1 loci (IFNG, IL10, TNF and TBX21) (**Figure 4.6.1.D**) between the healthy control group compared to the allergic asthmatic group.

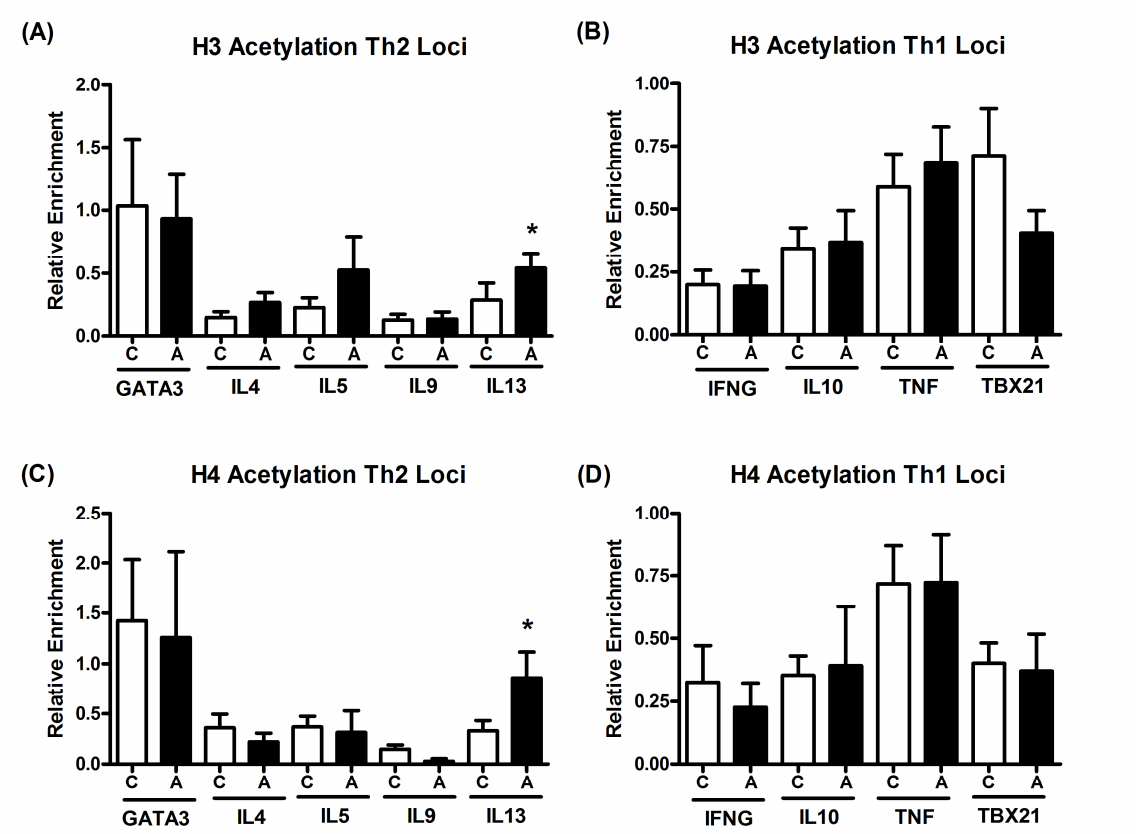


Figure 4.6. 1. A comparison between healthy control children (white bars) and allergic asthmatic children (black bars) on the basis of histone acetylation in peripheral CD4$^+$ cells. (**A**) Acetylation at histone H3 for Th2 genes showing higher acetylation in the at the *IL13* locus in the allergic asthmatic children compared to the healthy controls (**B**) Acetylation at histone H3 for Th1 genes showing no differences in relative enrichment between healthy controls and allergic asthmatics (**C**) Acetylation at histone H4 for Th2 genes showing higher acetylation in the at the *IL13* locus in the allergic asthmatic children compared to the healthy controls (**D**) Acetylation at histone H3 for Th1 genes showing no differences in relative enrichment between healthy controls and allergic asthmatics. * P<0.05.

4. Discussion

4.1. Epigenetic Mechanisms in allergic disease

Epigenetic regulation is divided into different mechanisms affecting different parts of the transcription and translation machinary (Bégin and Nadeau 2014; Amarasekera et al. 2014b; Lovinsky-Desir et al. 2014; Singh et al. 2014). The percent of methylation of a gene affects the accessibility of transcriptions factors to the gene and thus the transcription of the gene. Furthermore, another epigenetic mechanism plays a very important role in the repression or suppression of genes, which is micro RNAs (miRNA). MiRNA is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals, and some viruses, which functions in post-transcriptional regulation of gene expression (Bartel 2009). MiRNAs regulate the translation of different genes mainly in a negative way. They cause the silencing of the gene-transcription as their main mechanism (Ariel and Upadhyay 2012; Tomankova et al. 2012). The third epigenetic mechanism is histone modifications. Histone modifications cover different modifications from histone methylation to histone acetylation and further to histone phosphorylation and ubiquitination. Each of these different histone modifications causes a different effect on the gene transcription. In general, all histone modifications happen on the lysine residue on the N-terminal tail of the histone protein, either on H2A, H2B, H3 or H4 histones (Wilkinson and Gozani 2014). For instance, histone methylation at the lysine number 4 in histone H3 can cause gene activations, whereas the same methylation but on lysine number 9 on the same histone H3 can cause gene silencing (Benard et al. 2014). Furthermore, histone acetylation on different lysine residues on different histones can cause gene activation all the time (Barnes et al. 2005). These acetylated histones play a very important role in the development of a disease or the repression of it. In this study the focus was on histone acetylation as a major epigenetic modification causing alteration in allergic development and progression.

4.2. Effect of environmental factors on allergy pathophysiology

Allergy is a hypersensitivity disorder of the immune system. Its Symptoms include red eyes, itchiness, and runny nose, eczema, hives, or an asthma attack. Allergies can play a major role in conditions such as asthma. Allergic Asthma is a medical condition that causes narrowing of the small airways in the lungs. Typically, asthma patients develop wheezing and have increased mucous production in their lungs and they can have episodes of increased shortness of breath, often triggered by allergic reactions. Allergic asthma is a disease of chronic inflammation and hyperresponsiveness that affects the airways but not the alveoli or the parenchyma of the lung itself (Pavord and Wardlaw 2010)

There has been a drastic increase in the prevalence of asthma and allergic disease in the past two decades, especially in the western countries (Braman 2006). There are many factors that affect the prevalence of asthma.

The hygiene hypothesis attributes this increase in prevalence to environmental factors, namely the decrease in exposure to microbial compounds and or infectious diseases (Mutius 2007; Czeresnia 2005). Supporting this, epidemiological studies have shown that various immunological and autoimmune diseases are much less common in the developing world than the industrialized world and people emigrating from developing to industrialized countries increasingly develop immunological disorders in relation to the length of time since the immigration (Gibson et al., 2003).

There are many factors that affect the hygiene hypothesis and their major mechanism of action is through epigenetic regulation of different genes and factors (Lo and Zhou).

4.3. Establishment and validation of chromatin immunoprecipitation (ChIP) protocol

4.3.1. Establishment of the ChIP protocol

The chromatin immunoprecipitation (ChIP) protocol was adapted from different published data (Collas; Hezroni et al. 2011; Strenkert et al. 2011) and adjusted to be suitable for human $CD4^+$ cells as the main vessel for alteration in allergic diseases.

The choice of $CD4^+$ cells instead of whole PBMCs was based on the vast number of publications and reviews that show that $CD4^+$ cells are the most important cell type in the whole allergy and asthma pathogenesis and on that basis $CD4^+$ cells were chosen for our study (Steinke and Lawrence 2014; Yamane and Paul 2013; Zhang et al. 2013). During Chromatin-Immunoprecipitation there are several critical steps like cross-linking of DNA and protein (fixation) and shearing of the chromatin. It is known that shearing efficiency is depending on fixation, SDS concentration in the used buffer, the cell/tissue type and the used sonication device. From the literature it was already known that $CD4^+$ T cells are relative sonication resistant due to the compact chromatin structure. First a tip sonicator was tested for shearing and the chromatin was successfully sheared after 30 cycles with a 1% SDS concentration in the lysis buffer II. Other sonication times or SDS concentrations were not applicable or functional for a sufficient $CD4^+$ shearing. The use of the bioruptor was chosen to minimize handling mistakes that was found in the tip-sonicator, to sonicate up to 6 samples at once and to have a consistent sonication result, which was not the case by the tip-sonicator.

4.3.2. Validation of the ChIP protocol

After establishment of the protocol a validation of the protocol was performed. A six step validation protocol was set and conducted on human samples. The first step in the validation was to set a reference range for all different genes that were measured in the ChIP protocol. 14 different Th1, Th2, Th17 and Treg genes were picked and promoter primers were designed and validated. The reference range was set for both Histone H3 and H4 acetylation ChIP. The reference range was set using the lower and upper 95% Cl of the geometrical mean of each gene after ChIPing and measuring the percent enrichment of 10 different healthy adults for each of the established gene assays. The reference range represents the normal range that the enrichment of each gene should lay within. If the enrichment percentage goes beyond of this range either in a positive or negative way, this represents pathological conditions that represent the progression of a certain disease. The reference range represents the normal enrichment values that are connected with $CD4^+$ cells on both histones H3 and H4.

4.3.2.1. Reference Range and Lower Limit of the Blank

Beside the reference range, the lower limit of the blank (LOB) was also measured. The LOB represents the lowest measurable value after measuring 5 blank samples ± 2 SD. The LOB should be zero in the optimal case and as close to zero as possible in practice. Once the LOB shifts from zero away, this means that the background measurement of the whole test is as high as the LOB which may give a false positive or false negative. In this ChIP method, the LOB for most of the measured genes was below 0.001 and only for some loci like IL-10 it was up as 0.01% enrichment. Beyond these values, no enrichment of acetylated histones at the analyzed loci is possible. This shows that the method, yield a very faint background that can be neglected in the real measurements.

4.3.2.2. Lower Limit of Quantification

Furthermore, another parameter was measured and set in the process of validation, which is the lower limit of quantification (LOQ). The LOQ is the lowest number of cells or quantity of an analyte which can be analyzed with a certain method which will still produce reliable data. Usually for ChIP are needed around 2 million cells even if ChIP with lower cell numbers has been described as in μChIP (Collas). In the context of the ChIP validation the number of cells was reduced to $1X10^5$ without affecting the enrichment values significantly. Lower cell numbers lead to severely reduced enrichment values at all analyzed loci (figure 3.3.31). Nevertheless, with the validated ChIP protocol with cell numbers of $1x10^5$ cells. This cell number can be isolated from 0.2 ml of blood making this method suitable for studies were only small amounts of blood are available e.g. studies with children.

4.3.2.3. Temperature effect and longtime stability

In addition to what was done, two very important steps were under investigation, the effect of temperature and the long time storage stability.

Chromatin immunoprecipitation can be temperature sensitive as the histones can be degraded or the epitope recognized by the antibody can be denatured if the sample is exposed to higher temperature – like room temperature – for longer time periods. For this reason many of the steps are carried out either in 4°C room or on ice. Nevertheless, during collection of sample within a cohort study it might happen that samples are not

immediately frozen. To analyze if this can interfere with the obtained results the chromatin was left for 24 hours on room temperature to see whether incubation of the chromatin on room temperature alone for a longer time can cause any effect or degradation of the histone marks. It was obvious that histone modifications are significantly affected by room temperature as seen in figure 3.3.5.1. There was a significant decrease in enrichment values for different loci tested. Among them *IL4* and *IL10* that lost significant enrichment values on the acetylation histone after left on room temperature for some time. This effect is likely due to the degradation of the whole chromatin structure and the modifications that can happen to the binding sites of the antibodies. It is unlikely that the effect is due to the degradation of the DNA itself as the DNA is relatively stable for very long time even in room temperature. Due to the observed changes to the final readout of the enrichment percentage chromatin immunoprecipitation should be carried out directly after isolation of the cells or thawing of the cells and possibly on ice and in cool place.

On the other hand, the cells lost some of their enrichment values after freezing and thawing. This was consistent to all gene loci analyzed. This effect does not seem to affect the final result as the cells will be received either frozen or fresh from the clinics and donors.

As samples for cohort studies are often frozen in RPMI media containing DMSO and stored at -80°C (or liquid nitrogen) before analysis is taking place. To test whether long storage conditions can have influences different epigenetic modifications like histone acetylation cells were frozen in FCS and 10% DMSO and stored for 7 and 30 days in -80°C condition and a comparison was done with freshly isolated chromatin from the same donors. As shown in figure 3.3.6.1., there was no difference between the freshly ChIPed and the stored chromatin for both time points, 7 and 30 days. The changes in the enrichment percentage were minimal as seen for *IL4* or *IFNG* but no significance was reached. It was obvious that longer storage of intact cell, which is the main player for a successful ChIP, does not have any effect on different epigenetic modifications including the histone marks. This can be true for longer time points beyond 30 days but

a comparison between freshly isolated and stored cells for longer time periods were not tested in this study

For the final validation step, an internal standard to prove the stability and redundancy of our method was implemented. For this purpose a ready to ChIP chromatin from a leukemia cell-line, which is the closest relative of our main focus in this study, the $CD4^+$ cells, was used as our internal standard. Each patch of this chromatin can be divided into 10 different aliquots which can be measured once and have its own range and measurement. Moreover, this chromatin is used every 5^{th} or 6^{th} runs to ensure that our method runs without any problems and complications.

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4.4. Effect of Effect of Dietary factors status during pregnancy on histone acetylation in cord blood $CD4^+$ cells

After establishing and validating the ChIP method, samples from two different Australian cohort study were analyzed.

4.4.1. Effect of folic acid status during pregnancy on histone acetylation in cord blood $CD4^+$ cells

The aim of this study was to analyze the role of gestational folate exposure on epigenetic marks at allergy-relevant gene loci in $CD4^+$ T cells from the cord blood of neonates.

Classical epigenetic studies in mice demonstrate the fetal epigenetic profile is particularly susceptible to environmental disruption (Gonda et al. 2012; Hollingsworth et al. 2008) and there is accumulating evidence for this in humans {Hinz 2012 #173).

In general, the effect of folate acid during pregnancy is still under a lot of discussion and debate. Folic acid is very important for the development of the neural tube and protection against neural tube defects that may arise from folic acid deficiency (Jagerstad 2012). On the other hand there is increasing evidence that excessive folic acid supplementation during pregnancy can promote other diseases in offspring. For example, excessive maternal folate supplementation causes glucose intolerance to insulin in mice male offspring (Huang et al. 2014). Furthermore, high supplementation of folic acid during pregnancy can cause some adverse effects and increases in the

severity of some diseases like bronchitis in infants (Veeranki et al. 2014). These effects does not stop within the small range but there has been increasing evidence that excessive folic acid supplementation during pregnancy can cause an increase in allergic diseases later in life in the offspring (Bekkers et al. 2012; Dunstan et al. 2012; Haberg et al. 2011; Hollingsworth et al. 2008).

To analyze if gestational folate can modify epigenetic marks at allergy-relevant gene loci – which might promote the development of an allergy-prone immune phenotype later in life – $CD4^+$ T cells were isolated from neonates born to mothers with either low or high serum folate concentrations and histone H3 and H4 acetylation was analyzed at Th1, Th2, Th17 and Treg specific gene loci. To focus on the effect of folate children which developed allergy at one year of age were excluded from the study population. Analysis of H3 and H4 acetylation in cord blood $CD4^+$ T-cells revealed a significant association of high maternal serum folate levels and increased acetylation of H3 and H4 histones at the *GATA3* locus and H4 at the *IL9* locus in the cord blood $CD4^+$ cells. Additionally, there was a consistent tendency for increased acetylation of histone H3 at the *IL9* locus although this did not reach full significance. Furthermore, we found a tendency for lower histone acetylation at the *IFNG* locus in the HF group compared to the LF group. IFNG is mainly produced by Th1 cells, and this locus is known to be hypoacetylated in Th2 cells (Fields et al. 2002). Collectively these findings suggest that exposure to high gestational folate is associated with more transcriptionally permissive chromatin at Th2 associated genes in $CD4^+$ T-cells. The major questions that raises is, how does folate as a methyl donor and its main function is DNA methylation (Hollingsworth et al. 2008) affect histone acetylation? There are different possible mechanisms how a methyl donor like folate can cause histone modifications. For one thing, DNMT1 can cause hypermethylation at the E2F1 motif causing the deacetylation at histone H3 (Li et al. 2014). Furthermore histone deacetylation is linked with DNA methylation through methyl-CpG-binding protein MeCP2 using transcriptional-repression domain (TRD) that can function at a distance in vitro and in vivo (Nan et al. 1998; Wade et al. 1999). Moreover, DNA demethylase MBD2 is a candidate transcriptional activator. MBD2 is associated with histone deacetylase HADC1 through

MeCP2 as well. This association caused the inhibition histone acetylation completely (Ng et al. 1999; Zhang et al. 1999). It is obvious that DNA methylation is very tightly related with histone deacetylation through MeCP2 and MBD complex proteins with the major DNA methyltransferase DNMT1 and histone deacetylase HDAC1 and 2 (Fuks et al. 2000; Rountree et al. 2000) and this can be a very possible mechanism how folate can modify histones on the acetylation level through simple DNA methylation. Nevertheless, this should be investigated further to see if folate works through the same mechanisms or are there any other underlying mechanisms causing this effect.

Looking at the other epigenetic modification that was measured for this cohort (Data are a courtesy of Dr. David Martino, Manori Amarasekera, and Prof. Susan L. Prescott), whole genome DNA methylation was measured and the focus was at the promoter region not the whole DNA body.

DNA methylation in the promoter region did not appear to be associated with folate status for the limited number of loci tested. This was unexpected given the clear role of folic acid as a co-factor for the one-carbon metabolism. One reason for this observation might be that the methylation assays in the current study were restricted to short (~300bp) regions in gene promoters. There is now a growing appreciation that DNA methylation in gene promoters tends to be more closely associated with the differentiation status of cells, whilst disease-linked loci are increasingly reported to occur within gene bodies (Xu et al. 2013) or localized around CpG islands (Irizarry et al. 2009). Nevertheless, *ZFP57* was identified as a major loci affected by folate status in the high folate group (Amarasekera et al. 2014a). On the level of histone acetylation, different Th1, Th2, Th17 and Treg gene loci were analyzed. These findings both on the acetylation and methylation levels add new findings as well as new questions to the paradigm of the fetal epigenome and the influence of the environment *in utero*. Whether the effects of maternal folate supplementation on the fetal epigenome increase the risk of developing allergic diseases in later life remains to be elucidated.

4.4.2. Effect of Vitamin D status during pregnancy on histone acetylation in cord blood $CD4^+$ cells

During the analysis of the effects of folate it was found that women with a high folate level had also high levels of vitamin D while women with low folate levels tend to have low vitamin D levels. For this reason the analysis for the effect of folate on histone acetylation was adjusted to vitamin D. As described previously vitamin D interacts with the epigenetic machinery and thus vitamin D might also influence histone acetylation in cord blood $CD4^+$ cells. The analysis of histone H3 and H4 acetylation in the high vitamin D group versus the low vitamin D group revealed that - similar to the high folate group - the high vitamin D group exhibited increased acetylation at histone H3 at the *GATA3* and *IL9* loci and for the *IL9* locus at histone H4. It seems that Th2 genes are poised for transcription in $CD4^+$ T cells from neonates due to high vitamin D levels (Sloka et al. 2011). The consequence of the higher histone acetylation at Th2 gene loci could be a polarization into a Th2 direction which would make these children more likely to develop allergic diseases. Interestingly, it has been described that that vitamin D supplementation or high vitamin D status during pregnancy can cause a severer allergic and asthma conditions (Chen et al. 2014). Not only lung allergic disease was worsened but also the risk of infantile eczema was higher after supplementation with vitamin D during pregnancy (Miyake et al. 2014). Nevertheless, the effect of vitamin D on asthma and allergy development is contradictory. Furthermore, low vitamin D during pregnancy can cause a worsening of the neonatal allergic disease through an induction of Th2 cells and eosinophils (Vasiliou et al. 2014). Moreover, in COPSAC birth control study among other studies, it was obvious that Cord blood 25(OH)-Vitamin D deficiency is associated with increased risk of recurrent troublesome lung symptoms till age 7 years (Chawes et al. 2014; Rajabbik et al. 2014).

Furthermore, multifunctional enhancers regulate VDR gene transcription, and 1,25(OH)2D3 induce the accumulation of VDR and up-regulate histone H4 acetylation at conserved regions in the human VDR gene (Zella et al. 2010). Ligand-dependent HDAC-containing complex binds with relB promoter and VDR in dendritic cells (DC). Experimental evidence showed that HDAC3 is involved in negative regulation of relB in DC stimulated with LPS resulting in dissociation of VDR/HDAC3 from the relB promoter. This demonstrates the importance of vitamin D-mediated chromatin

remodeling in regulation of DC function (Dong et al. 2005). In addition to that, VDR inhibits NF-κB function through SIRT1 and 1,25D signaling, suggesting the role of 1,25D-mediated deacetylation of NF-κB through its interaction with SIRT1 (Lavu et al. 2008). This may have implications in epigenetic regulation of steroid resistance and inflammatory response in patients with asthma and COPD where vitamin D insufficiency/deficiency occurs. In addition, this may play a role in epigenetic changes associated with vitamin D insufficiency/deficiency in addition to high methyl donor diet and environmental effects in utero which would result in susceptibility to chronic lung diseases later in adult life.

The mechanism by which vitamin D could modify histone acetylation is not completely clear. Vitamin D interacts with the vitamin D receptor which migrates toward the nucleus and interacts directly with the promoter region of the different genes (Fetahu et al. 2014). In addition to that, there is evidence that the vitamin D receptor due to the uptake of vitamin D activates different histone acetyltransferases and histone deacetylases (Karlic and Varga 2011), that can cause the previous investigated results (Sundar and Rahman 2011). Furthermore, vitamin D plays a very important role in the pathogenesis of many diseases. Hypermethylation of key placental genes involved in vitamin D metabolism suggests uncoupling of processes that may interfere with placentation and availability of vitamin D at the maternal-fetal interface (Anderson et al. 2014). Which enzymes are activated in this process, and how does vitamin D affects exactly the epigenome of the fetus is still to be further deeply investigated.

4.4.3. Effect of fish oil supplementation during pregnancy on different histone acetylation marks

Another dietary factor which might influence of allergic disease is fish oil. In an Australian cohort pregnant women were either supplemented with fish oil or olive oil as a placebo according to Dunstan et.al (Dunstan et al. 2003). From these 98 mother/fetus pairs 70 samples were picked up representing 34 placebo supplemented mothers and 36 fish oil supplemented mothers. From cord blood mononuclear cell fraction, $CD4^+$ cells were isolated and then ChIPed for the main asthma and allergy associated genes (IL4, IL5, IL9, IL10, IL13, IFNG, TNF, IL17A, GATA3, TBX21, and FOXP3). Furthermore,

another candidate gene was picked and an assay for H3 and H4 acetylation was established according to an earlier observation by the group of Susan Prescott, that protein kinase czeta (PRKCz) can be a very important predictive factor by allergen prevention (Prescott et al. 2007). Fish oil proves once again that it can attenuate inflammatory process through ω-3 fatty acids contained in it. Fatty acids in general cause a general DNA hypermethylation which can regulate different genes including inflammatory cytokines and genes (Burdge and Lillycrop 2014). Whether histone acetylation is affected through the same mechanisms illustrated earlier is still to be revealed.

Protein kinase c zeta is a member of the protein kinase and it plays an important role in many different functions in the human system. It plays an important role in the memory mechanisms (Volk et al. 2013) as well as its involvement in the regulation of cardiac glucose and long-chain fatty acid uptake (Habets et al. 2012). Recently, D'Vaz N et. al has shown that neonatal protein kinase zeta expression in T-cells can predict the development and severity of infant allergic disease (D'Vaz et al. 2012a). In this analysis there was a significant reduction in the relative enrichment of *IL13* in both histones H3 and H4 in the fish oil group compared to the placebo group. Furthermore, there was a reduction in the acetylation of *TBX21* at histone H3 in the fish oil supplemented group compared to the placebo group. These findings correlate to what Dunstan et. al found in 2003 in the cytokine analysis of the same cohort after stimulation with different stimuli. They found that in the fish oil supplemented group there was a reduction in the cytokine production on the level of IL13 as well as IFNG in comparison to the placebo group (Dunstan et al. 2012). There is more evidence showing that fish oil supplementation during pregnancy is associated with decreased mRNA levels of Th2-related molecules like *IL4* and *IL13* in the fetus and decreased maternal inflammatory cytokines. This effect can be TGFβ mediated (Krauss-Etschmann et al. 2008).

Omega-3 (n-3) polyunsaturated fatty acids (n-3 PUFAs) the major component of fish oil has well documented anti-inflammatory properties, and consequently therapeutic potential in chronic inflammatory diseases. There is strong evidence indicates n-3 PUFAs as beneficial as a dietary supplement in certain diseases such as rheumatoid

arthritis (Yates et al. 2014). Klemens and colleagues looked at five randomised controlled trials (n = 949). N-3 PUFA supplementation during pregnancy reduced 12-month prevalence of positive egg SPT (two trials, 12/87 versus 32/100, OR 0.33, 95% CI 0.16, 0.70) and childhood asthma (two trials, 10/303 versus 17/179, OR 0.349, 95% CI 0.154, 0.788) and significantly reduced cord blood interleukin-13 levels (Klemens et al. 2011).

Fish oil or its components of omega-3 fatty acids affect the inflammatory response by different ways. They can affect the COX and LOX pathways causing the induction of anti-inflammatory mediators as prostaglandin E3 (PEG3) (Wendell et al. 2015). Furthermore, omega-3 fatty acids causes a change in the production of leukotriene B4 and B5 by stimulated neutrophils in patients with colorectal cancer causing a resolution of the inflammation (Sorensen et al. 2014).

On the other hand, and in this study, fish oil supplementation caused a change in the epigenetic profile of the $CD4^+$ cells. This change cannot be due to any of the earlier described mechanisms. There is new evidence that fish oil supplementation changes the epigenome on the level of DNA methylation (Amarasekera et al. 2015). More studies showed that fish oil and omega-3 supplementation causes a change in the methylation patterns at different loci suggesting an epigenetic mechanism for fish oil (Aslibekyan et al. 2014). Furthermore, leptin promoter is hypoacetylation at histones H3 and H4 paralleled with an increase in binding of histone deacetylases (HDACs) 1, 2 and 6 in the diet induced obese mice. These modifications may serve a feedback role to maintain leptin concentrations within a normal range. The regulation of leptin transcriptional expression by n-3 PUFAs is mediated, at least in part, by epigenetic targets, such as MBD2 and histone modifications (Shen et al. 2014).

In addition to these findings *PRKCz* were acetylated at different promoter regions at histone H3. PRKCz was introduced by D'Vaz as a predictor of development and severity of infant allergic disease. Protein kinase C zeta (PRKCz) is a member of the protein kinase C family that controls the function of other proteins by phosphorylation of the serine or threonine residues in these proteins. PRKCz's function in different physiological actions is not fully understood. It plays an important role in insulin-

stimulated glucose transport. Inhibition of the PRKCz enzyme inhibits insulin-stimulated glucose transport while activation of PRKCz increases glucose transport (Bandyopadhyay et al. 2002). In Allergy, the role of PRKCz is not well known. There are hints that protein kinase c zeta along with other members of the PKC family play a very important role in the inhibition of eosinophils migration to the lungs during asthma (Langlois et al. 2009). Moreover the inhibition of PRKCz caused drastic changes in the functions and effectiveness of the eosinophils (Kato et al. 2005b). On the other hand PRKCz affects neutrophils function as well by attenuating PMA-induced O(2)(-) generation by neutrophils (Kato et al. 2005a).

For those 70 samples, all the clinical data were available including asthma prevalence, food allergy or even eczema and wheezing development by those children. For that matter a more complexed analysis was done using a linear-regression analysis looking at the correlation between the disease status later in life and the acetylation status of different allergy related loci. First I looked at the correlation between skin prick test (SPT) and the acetylation status at both H3 and H4 histones. We were able to identify three genes that were significantly positively correlated with the development of a positive skin prick test later in life. The first one was *FOXP3*, the major transcription factor for T-regulatory lymphocytes. This means there was a higher acetylation at the *FOXP3* locus in the SPT positive group compared to the negative group. There are few publications about the effect of *FOXP3* and its epigenetic modification on the development of SPT. *FOXP3* expression is higher in children with a positive SPT against egg and milk (Sicherer et al. 2010). Whether this is a spontaneous effect by the induction of the allergic reaction or is it epigenetically predisposed is still to be cleared. Furthermore, we were able to see a significant higher enrichment at both *PRKCz* and TBX21acetylated loci with the incidence of SPT later in life. Nevertheless, this correlation was not able to prove any of these loci to be a good prognostic marker for skin prick test, as the correlation was not significant. Through this analysis, I was able to identify both H3 acetylation at the *IL5* locus and H4 acetylation at the *IFNG* locus as good prognostic markers for wheezing in later life. It is known that IL5 is on the main cytokines playing a very important role in the pathology of asthma. IL5 is linked with

the exacerbation of wheezing in asthma and once a bacterial or viral infection is there (Choi et al. 2009; Kato et al. 2011). It seems that *IL5* locus is acetylated and will be transcribed excessively once a viral or bacterial lung infection is existent (Kato et al. 2011). On the other hand IFNG has been described to be produced as well in viral infections (Drysdale et al. 2012). Moreover, it is know that certain polymorphisms in the IFNG gene like the polymorphism of *IFNG* +874 T/A cause exacerbation of wheezing in asthmatic children (Nuolivirta et al. 2009). Concomitant with these findings it seems that acetylation at the *IFNG* promoter in cord blood $CD4^+$ cells can be a good predictor of development of wheezing later in life. In the same context histone H4 acetylation at the *IFNG* locus was highly positively correlated with the development of asthma later in life. Nevertheless, this correlation is abolished when looking at food allergy, eczema or even in a more universal manner, at any allergic disease.

4.5. A comparison analysis between healthy vs. allergic asthmatic children on the level of histone marks

To investigate if an allergic disease like asthma is associated with changes in histone acetylation a sub-population from the CLARA study (Raedler D 2014). In this study 230 children were recruited either suffering from allergic asthma or healthy controls. From those 230 a sub-group of 28 children was selected. In this sub-group were 15 asthmatics vs. 13 healthy controls. $CD4^+$ cells were isolated from children PBMCs either healthy controls or allergic asthmatics. The cells were then fixated with formaldehyde, sonicated with a Bioruptor for 30 cycles (30 sec on, 30 sec off, high power) and ChIPed to measure histone acetylation on both histone H3 and H4. Analyzed were Th1 and Th2 gene loci for both H3 and H4 acetylation. For Th1 genes histone acetylation at the *IFNG, IL10, TNF, and TBX21* were measured. No differences of the acetylation levels at H3 and H4 histones could be detected. Furthermore, we looked at the Th2 genes and for that regard we analyzed the histone acetylation marks for *GATA3, IL4, IL5, IL9 and IL13* genes. There was no difference between healthy controls and allergic asthmatic children on the level of all Th2 genes except the *IL13* locus. *IL13* is considered one the major Th2 cytokine genes and plays a very important role in the development of asthma and the pathogenesis of asthma (Corren 2013; Rayees

et al. 2014). Furthermore, *IL13* gene is epigenetically regulated though HDACs and DNMTs (Su et al. 2008). DNA methylation may affect *IL13* locus which may cause modification at airflow and airway reactivity (Patil et al. 2013). Interestingly, the amplicon used in this study for analyzing the level of histone acetylation at the *IL13* promoter is spanning the *IL13* SNP rs1881457. This SNP was previously associated with atopy and asthmatic symptoms (Beghé et al. 2010; Hagner et al. 2013). For further studies, it would be interesting to investigate if this genotype influences histone acetylation at the *IL13* promoter. Furthermore, more genes were analyzed for the same cohort (Courtesy of Diana Rädler PhD thesis). Nevertheless, a very interesting finding was seen looking at the Treg locus *FOXP3*. It was found that especially histone H3 acetylation at this locus is increased in the group of asthmatic children compared to healthy controls. *FOXP3* encodes the master transcription factor for the differentiation of Treg cells and the expression level of this gene is crucially connected to Treg cell numbers. It has been described that differentiation of Foxp3 Tregs is associated with strong increase of histone H3 acetylation but with only marginal effects on histone H4 acetylation at the *FOXP3* locus (Floess et al. 2007), indicating a crucial link between histone H3 acetylation at the *FOXP3* locus and Treg cells. If increased acetylation of histone H3 at the *FOXP3* locus is promoting the differentiation of Treg cells, higher numbers of these cells would be expected in the asthmatic group. Higher numbers of Foxp3 positive Treg cells were indeed detected in the asthmatic group compared to the healthy control group (Raedler D 2014). These results are contradictory as there are different evidence and publications suggesting a lower number of T-regulatory cells in asthmatic children compared to the healthy control (Kawayama et al. 2013; Stelmaszczyk-Emmel et al. 2013). On the other hand there are other clinical data showing higher Treg numbers in the lungs of asthmatic children when compared to healthy controls (Sjåheim et al. 2013; Smyth 2010). It remains an open question what role does $FOXP3^+$ T-cells have in asthma development.

5. Conclusion and Summary

In this study chromatin immunoprecipitation was established for using the method in cohort studies were only small amount of cells are available and the samples are routinely frozen and stored at low temperature. Additionally, the method was validated for that purpose. This method was validated using different well known validation protocols. Three different cohorts were analyzed using this method with three different perspectives.

In the first cohort, the effect of folate status during pregnancy upon acetylation of Th1, TH2, Treg and TH17 cell loci in cord blood $CD4^+$ T cells was investigated. For that purpose the two extremes very low vs. very high folate levels in maternal blood were picked and both DNA methylation and histone acetylation were investigated.. *GATA3* and *IL9* were significantly more acetylated at both histones H3 and H4 in the high folate group compared to the low folate group. This suggests that folate status during pregnancy can modify the epigenome toward a more Th2 phenotype supporting the development of allergic disease later in life.

On the other hand, supplementation with fish oil during pregnancy showed adversary effects to what folate showed by causing the reduction in acetylation at the *IL13* locus at both H3 and H4 histones. Parallel to that there was a reduction in the histone acetylation in H3 histone at the *TBX21* locus. This proves to be consistent with the protein and mRNA data obtained from the same individual 11 years ago (Dunstan et al. 2003). Fish oil proves once again that it can attenuate inflammatory process through ω-3 fatty acids contained in it. Fatty acids in general cause a general DNA hypermethylation which can regulate different genes including inflammatory cytokines and genes (Burdge and Lillycrop 2014). Whether histone acetylation is affected through the same mechanisms illustrated earlier is still to be revealed.

In the same fashion, a comparison between allergic asthmatics with healthy controls was explored. This picture looks more complex. It is not a single factor that causes these epigenetic modifications. Not all the allergy/asthma related genes were influenced in that scenario but there was susceptible gene, among them were *IL13* and *FOXP3* (FOXP3 data are courtesy of Diana Raedler PhD thesis). Both were significantly more acetylated at histone H3 in asthmatic children in comparison with healthy controls.

Moreover, the acetylation at histone H4 for *IL13* locus was as well increased in the asthmatic children compared to their fellow healthy controls.

The effect of environmental factors on the development of allergic disease through the development and alteration of the human epigenome is now a major mechanism of disease development. How these effects and what are the most important marks are, is still an open question.

There are still some open questions to be answered and they are of a great importance for future planning.

1- Folate is considered a methyl donor and affects DNA methylation. In this study we have seen another effect of folate comprising histone acetylation as a key factor driving the Th2 machinery. How folate as a methyl donor does affect histone acetylation remains to be clarified.

2- Fish oil with all the different ω-3 fatty acids is considered with different publications a protector against development of allergic disease. In this study we were able to show that fish oil supplementation is associated with histone acetylation and this was further correlated to allergic disease development later in life. The remaining question is how a fatty acid that is usually incorporated into cell membrane alters the epigenome of an individual causing the silencing or activation of certain genes.

3- It has been shown by many investigators that epigenetic modifications work not lonely but are interlinked with each other. Several studies showed interacted mechanisms between different epigenetic modifications. It is very important to have more detailed studies to shed more light on that prospective to understand more the complexity of the human genome.

At the end, in this study one small step toward a better understanding of our complexity has been made and how does a small part of the human epigenome work has been revealed.

5.1. Zusammenfassung

In dieser Doktorarbeit wurde die Chromatin Immunoprecipitation zur Anwendung in Kohortstudien etabliert und validiert. Die für die Kohortstudien verwendeten Proben haben in der Regel eine geringe Zellzahl, die routinemäßig eingefroren und für längeren Zeitraum gelagert werden.

Dieses Verfahren wurde mit verschiedenen bekannten Validierungsprotokollen validiert. Anschließend wurden mit Hilfe des Chromatin Immunoprecipitation drei verschiedene Kohorten an drei verschiedenen Perspektiven analysiert.

In der ersten Kohorte wurde die Wirkung des mütterlichen Folsäure-Status während der Schwangerschaft bei der Acetylierung von TH1, TH2, Treg und TH17 Gen Loci im Nabelschnurblut von CD4+ T-Zellen untersucht. Dazu wird der sehr niedrigen Folsäure-Status mit der sehr hohen Folsäurespiegeln im Blut der Mutter bezüglich der DNA-Methylierung und der Histon-Acetylierung verglichen und untersucht. GATA3 und IL9 wurden an beiden Histonen H3 und H4 in der hoch Folsäuregruppe im Vergleich zur niedrig Folat-Gruppe deutlich mehr acetyliert. Dies bedeutet, dass der Folsäure-Status das Epigenom in Richtung Th2-Phänotyp während der Schwangerschaft verschieben und die Entwicklung der allergischen Erkrankungen später im Leben unterstützen könnte.

Auf der anderen Seite, hat die Supplementierung mit Fischöl während der Schwangerschaft andere Wirkungen im Vergleich zu dieser aus Folat gezeigt. Im Gegensatz zur Placebo Gruppe wurde in der Fischöl Gruppe die Acetylierung am IL13 Locus sowohl auf H3 als auch auf H4-Histonen verringert. Parallel dazu, ist die Histon-Acetylierung auf H3-Histon am TBX21 Locus in der Fischöl Gruppe auch gesunken.

Dies erweist sich im Einklang mit den Protein und mRNA Daten aus den gleichen Probanden vor 11 Jahren (126). Diese Studie beweist nochmal dass die Entzündungsprozesse durch die im Fischöl enthaltenden ω-3 Fettsäuren zu dämpfen. Fettsäuren können in der Regel zu einer allgemeinen DNA-Hypermethylierung, einschließlich verschiedene inflammatorischen Zytokinen und Genen, durchführen (183). Ob Histonacetylierung durch die gleichen zuvor dargestellten Mechanismen reguliert wird, ist es noch enthüllen.

In gleicher Weise wurde ein Vergleich zwischen allergischem Asthmatiker und gesunden Kontrollen durchgeführt. Dieses Bild sieht komplexer aus. Es ist nicht nur ein einzelner Faktor sondern verschiedene Faktoren, die diese epigenetischen Modifikationen verursachen. Nicht alle Allergie / Asthma-Gene wurden in diesem Szenario beeinflusst. Es gab Gene, die anfällig sind. Unter diesen waren IL13 und FOXP3 (FOXP3 Daten sind mit freundlicher Genehmigung aus der Dissertation von Diana Raedler). Beide wurden an Histon H3 in asthmatischen Kindern im Vergleich zu gesunden Kontrollen deutlich mehr acetyliert. Darüber hinaus war die Acetylierung auf Histon H4 an der IL13 Locus auch in den asthmatischen Kindern im Vergleich zu ihren gesunden Kontrollen erhöht.

Die durch die Umweltfaktoren veränderten menschlichen Epigenom ist ein Hauptmechanismus der allergischen Krankheitsentwicklung.

Folsäure wird als Methyldonor betrachtet. Sie beeinflusst die DNA-Methylierung. In dieser Studie haben wir einen weiteren Effekt von Folsäure entdeckt. Es kann auch die Histonacetylierung als Schlüsselfaktor für den Antrieb des Th2 Phänotyps beeinflussen. Wie dies Methyldonor Folat die Histonacetylierung beeinflusst und verändert ist noch zu klären. Darüber hinaus wird in dieser Studie gezeigt, dass die Fischöl-Supplementierung mit der Histonacetylierung verbunden ist. Dies war mit den später im Leben entwickelten allergischen Krankheiten korreliert. Es bleibt nun die Frage zu klären, wie eine Fettsäure, die in der Regel in Zellmembranen eingebaut wurde, das Epigenom verändert.

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7. Verzeichnis der akademischen Lehrer

7.1. Amman Universität

Meine akademischen Lehrer waren die Damen und Herren Professoren bzw. Privat-Dozenten in Marburg:

Abbas, Al Bazaz, Al Aghbar, Atwan, Dawood, Gharibeh, Khalaf, Maraqa, Mursi, Oraikat, Othman, Shalaan, Shakia.

7.2. Philipps Marburg Universität

Meine akademischen Lehrer waren die Damen und Herren Professoren bzw. Privat-Dozenten in Marburg:

Daut, del Rey, Eilers, Garten, Jacob, Kirchner, Lill, Liss, Löffler, Lohoff, Moll, Müller, Petz, Renkawitz-Pohl, Renz, Röhm, Röper, Schäfer, Seitz, Schäfer, Weih