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PROGRAMA DE PÓS-GRADUAÇÃO EM SAÚDE DA CRIANÇA E DO  
ADOLESCENTE

DOUGLAS DA SILVA LIMA

**ANÁLISE DO PERFIL DE METILAÇÃO DO GENE *SMAD3* ASSOCIADO À ASMA  
EM CRIANÇAS**

Porto Alegre

2023

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A apresentação desta dissertação é requisito parcial para título de mestre do Programa de Pós-Graduação em Saúde da Criança e do Adolescente, da Universidade Federal do Rio Grande do Sul.

Orientador: Prof. Dr. Marcelo Zubaran Goldani

Coorientadora: Prof.<sup>a</sup> Dr<sup>a</sup> Mariana Bohns Michalowski

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## RESUMO

**Introdução:** O padrão de saúde dos indivíduos pode ser influenciado por eventos epigenéticos no período gestacional, e seus efeitos se manifestam durante todo o ciclo vital. Esses eventos se associam a manifestação de sintomas respiratórios nos primeiros anos de vida, bem como fatores de risco ambientais, genéticos e comportamentais podem também influenciar a função pulmonar em crianças. A epigenética caracteriza-se por eventos genéticos que podem ser transmitidos, reversíveis e diretamente influenciados pelo ambiente, mas sem causar alteração na sequência nucleotídica do DNA. Estes eventos podem estar associados ao desenvolvimento da asma, sendo que alguns estudos têm relatado uma associação entre sibilância e asma com mudanças no perfil de metilação do DNA. A hipermetilação da região promotora do gene *SMAD3* está associada à asma e pode ser influenciada por fatores ambientais. **Objetivo:** Analisar o perfil de metilação da região promotora do gene *SMAD3* em crianças oriundas de diferentes ambientes intra uterinos e sua associação com dados clínicos obtidos através de espirometria e pelo questionário ISAAC (International Study of Asthma and Allergy in Childhood). **Métodos:** Trata-se de um estudo longitudinal, utilizando amostras de conveniência. Os pacientes incluídos na pesquisa foram divididos em três grupos: filhos de mães tabagistas (TBC) (n=10), crianças pequenas para idade gestacional devido à restrição de crescimento intrauterino idiopática (RCIU) (n=06) e um grupo controle (CTR) (n=10). Para avaliação do perfil de metilação foi realizada extração do DNA das amostras de células epiteliais da mucosa oral ao nascimento e na idade pré-escolar. As amostras foram avaliadas por PCR em tempo real (RT-PCR) através da metodologia de HRM (*High Resolution Melting*). **Resultados:** O grupo TBC apresentou uma porcentagem média de metilação entre 2,5 e 50% e os grupos RCIU e CTR uma média entre 20 e 100% em todo o período. Houve uma significância estatística quanto a porcentagem de metilação entre os grupos TAB e RCIU ao nascer. Não houve diferença estatística entre os grupos pré-escolares. **Conclusões:** Não encontramos uma diferença significativa nos resultados da espirometria ou questionário ISAAC entre os grupos, mas observamos uma diferença significativa da metilação do gene *SMAD3* entre os grupos TBC e RCIU ao nascimento ( $p= 0.007$ ), entretanto essa diferença desapareceu na idade pré-escolar. O impacto desses resultados e sua relação com a evolução da saúde das crianças e a incidência de asma a longo prazo permanece por esclarecer.

**Palavras-chave:** Asma. Criança. Epigenética. Metilação de DNA.

## ABSTRACT

**Introduction:** The health pattern of individuals can be influenced by epigenetic events in the gestational period, and their effects are manifested throughout the life cycle. These events are associated with the manifestation of respiratory symptoms in the first years of life, as well as environmental, genetic and behavioral risk factors that can also influence lung function in children. Epigenetics is characterized by genetic events that can be transmitted, reversible and directly influenced by the environment, but without causing alteration in the nucleotide sequence of DNA. These events may be associated with the development of asthma, and some studies have reported an association between wheezing and asthma with changes in the DNA methylation profile. Hypermethylation of the promoter region of the SMAD3 gene is associated with asthma and may be influenced by environmental factors. **Objective:** To analyze the methylation profile of the promoter region of the SMAD3 gene in children from different intrauterine environments and its association with clinical data obtained through spirometry and the ISAAC questionnaire (International Study of Asthma and Allergy in Childhood). **Methods:** This is a longitudinal study using convenience samples. The patients included in the research were divided into three groups: children of smoking mothers (TBC) (n=10), children small for gestational age due to idiopathic intrauterine growth restriction (IUGR) (n=06) and a control group (CTR) (n=10). To evaluate the methylation profile, DNA was extracted from samples of epithelial cells from the oral mucosa at birth and at preschool age. The samples were evaluated by real-time PCR (RT-PCR) using the HRM (High Resolution Melting) methodology. **Results:** The TBC group had an average percentage of methylation between 2.5 and 50% and the IUGR and CTR groups an average between 20 and 100%. There was statistical significance regarding the percentage of methylation between the TBC and IUGR groups at birth. There was no statistical difference between preschool groups. **Conclusions:** We did not find a significant difference in the results of the spirometry or ISAAC questionnaire between the groups, but we observed a significant difference in the SMAD3 gene methylation between the TBC and IUGR groups at birth ( $p= 0.007$ ), however this difference disappeared in the pre-schoolers. The impact of these results and their relationship to the evolution of children's health and the long-term incidence of asthma remains to be clarified.

**Keywords:** Asthma. Child. Epigenetics. DNA Methylation.

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## LISTA DE ABREVIATURAS E SIGLAS

DNA	Ácido desoxiribonucleico
HRM	<i>High resolution melting</i>
TBC	Filhos de mães gestantes tabagistas
OMS	Organização Mundial da Saúde
RCIU	Restrição de crescimento intrauterino
PCR	Polimerase chain reaction
PIG	Pequeno para idade gestacional
SPSS	Statistical Package for the Social Sciences
SMAD3	SMAD family member 3
UTIP	Unidade de terapia intensiva pediátrica
CpG	Dinucleotídeos citosina-fosfatoguanina
CTR	Grupo controle
gDNA	DNA genômico
LPT	Laboratório de pediatria translacional
IVAPSA	Impacto das Variações do Ambiente Perinatal sobre a Saúde da Criança
DPOC	Doença pulmonar obstrutiva crônica

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## 1 INTRODUÇÃO

No mundo, a asma afeta mais de 300 milhões de pessoas anualmente (GINA, 2023). É uma das doenças crônicas mais prevalentes na infância (CARDOSO *et al.*, 2017). A gravidade dos sintomas tem sido associada com a frequência e intensidade dos episódios de sibilância (HALLIT *et al.*, 2018). O desenvolvimento de certas doenças pode estar relacionado a eventos ocorridos no início da vida (HORTA; VICTORA, 2013). Estudos demonstram que o ambiente intrauterino pode influenciar no crescimento e no desenvolvimento fetal e pós-natal, assim como no risco de desenvolver doenças comuns não transmissíveis na idade adulta (GODFREY *et al.*, 2013). A exposição da criança a um ambiente intrauterino favorável parece estar relacionada a uma maior tolerância aos diversos ambientes durante a vida e em suas repercussões na relação saúde-doença. Contudo, o desenvolvimento humano é consequência não só do ambiente, mas também de modificações epigenéticas (SILVEIRA *et al.*, 2007).

Segundo Cavalli e Heard (2019), epigenética é o estudo de moléculas e mecanismos que podem perpetuar estados alternativos de atividade gênica no contexto da mesma sequência de DNA. Essa perspectiva visa abranger: herança mitótica e persistência de atividade genética ou estados de cromatina por períodos de tempo, mesmo sem divisão celular (CAVALLI; HEARD, 2019).

Estudos têm demonstrado que a metilação de genes relacionados à resposta inflamatória, à remodelação das vias aéreas e ao metabolismo do tecido pulmonar está associada ao desenvolvimento e à gravidade de doenças pulmonares, como a asma e a doença pulmonar obstrutiva crônica (DPOC). Por exemplo, a hipermetilação de genes reguladores da resposta inflamatória tem sido observada em pacientes asmáticos e está associada a uma resposta inflamatória exacerbada nos pulmões (PACIFICO *et al.*, 2019; HAN *et al.*, 2020). Além disso, a metilação anormal de genes envolvidos no metabolismo do tecido pulmonar pode influenciar o equilíbrio oxidativo-nitrosativo e a resposta adaptativa às condições hipóxicas no pulmão (MURGIA *et al.*, 2017; TIGAN *et al.*, 2020). Essas alterações epigenéticas podem afetar a expressão gênica e contribuir para a progressão e a patogênese de doenças pulmonares.

O perfil de metilação de alguns genes estão associados à asma, dentre eles, destaca-se o gene *SMAD3* que atua na via de sinalização do fator de crescimento,

ajusta a atividade gênica e a proliferação celular e apresenta interações moleculares significativas com genes relacionados à asma (EDRIS *et al.*, 2019; PENG *et al.*, 2019). Além disso, atua no remodelamento das vias aéreas por meio de fibroblastos, regulando o processo de fibrose e a resposta imune (LUND *et al.*, 2018; QI; XU; KOPPELMAN, 2019; ZHANG *et al.*, 2021). A hipermetilação da região promotora desse gene está ligada a casos de asma infantil, principalmente em pacientes cujas mães também são asmáticas, sugerindo que a asma pode ser herdada em parte por esse mecanismo epigenético (DE VRIES *et al.*, 2017; REESE *et al.*, 2019).

## 2 REVISÃO DA LITERATURA

### 2.1 DOENÇAS RESPIRATÓRIAS

As doenças da infância são de etiologia multifatorial, sendo que crianças menores de cinco anos são especialmente suscetíveis a desenvolver complicações respiratórias. As infecções respiratórias são responsáveis pela maior parte de internações hospitalares nesse período (AUGUSTO; DOMINGOS, 2017).

Dentre as doenças respiratórias a asma é a doença crônica de maior prevalência na infância (FORNO; CELEDON, 2019). Atualmente, estima-se que mais de 300 milhões de pessoas no mundo sofram desta doença (GINA, 2023). A prevalência da asma varia de acordo com o país e região, com taxas mais altas em países desenvolvidos. A Organização Mundial da Saúde (OMS) calcula que a asma cause cerca de 250.000 mortes por ano (WHO, 2023). O aumento da prevalência de doenças alérgicas cresce em escala global. Nos Estados Unidos são gastos anualmente U\$18 bilhões no tratamento das manifestações alérgicas (PENG *et al.*, 2019). Estima-se que ocorram cerca de 2.500 mortes por asma anualmente no Brasil (Ministério da Saúde, 2023).

Na prática clínica, a asma é caracterizada por sibilância, falta de ar, aperto no peito e tosse, que podem variar ao longo do tempo em sua ocorrência e intensidade. Alguns sintomas são transitórios e tendem a desaparecer na idade escolar, outros sintomas persistentes devem permanecer até a idade adulta, incluindo remodelagem da unidade respiratória (THIBEAULT; LAPRISE, 2019). No entanto, o diagnóstico de asma não pode se basear em apenas uma característica ou um episódio. Aproximadamente 30% das crianças terá pelo menos um episódio de sibilância durante a vida e crianças com asma tendem a ter uma diminuição da função pulmonar. Se as manifestações da doença não forem tratadas corretamente, pode ocorrer um declínio da função pulmonar na fase adulta (McGEACHIE *et al.*, 2016). O início e a progressão clínica estão fortemente ligados às exposições ambientais e à suscetibilidade genética (FIGURA 1) (POPOVIC *et al.*, 2019).



FIGURA 1 - Principais fatores epigenéticos que compõem fatores de risco para asma.  
Fonte: HERNANDEZ-PACHECO; KERE; MELÉN, 2022.

Mesmo que o diagnóstico de asma seja categoricamente dado apenas da idade escolar em diante, o processo de doença inicia-se antes com episódios de sibilância que começam na primeira infância. A sibilância na primeira infância, especialmente quando acompanhada de sensibilização alérgica e outras condições atópicas tem demonstrado ser um forte preditor para asma (POPOVIC *et al.*, 2019). O histórico de alergia na família sugere uma forte relação com a base genética. No entanto, a relação entre suscetibilidade à doença alérgica e a gravidade da asma ainda não está bem estabelecida (PENG *et al.*, 2019).

## 2.2 AMBIENTES INTRA UTERINOS

A influência de determinados fatores ambientais no início da vida pode estar relacionada com alterações no desenvolvimento (SILVEIRA *et al.*, 2007). Manifestações de sintomas respiratórios nos primeiros anos de vida, bem como fatores de risco ambientais, genéticos e comportamentais, podem influenciar na função pulmonar em crianças (POPOVIC *et al.*, 2019). Algumas mudanças epigenéticas iniciam no período intrauterino e podem variar ao longo da vida, sendo que, em alguns casos, essa informação é passada de uma geração para a outra (DEVRIES *et al.*, 2017; EDRIS *et al.*, 2019).

A exposição intra-uterina à fumaça do tabaco é um fator de risco à saúde do feto e pode induzir a problemas durante a gravidez, como malformações da placenta e prematuridade, também podendo acarretar em problemas de longo prazo para a criança, sendo que o tabagismo passivo pode ser responsável por anormalidades morfológicas pulmonares (hipoplasia pulmonar, redução da elasticidade, aumento da deposição de colágeno e alteração da estrutura alveolar) e distúrbios funcionais (complacência reduzida, aumento da resistência das vias aéreas, hiper-reatividade brônquica) (BOSDURE; DUBUS, 2006). A exposição materna à nicotina do tabaco é conhecida por induzir vasoconstrição na vasculatura placentária, além de promover taquicardia na mãe e no feto (LEOPÉRCIO; GIGLIOTTI, 2004; MACHAALANI *et al.*, 2014). Dessa forma, a exposição à nicotina durante a gravidez continua sendo um problema de saúde pública generalizado, impactando nas saúdes fetal e pós-natal (WONG *et al.*, 2015). O tabagismo durante a gestação provoca alterações na função imune do feto e alterações na função pulmonar no período neonatal, estando dentre os principais determinantes gestacionais com impacto direto na função pulmonar de lactentes e escolares (FRIEDRICH *et al.*, 2007).

Recém-nascidos com restrição de crescimento intrauterino (RCIU) idiopático representam um grupo importante para estudo no qual se observa um crescimento mais rápido (“catch up”) no período pós-natal, uma condição associada a complicações metabólicas e vasculares a longo prazo (RENZ, 2019). As alterações desta condição podem levar a modificações na utilização da glicose fetal e da homeostase da insulina, alterando mecanismos metabólicos essenciais para os desfechos em saúde e doenças pós-natais a curto e longo prazo (DEVASKAR; CHU, 2016). A restrição do crescimento pode forçar adaptações do corpo, como calibre menor das vias aéreas, levando a menor função pulmonar e, por consequência, aumentando o risco de asma (DEKKER *et al.*, 2019). Outro aspecto relevante descrito por Barker e colaboradores foi a ligação entre baixo peso ao nascer e depleção da função pulmonar na idade adulta (BARKER *et al.*, 1991; BARKER *et al.*, 2002). Os órgãos apresentam uma capacidade limitada de se recuperar de efeitos deletérios durante o desenvolvimento intrauterino, que podem persistir e afetar a saúde durante a vida adulta e, dessa forma, o pulmão pode ser prejudicado por tais condições durante o desenvolvimento fetal resultando em alterações na estrutura pulmonar e comprometendo a função respiratória durante a vida pós-natal (BRIANA; MALAMITSI-PUSCHNER, 2013).

### 2.3 EPIGENÉTICA E METILAÇÃO DO DNA

A epigenética é o estudo de moléculas e mecanismos que podem perpetuar estados alternativos de atividade gênica, no contexto de uma mesma sequência de DNA (CAVALLI; HEARD, 2019), os fatores epigenéticos podem influenciar nos desfechos de saúde e doença e sugerem inclusive características transgeracionais (HERNANDEZ-PACHECO; KERE; MELÉN, 2022). Como os aspectos ambientais e de desenvolvimento têm impacto na regulação epigenética, esse mecanismo tem recebido atenção especial recentemente na etiopatogenia da asma. (FORNO et al., 2017; EDRIS et al., 2019).

As alterações podem ocorrer através da metilação do DNA, modificações pós-traducionais de histonas e de RNAs não codificantes (BIRD, 2002), sendo de extrema importância para o controle da expressão gênica, *imprinting* genômico, inativação do cromossomo X e também especificação das células (HUANG et al., 2015). A metilação do DNA é, provavelmente, a mais extensamente estudada e se refere a adição de um grupo metil na posição C5 da citosina para formar 5-metilcitosina e é encontrado predominantemente nos sítios dinucleotídeos citosina-fosfatoguanina (CpG) (FIGURA 2) (FORNO; CELEDON, 2019; POPOVIC et al., 2019).

*As ilhas CpG são encontradas frequentemente nas regiões 5' regulatórias dos genes e aproximadamente 60% dos promotores dos genes estão nessas ilhas (GAL-YAM et al., 2008). Apesar da maior parte dos sítios CpG no genoma estarem metilados, cerca de 90% das ilhas permanecem desmetiladas durante o desenvolvimento e em diferentes tecidos (GAL-YAM et al., 2008; SHARMA et al., 2010). Entretanto, algumas ilhas CpGs de regiões promotoras tornam-se metiladas durante o desenvolvimento, o que resulta em silenciamento transcricional a longo prazo (GAL-YAM et al., 2008). Alguns estudos descrevem que a hipermetilação de regiões promotoras causa silenciamento do gene, enquanto a hipometilação intragênica leva à ativação do gene (HON et al., 2012).*



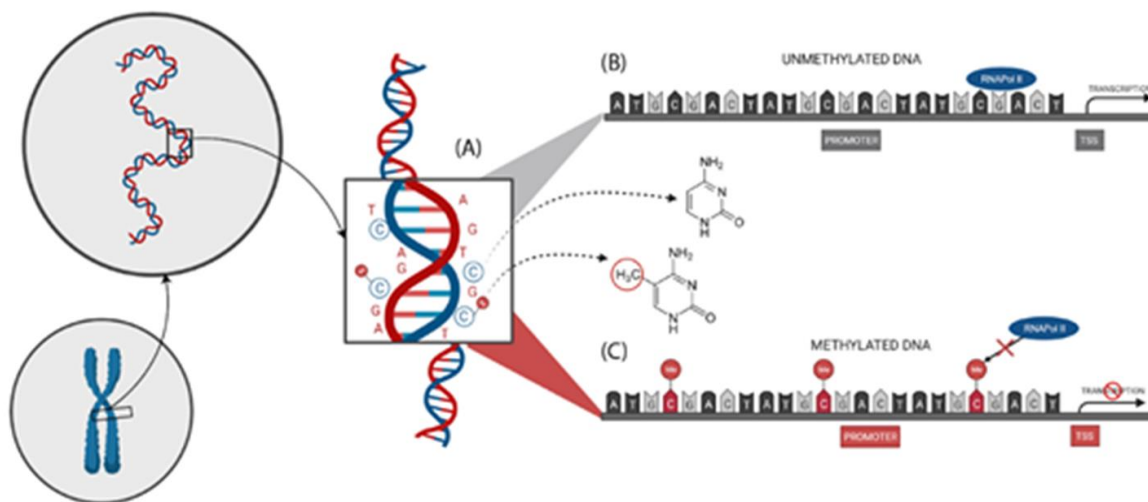


FIGURA 2 - (A) Adição do grupo metil ao carbono 5 da citosina na citosina-fosfato-guanina (CpG); (B) Nenhuma metilação CpG na região promotora do gene leva à transcrição ativa; (C) A metilação na região promotora dos genes leva à repressão transcricional; Nota: TSS: local de início da transcrição; RNA pol II: RNA polimerase II.

Fonte: De autoria própria

## 2.4 ASMA E METILAÇÃO DO GENE *SMAD3*

PENG e colaboradores (2019) observaram uma tendência longitudinal temporal de sensibilização alérgica, reforçando a ideia de que o perfil de metilação ao nascer pode ser um marcador de vulnerabilidade alérgica durante a infância (PENG *et al.*, 2019). Alguns estudos relatam uma associação entre sibilância e asma com mudanças na metilação de DNA (DEVRIES *et al.* 2017; POPOVIC *et al.*, 2019), reforçando a perspectiva de que fatores epigenéticos podem estar associados ao desenvolvimento desta patologia (PENG *et al.*, 2019).

Mudanças no perfil de metilação do gene *SMAD3* têm sido associadas a sibilância e asma. *SMAD3* (SMAD Family Member 3) é um gene codificador de proteínas localizado no cromossomo 15 (loci 15q 22.33) e é composto por 6.464 pares de bases. Desempenha um papel importante na regulação da resposta imune e atua em conjunto com outras proteínas para promover a regulação da fibrose pulmonar (DEVRIES *et al.*, 2018; ZHANG *et al.*, 2018). Ele está envolvido na via de sinalização

do fator de crescimento, regulando a atividade gênica, proliferação celular e na remodelação das vias aéreas através dos fibroblastos, ativando a produção de colágeno tipo 1 (Figura 3) (PENG *et al.*, 2019; ZHANG *et al.*, 2021). A hipermetilação da região promotora deste gene está associada à asma, particularmente em filhos de mães asmáticas (DEVRIES *et al.*, 2017). De acordo com DeVries e colaboradores (2017), crianças de mães asmáticas apresentaram níveis de metilação elevados na região promotora do gene *SMAD3* ao nascimento.

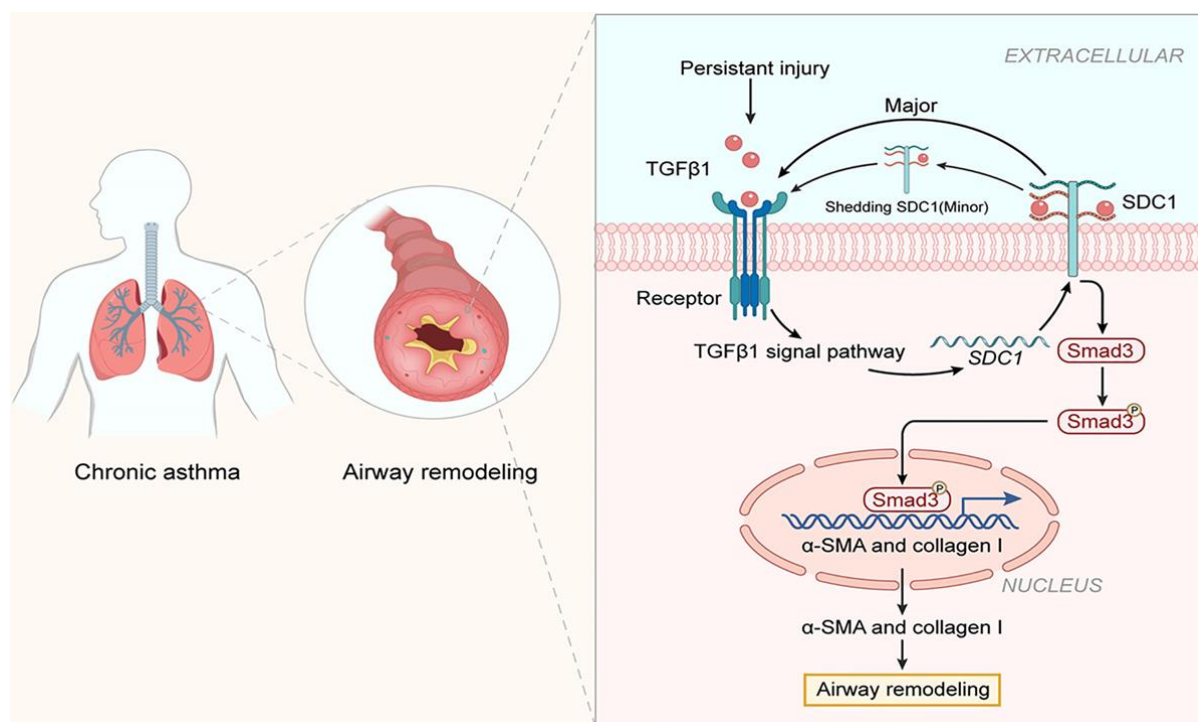


FIGURA 3 - Mecanismo onde o gene *SMAD3* atua na remodelagem das vias aéreas em pacientes asmáticos.

Fonte: ZHANG *et al.*, 2021

A metilação da região promotora do gene *SMAD3* foi identificada relacionada à asma atópica induzida por rinovírus em crianças, onde este gene também está envolvido na regulação da resposta imune (LUND *et al.*, 2018). O mesmo gene apresentou maior número de interações moleculares com outros genes ligados ao desenvolvimento da asma (DEVRIES *et al.*, 2017). Sugere-se que pode ser possível detectar uma tendência à asma infantil ou adulta no início da vida através do padrão epigenético

quando associado com fatores de imunidade e vias pró-inflamatórias (SHEIKPOUR *et al.*, 2021).

Não há na literatura estudos que tenham analisado especificamente a metilação do DNA de genes relacionados à asma em uma abordagem longitudinal (desde o nascimento até a idade pré-escolar). Assim, o objetivo deste estudo foi analisar e comparar o perfil de metilação da região promotora do gene *SMAD3* em crianças expostas a três diferentes ambientes intrauterinos, em dois momentos distintos de seu desenvolvimento.

### 3 JUSTIFICATIVA

A determinação dos níveis de metilação em filhos com restrição de crescimento intrauterino (RCIU) e filhos de mães tabagistas é um universo pouco explorado. Muitas perguntas ainda estão em aberto, tais como a investigação das mudanças do perfil de metilação entre estes grupos e se existe alguma alteração epigenética pós-neonatal que pode regular esta alteração.

Estudos com essa temática permitem conhecer estas mudanças, além de fornecerem base para melhor compreender as possíveis mudanças e fatores de risco, possibilitando relacionar tais dados com o perfil de saúde do indivíduo.

Este estudo considera que os efeitos do ambiente podem modificar aspectos epigenéticos do DNA e que a asma pode estar relacionada com a variação da metilação de determinados genes. Não há na literatura nenhum estudo que avalie especificamente a metilação da região promotora do gene *SMAD3* relacionado à asma de maneira longitudinal.

#### **4 HIPÓTESE**

Fatores ambientais durante a gestação podem oferecer padrões de metilação peculiares de genes favorecendo o surgimento de asma durante a primeira infância.

## 5 OBJETIVO

### 5.1 OBJETIVO GERAL

Analisar a variação do perfil de metilação da região promotora do gene *SMAD3* em crianças em dois momentos distintos: ao nascer e em idade pré-escolar.

### 5.2 OBJETIVOS ESPECÍFICOS

- a) Comparar a porcentagem de metilação do gene associado à asma entre os grupos tabaco, restrição de crescimento intrauterino e grupo controle em dois momentos distintos: ao nascer e em idade pré-escolar;
- b) Associar o perfil de metilação do gene *SMAD3* com as variáveis espirometria e questionário ISAAC, na idade pré-escolar.

## 6 METODOLOGIA

### 6.1 POPULAÇÃO DE ESTUDO

Vinte e seis crianças da coorte IVAPSA foram incluídas e avaliadas ao nascer e na idade pré-escolar até os 5 anos de idade (RIBAS WERLANG *et al.*, 2019), sendo: dez filhos de gestantes fumantes (TBC), seis crianças com restrição de crescimento intrauterino idiopático (RCIU), e dez crianças em um grupo controle de (CTR).

### 6.2 COLETA E PROCESSAMENTO DE DADOS

#### 6.2.1 Análise de In Sílico

Todo o processo de projeção dos primers para a PCR específico para metilação (MSP) foi dividido em cinco etapas: i) Seleção da sequência do gene no banco de dados (<https://www.ncbi.nlm.nih.gov/gene/> ii) identificação do região promotora através do EPD - Eukaryotic Promoter Database ([epd.epfl.ch/index.php](http://epd.epfl.ch/index.php)); iii) identificação de possíveis sítios de ligação de fator de transcrição em sequências de DNA na região promotora através do ALGGEN PROMO (<http://alggen.lsi.upc.es/cgi-bin/promo>) iv) identificação das ilhas CpG através do software Methyl Primer express , v) projeção do primer para região metilada e não metilada através do site Primer3 (<https://primer3.ut.ee/>).

#### 6.2.2 Coleta de células da mucosa oral e extração de DNA

O DNA genômico das crianças (gDNA) foi obtido a partir de células epiteliais da mucosa, coletadas com auxílio de swabs estéreis. A extração do DNA genômico foi realizada utilizando um protocolo previamente estabelecido (GARBIERI *et al.*, 2017) quantificado no NanoDrop® (Thermo Fisher Scientific, Massachusetts, EUA) e armazenado em freezer a - 20°C. As análises moleculares foram realizadas no Laboratório de Pediatria Translacional (LPT), localizado no HCPA.

### 6.2.3 Tratamento do DNA com bissulfito de sódio

O DNA (uma quantidade de 400 ng) foi tratado com o kit EZ DNA Methylation® seguindo as recomendações do fabricante. Nesta etapa ocorre inicialmente a sulfonação da citosina (adição de grupamento sulfito), resultando numa citosina sulfonada que sofre desaminação dando origem à uracila sulfonada. No último passo, ocorre a dessulfonação alcalina da uracila sulfonada, resultando em uracila. Quando a citosina está metilada a sulfonação é impedida, não ocorrendo a transformação de citosina em uracila. De uma forma geral, o tratamento de bissulfito converte citosina não metilada em uracila e não promove alterações na citosina metilada.

### 6.2.4 Análise da metilação por HRM (High Resolution Melting)

A análise do perfil de metilação foi realizada pelo método High Resolution Melting (HRM) no equipamento StepOne Real-Time PCR System (Applied Biosystems®). As sequências de *primers* utilizadas são descritas na tabela 1. Como controles metilado e não metilado, foi usado o kit de controle Cells-to-CpG gDNA Control Kit Zymo Research®.

TABELA 1 - Sequências de primers: metilados (M) e não metilados (U) da região promotora do gene *SMAD3*.

Target gene	Orientation	Primer sequence *	Amplification Product	CpG's inside Amplicon
<b><i>SMAD3</i> - M</b>	Forward	5'-ATATCGGTTAGTCGGTTGC--3'	138	10
	Reverse	5'-AAACTCCGCGACTTTTCTC-3'		
<b><i>SMAD3</i> - U</b>	Forward	5'-TGGTTAGTTGGTTGTGGGAG-3'	138	—
	Reverse	5'-AACTCCACAACCTTTTCTCCC-3'		

(\*) Fonte: De autoria própria



Uma curva padrão com uma razão de metilação conhecida nas proporções de 0%, 2,5%, 20%, 50% e 100% foi utilizada em cada ensaio. A PCR foi preparada com um volume final de 10 $\mu$ L, contendo 5 $\mu$ L de MeltDoctor (Applied Biosystems), 1  $\mu$ l de primer a 10 nmol e 1 $\mu$ L ( 10 ng/ $\mu$ L) de amostras de DNA tratado com bissulfito. As condições de reação da PCR seguirão os seguintes parâmetros: 95°C por 10 min, 40 ciclos a 95°C por 15 segundos e 60°C por 1 minuto, seguido por uma etapa HRM de 95°C por 10s, 60°C por 1 minuto, 95°C por 15s e 60°C por 15s, com uma taxa de incremento de 0,2°C para aquisição de dados.

Os pontos da curva abaixo de 20% foram considerados não metilados e os pontos acima desse valor foram considerados metilados.

### 6.3 FUNÇÃO PULMONAR

Foi utilizado o questionário ISAAC (International Study of Asthma and Allergies in Childhood) (Apêndice 1) e foram aplicados pontos de corte para asma, rinite e eczema (ASHER *et al.*, 1995; SOLÉ *et al.*, 1998, VANNA *et al.*, 2001; YAMADA *et al.*, 2002). A função pulmonar foi avaliada por espirometria apenas em crianças pré-escolares (WINCK *et al.*, 2016). O índice de Tiffeneau-Pinelli foi considerado para risco aumentado para asma (crianças que apresentaram padrão obstrutivo) nos resultados dos dados de espirometria (BEEKMAN *et al.*, 2014).

### 6.4 AMOSTRA

#### 6.4.1 Critérios de inclusão

Amostras de DNA genômico (com quantidade viável de DNA para o estudo) coletadas de crianças participantes das fases 1 e 2 do projeto IVAPSA.

#### 6.4.2 Critérios de exclusão

Foram excluídas as amostras de DNA com qualidade inadequada para realização do perfil de metilação da região promotora do gene *SMAD3*.

## 6.5 ANÁLISES ESTATÍSTICAS

As variáveis categóricas foram descritas por frequências absolutas e relativas. As variáveis contínuas foram expressas como média e desvio padrão ou mediana e intervalo interquartilico. O teste de Kruskal-Wallis foi utilizado para comparar os grupos seguido do teste de Dunn para comparações múltiplas em variáveis não paramétricas. A correlação entre a porcentagem de metilação e os resultados da espirometria foi avaliada por meio do coeficiente de correlação de postos de Spearman.

Todas as análises estatísticas foram realizadas com o pacote estatístico SPSS v.29 para Windows (SPSS Inc., Chicago, IL, EUA). Os resultados foram estatisticamente significativos quando o valor de  $p < 0,05$ .

## 6.6 CONSIDERAÇÕES ÉTICAS

Este estudo foi aprovado pelo Comitê de Ética do Hospital de Clínicas de Porto Alegre (CEP-HCPA) sob o número 2019-0650.

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## 7 RESULTADOS

### 7.1 ARTIGO DE REVISÃO

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#### Title

**Epigenetics, hypersensibility and asthma: what do we know so far?**

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### Running head and abbreviations

**Epigenetics, hypersensibility and asthma:** DNAm (DNA methylation), DOHaD (Developmental Origins of Health and Disease); CpG (cytosine-phosphate-guanine); EWAS (epigenome-wide association study); ALSPAC (Avon Longitudinal Study of Parents and Children; TSS (transcription start site); RNA pol II (RNA polymerase II); AECs (airway epithelial cells); IL5RA (interleukin 5 receptor subunit alpha); *EPX* (eosinophil peroxidase); *EVL* (Enah/Vasp-like); *SMAD3* (SMAD family member3); *ADAM33* (ADAM metallopeptidase domain33).

**Abstract**

In this review, we describe recent advances in understanding the relationship between epigenetic changes, especially DNA methylation (DNAm), with hypersensitivity and respiratory disorders such as asthma in childhood. It is clearly described that epigenetic mechanisms can induce short to long-term changes in cells, tissues and organs. Through the growing number of studies on the Origins of Health Development and Diseases, more and more data exist on how environmental and genomic aspects in early life can induce allergy and asthma. The lack of biomarkers, standardised assays and access to more accessible tools for data collection and analysis are still a challenge for future studies. Through this review, we draw a panorama with the available information that can assist in the establishment of an epigenetic approach for the risk analysis of these pathologies.

**Keywords:** Asthma. Children. DNA methylation. Epigenetics.

## Introduction

According to Cavalli and Heard (2019), epigenetic means "*The study of molecules and mechanisms that can perpetuate alternative states of gene activity in the context of the same DNA sequence*". This perspective aims to cover: transgenerational inheritance, mitotic inheritance and persistence of genetic activity or chromatin states alterations for periods of time, even without cell division. It can also be related to environmental exposures leading to structural damage or functional changes in cells, tissues and organs <sup>2-4</sup>. In this context, the epigenome represents the interface between gene and environment and provides a particular opportunity to recognize this intersection of causal components <sup>5</sup>.

The relationship between epigenetic changes and their outcomes was first described by Conrad Waddington, in the 1940s, introducing the theory of "Epigenetic Landscape" <sup>1</sup>. From there, numerous studies were developed and recent researchers were able to design how a single-cell unit would become in a complex of differentiated cells, with specific functions, expressing specific sets of genes as a result of cellular identity mechanisms. Further, we are able to understand how phenotype becomes an aftermath of genotype and its interplay with the environment <sup>2</sup>.

The trajectory of Developmental Origins of Health and Disease (DOHaD) studies suggest a mesh with those causal components and epigenetic variants, considering that the in-utero and post-natal life can induce biological changes leading to distinct outcomes <sup>4</sup>.

## Epigenetics and DNA methylation

The importance of epigenetic events is based on its long-lasting effects, starting from early life (gestation) until adulthood <sup>6</sup>. Besides, the epigenetic regulation is highly specific, may induce short and long-term changes in gene expression and may be passed on from one generation to another <sup>7</sup>. For this reason, they provide a baseline of the environmental influence on gene expression and disease hazard <sup>8</sup>.

There are three main biomarkers that play crucial roles in understanding the importance of epigenetics aspects: DNA methylation, alterations of chromatin states and action of non-coding RNAs <sup>12</sup>. Among possible epigenetic biomarkers, DNA methylation (DNAm) is likely the most prominent and frequent approach <sup>9</sup>. DNAm consists of the addition of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine, and is predominantly found at cytosine-phosphateguanine (CpG) dinucleotide sites <sup>5,10</sup>. Initially, DNAm was proposed as the only carrier of epigenetic information, but later it was accepted that chromatin, proteins and non-coding RNAs also act in this process. Likewise, variations in histones can influence the local structure of chromatin, directly or indirectly. Those variations can be inheritable, but reversible and may vary in different parts of the genome at different stages of the life cycle. Recent studies of cell reprogramming have shown that DNAm and chromatin can also behave as epigenetic barriers, preventing alterations in gene expression and cell identity. Nevertheless, chromatin states may impact transcriptional factors binding and DNA sequence polymorphism, targeting the balance of genomic mutability and stability <sup>2,4</sup>. Altogether, those mechanisms compound epigenetic regulation that controls several processes, like cellular response to endogenous and exogenous stimuli, leading to healthy conditions or diseases (FIGURE 1) <sup>6</sup>. As an example, cell differentiation in embryogenesis is triggered by DNAm <sup>1</sup>.

Human health is influenced by the exposure to the environment. In this perspective, the in-utero life must be imperatively considered, once exposures through this period may disturb the immune system, lung growth and respiratory performance later in life <sup>6</sup>. It has been shown that extrinsic factors during the perinatal period, such as adverse environment and maternal depression may impact on the DNAm of the offspring and future outcomes inducing physical and psychiatric diseases. Otherwise, a good standard of *nurturing may lead to healthier outcomes, inducing a “reshape”* effect of DNAm during lifetime <sup>4,10</sup>. It is also important to highlight that genetic variants can explain how environmental factors impact on epigenetic modifications <sup>3</sup>. In this scenario, environmental exposures are likely to contribute to the increase in allergic diseases<sup>11</sup>.

### **Epigenetic paths for hypersensitization**

Allergic diseases are increasing on a global scale. In the United States, US\$ 18 billion are spent annually on their treatment <sup>12</sup>. As previously mentioned, the early years of life play a decisive role for atopic diseases <sup>9</sup>.

Methylation patterns and gene expression changes are well connected to persistent atopic asthma in respiratory epithelia of American children and some genes are significantly correlated to immune response <sup>13</sup>. In a recent trial by Popovic *et al*, 2019, a case-control study nested in a birth cohort, with 136 children between 6 and 18 months old, to figure the association of early childhood wheezing and DNAm, has observed a longitudinal temporal tendency towards allergic sensitization, reinforcing the idea that the methylation profile at birth can be a marker of allergic vulnerability during childhood <sup>4</sup>. DNAm is known to be associated with asthma, which can be

triggered by smoking, air pollution, indoor contaminants and allergens. Passive tobacco exposure decreases lung function and increases the risk of respiratory diseases. Also, prenatal tobacco smoking exposure increases the risk for the development of asthma on the neonate <sup>15</sup>. In a similar manner, air pollutants or allergens can go through the airway epithelial barriers and reshape immune responses <sup>14</sup>. Likewise, air pollution as well as viral infections and intestinal and pulmonary dysbiosis, can induce sensitization, exacerbations or trigger the onset of asthma <sup>6</sup>.

In 2018, Yang *et al.* used nasal epithelium samples from African-American children with allergic asthma to compare methylation profiles of genomic DNA. They were able to identify 186 genes with methylation changes, including genes related to atopy, asthma, immunity, airflow obstruction and epigenetic regulation. By showing that the epigenetic marks on the respiratory epithelium are related to allergic asthma, this study provided a basis for studies of nasal DNA in larger populations <sup>16</sup>. In another study, DeVries *et al.* suggest that childhood asthma engages epigenetic alterations in immunoregulatory and pro-inflammatory pathways <sup>17</sup>. Thus, asthma has an epigenetic regulatory mechanism influenced by genetic variability and environmental exposures, that can be used as a strong biomarker <sup>12</sup>.

An important issue in these studies is the origin of the tissue being analysed. Most allergic biomarkers to date were extracted from whole blood DNA. Also, cord blood immune cells were adopted to establish (in an effort to predict) DNAm signatures related to allergy and asthma <sup>17</sup>. Although manifested at birth, these epigenetic signatures are still able to change, determined by physiological pathways. It means that some gene alterations might be transient or disease related. Therefore, DNAm mechanisms may present some malleability during lifespan. Besides, most indicators

of allergic disease and asthma are related to epigenetic ageing of cells collected from nasal epithelia <sup>5</sup>. Data on the differences and changes in the methylation profiles of these different tissues and their implication in hypersensitivity reactions are still very few.

### **Asthma: an epigenetic outcome**

Asthma is one of the most common pulmonary chronic diseases. It may start early in life and, if not handled properly, tends to cause loss of pulmonary function in adult life <sup>18</sup>. It is characterised by reversible airflow obstruction and airway inflammation with symptoms such as wheezing, shortness of breath, cough and chest tightness, that may vary over time and frequency, intermittent or persistently. Some of them are transient and tend to disappear at school-age. Other persistent symptoms are up to remain until adulthood <sup>11</sup>. The onset and clinical progression are strongly connected to environmental exposures and genetic susceptibility <sup>5,8</sup>. Environmental and genomic aspects as well as aberrant immune maturation early in life may engage the disease outbreak <sup>23</sup>.

In the past decade, the increase of morbidity and mortality related to respiratory diseases has reached the spotlights in several countries <sup>13</sup>. Epidemiological evidence has shown that approximately 30% of children will experience at least one episode of wheezing in their lifetime <sup>3</sup>. Worldwide, asthma is frequently diagnosed during pre-scholar years and affects more than 300 million people <sup>19</sup>. The estimated heritability of the disease in certain studies starts from 40 to 60% <sup>5,8</sup> and can reach as high as 80% <sup>20</sup>. However, the timeline and mechanisms of asthma onset are not well described yet



To keep homeostasis on human lungs, a healthy adaptation to the environment is needed and the lung epithelia must present a competent mucosal immunity. Respiratory system provides mucociliary clearance and a barrier against pathogens and other particles that may affect the alveolar gas exchange. An unbalanced immune system or deficient epithelial barrier can therefore result in respiratory illness, as the respiratory system remains in unprotected contact with exogenous factors. <sup>5,15</sup>

Protection factors are also described. Some exposures can promote healthier airway development even in the presence of a genetic predisposition to the disease. Recent studies propose that the use of fish oil and vitamin D throughout pregnancy may minimise the risk of early childhood wheeze. Also, children from a farm environment that are frequently exposed to allergens, bacteria, fungi and others from diverse microbiomes and the consumption of unprocessed cow milk may develop a strong protective barrier against asthma and allergy. Indeed, children from rural areas have shown a prevalence of allergic diseases lower than children from urban environments <sup>6,23</sup>.

Asthma management is based on the severity and risk profile of these children. Many recent studies have evaluated the relationship of genetic polymorphisms as potential risk factors for the onset and severity of asthma symptoms <sup>7,11,23</sup>. Even with the advance of asthma treatment, there is still a group of patients that faces a low response to traditional approaches. From this, a better understanding of asthma and allergy mechanisms through the observation of DNAm and gene expression may provide a clue about reliable biomarkers associated with therapeutic responses in this population. <sup>13</sup>.

There is a strong connection between asthma and DNAm<sup>4,5,26</sup> (Table 1). In 2019, Reese *et al.* conducted a study with newborns and school-age children. They evaluated the entire epigenome by observing the presence of CpG methylation in the blood in these two age groups both in prospective and cross-sectional analyses. In 8 cohorts of newborns (668 cases), 9 CpGs islands and 35 regions were differentially methylated in patients who developed asthma, while in older children, in a cross-sectional analysis, 179 CpGs islands and 36 differentially methylated regions were identified in these cases.

Another interesting fact is that DNAm is tissue specific. In the case of diseases whose local aspects are pronounced, such as asthma, the selection of tissues to carry out the DNA methylation profile can be an important variable in the analysis<sup>4</sup>. DNA methylation of the nasal epithelium may offer more information about changes in the airways, once whole blood DNA does not perform a trustworthy complete agreement for pulmonary sample studies<sup>27</sup>. Cardenas *et al.* (2019) conducted an epigenome-wide association study (EWAS) with nasal samples of 547 children to analyse current asthma, allergic sensitization and rhinitis, fractional exhaled nitric oxide (FeNO) and *the lung function, where diverse differentially methylated CpG's and regions were found related to these diseases, suggesting that the nasal cellular epigenome may be a good biomarker to airway inflammation in children. Also, in peripheral blood samples it is crucial to consider confounding factors, such as leukocytes and eosinophils, that may lead to biased results*<sup>3,5</sup>.

Some data contradict the tissue specificity theory in the analysis of patients with asthma. A recent study by Vieira Braga *et al* (2019) was designed to find differences between asthmatic and healthy patients. A wide overlapping of the cellular epigenetic

profile of the different locations of the respiratory tree was observed. In fact, a diversity of immune cells was found in different parts of the respiratory unit. Therefore, it could be observed that, in asthmatic patients, airway wall remodelling was highly correlated with the presence of a chronic inflammatory response, which may cause a cell-to-cell signalling network. <sup>22</sup>.

Also, variations on innate and adaptive immune responses appear to precede the diagnosis of asthma in children. This group of respiratory and immune modifications may characterise a window of susceptibility toward the disease and can contribute to asthma pathogenesis. The disease trajectory can also be affected by post-translational histones modification that influences genetic regulation and its possible outcomes <sup>26</sup>.

## **Genes in sight**

Several genes whose altered DNA methylation has been described in asthmatic individuals have different pathophysiological aspects and relationships with components and functions of the immune system <sup>29</sup>. Some of them deserve special attention (Table 1).

### *IL5RA*

The *IL5RA* gene (interleukin 5 receptor alpha subunit) is found on the human chromosome 3. Hypomethylation of the *IL5RA* gene in airway epithelial cells (AECs) and eosinophils has been shown to be a potential therapeutic target for asthma. In an EWAS, Arathimos *et al.* (2017) used data from the Avon Longitudinal Study of Parents and Children (ALSPAC), with more than 1,500 patients, identifying 302 CpGs related

to asthma and 405 to wheezing, with a robust overlap between them <sup>3</sup>. After adjusting for cell count, there was a difference of 2.49 [95% CI - 1.56, - 3.43] percent of methylation in patients with asthma. The *IL5RA* gene also showed an important link with the pathogenesis of asthma in multi-omics approach <sup>20</sup>. The hypomethylation of *IL5RA* gene on airway epithelial cells and eosinophils has shown to be a therapeutic target for asthma. The epigenetic finding allowed the study and incorporation of the monoclonal antibody Benralizumab <sup>20</sup> (Anti-IL-5Ra) in the clinical practice, a drug that binds to *IL5RA*, inducing a reduction in exacerbations and improvement in lung function in patients with severe asthma <sup>5,6,20,30</sup>.

### *EPX*

Also common in eosinophils and AECs, the *EPX* (eosinophil peroxidase) gene, is involved in granulocyte functions, notably neutrophils, cytokine production and signalling <sup>29</sup>. The enzyme expressed from eosinophil peroxidase is released to protect from parasitic infection and also allergic stimuli <sup>3</sup>. The gene is found clustered with other peroxidase genes on chromosome 17. Lower DNAm of the *EPX* is related to allergic asthma. In a study with 483 Puerto Rican children and adolescents, the methylome from nasal epithelial cells was studied. It was observed that 61% (48/79 CpGs), including multiple CpGs annotated to *EVL* and *EPX* genes were associated with asthma outcomes, as allergic asthma, environmental IgE sensitization, FeNO and total IgE (FDR < 0.05)<sup>5,16,27</sup>.

### *SMAD3*

The *SMAD3* (*SMAD Family Member 3*) is a protein-coding gene located at chromosome 20p13. It plays an important role in immune response regulation and

acts together with other proteins to promote fibrosis regulation <sup>26,32</sup>. The hypermethylation of the *SMAD3* gene promoter is associated with asthma, particularly in children of asthmatic mothers. A study of DeVries *et al.* (2017) has shown that children from asthmatic mothers had *SMAD3* gene methylated at birth. The methylation profile was analysed from cord blood samples of children from three different cohorts. Methylation levels measured by bisulfite sequencing over the entire DNA [Spearman correlation coefficient ( $\rho$ )=0.48,  $P=1.2\times 10^{-13}$ ] and at intermediate DNA methylation levels (8–92%,  $P=0.006$ ), *SMAD3* methylation was extremely correlated with asthma ( $\rho=0.46$ ,  $P=0.009$ ) <sup>21</sup>.

### **Future perspectives**

Studies on epigenetics have advanced, but despite this, it is not yet clear whether changes in these pathways have a direct causal relationship in certain diseases. For this reason, it is essential to better understand the connection between the pathogenesis of pathologies, their environmental and developmental influences. More systematic studies are needed to detect how DNAm paves the way for certain diseases <sup>21</sup>. However, integrative analysis with potent causal inference and longitudinal data is still a challenge <sup>6</sup>. Various limitations of epigenetic assays can interfere with study results, including tissue-specificity, cellular heterogeneity, sample quality and batch effects. Proper experimental design and data normalisation are essential to mitigate these effects <sup>37</sup>.

A relevant point in carrying out these analyses is the origin of the biological material used for the study. Although many surveys use blood, samples from other locations can also be used. Nasal epithelium, for example, has often been used to

study airway diseases, including cystic fibrosis, allergic sensitization, asthma, and other obstructive respiratory disorders. <sup>27</sup>. Nasal epithelia is easier to access than blood samples and may handle a profitable proxy for pathological changes in lung cells

6:28,

In addition to the origin of the biological material used, the association between environmental exposure and epigenetic variations is another challenge. The ability to compare two or more samples at different time points with the same individuals in the cohort can ensure a strong approach to data analysis <sup>3</sup>. Genetic predisposition, epigenetics and exposure are perfectly suited to be studied through birth cohorts. Currently, the influence of intergenerational events on epigenetic mechanisms that can induce changes in the health-disease pattern is also evidenced. Studies indicate that the health of offspring is influenced by intergenerational aspects related to *maternal and grandparent's exposures and health status* <sup>9</sup>.

*The development of an "asthma phenotype index", considering molecular and clinical criteria, with predictive values of management would be relevant for asthma diagnosis and modulated treatment* <sup>36</sup>. On the other hand, the small number of studies available today apply different designs and analysis techniques. This fact precludes robust conclusions about DNAm and the epigenetic mechanisms of asthma. <sup>27</sup>. Machine learning analytics can provide substantial benefit in analysing this heterogeneity, but little data is available to allow the use of this technology <sup>24</sup>. The characterization of epigenetic alterations with homogeneous approach and standardised techniques for disease outbreak and progress remains needed <sup>8</sup>. New studies in the areas of genomics, biochemistry and genetics can facilitate the understanding of how epigenetic mechanisms influence the evolution of these

patients, promoting safer and more economical clinical approaches <sup>2</sup>. In addition, these studies may seek to define how many aspects of life can influence the onset of inflammatory pathologies and asthma. Epigenetics studies fit a new perspective in understanding pulmonary diseases. It is possible that methylation profile analyses may soon be part of the diagnosis and risk stratification for childhood asthma.

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FIGURE 1

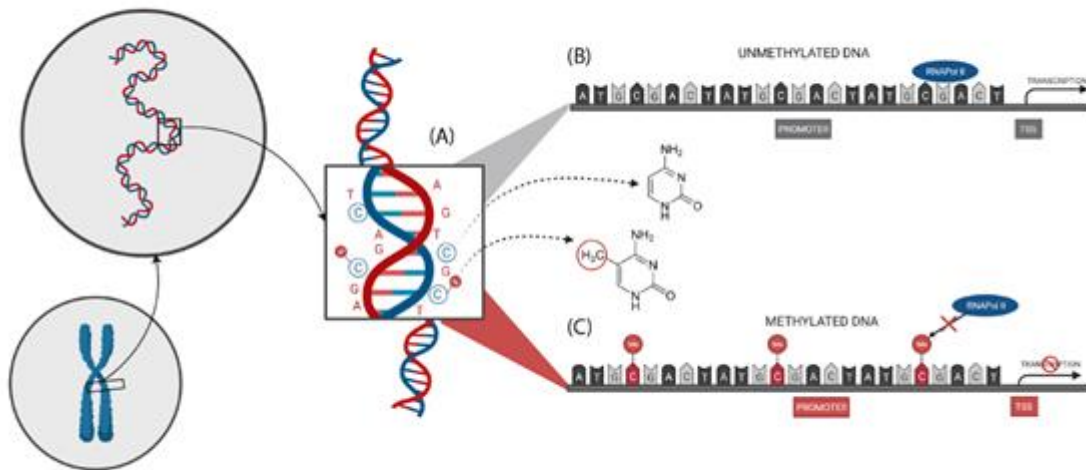


FIGURE1 - (A) A methyl group addition to the cytosine carbon 5 in cytosine-phosphate-guanine (CpG); (B) No CpG *methylation in the gene's promoter region leads to active transcription*; (C) Methylation in the promoter region of genes leads to transcriptional repression;

TSS, transcription start site; RNA pol II, RNA polymerase II.

Table 1: Characteristics of DNAm studies on asthma demonstrating heterogeneous techniques applied among the trials.

Author	Patients	Sample	Main Results
<b>Reese <i>et al.</i>, 2019</b>	n= 1299 Age= 7-17 years old	Whole blood	Identified epigenetic variations related to asthma in newborns and children.
<b>Cardenas <i>et al.</i>, 2019</b>	n= 1083 Age= 12 to 65 years old	Nasal swab cells	285 CpGs sites associated with asthma.
<b>Arathimos <i>et al.</i>, 2017</b>	n= 1529 Age = 7.5 years and 16.5years	Peripheral blood	<i>IL5RA</i> and <i>AP2A2</i> gene methylation related to asthma at 16.5 years old.
<b>Popovic <i>et al.</i>, 2019</b>	n= 136 Age= 6 to 18 months	Saliva	<i>PM20D1</i> gene hypermethylation associated with early childhood wheezing.
<b>Yang <i>et al.</i>, 2018</b>	n= 78. Age = 10 to 12 years old	Nasal epithelia	186 genes related to atopy, asthma, immunity, airflow

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obstruction and epigenetic regulation.

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<b>Ning <i>et al.</i>, 2019<sup>34</sup></b>	n= 182 children. 3 to 14 years old	Peripheral blood	<i>ADAM33</i> polymorphism is correlated with increased susceptibility to asthma.
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<b>Nicodemus- Johnson <i>et al.</i>, 2016</b>	n= 115 adults. 26 to 52 years old	Airway epithelial cells	Regulatory locus associated with asthma risk and epigenetic signatures of specific asthma endotypes.
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## 7.2 ARTIGO ORIGINAL

### **SMAD3 gene methylation profile and asthma in childhood: a longitudinal approach**

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**Abstract:**

Asthma is the most common chronic disease in childhood, and if not treated properly, may accelerate the loss of respiratory capacity in adulthood. Intrauterine and early postnatal exposures may play an important role in short- and long-term health outcomes. Epigenetic factors are associated with asthma development. DNA methylation of the SMAD3 (SMAD family member 3) promoter region is associated with asthma and can be influenced by genetic and environmental factors. To evaluate the variation in the methylation profile of the SMAD3 promoter region in children from different intrauterine environments over time, a longitudinal observational study was conducted with 26 children. They were divided into three groups: children of smoking pregnant women (TBC) (n = 10), small-for-gestational-age due to idiopathic intrauterine growth restriction (IGR) (n = 06), and control group (CTR) (n=10). Genomic DNA was obtained from epithelial mucosal cells at birth and at preschool age. The methylation profile was assessed by RT-PCR. Although there were no differences in spirometry results or ISAAC questionnaire among the groups, a significant difference was observed in SMAD3 gene methylation between the TBC and IGR groups ( $p < 0.05$ ) at birth. A difference was also observed in the rate of SMAD3 methylation in newborns exposed to smoking in the prenatal period and those born with IGR. This difference disappeared at the preschool age. The impact of these results and their relationship with the evolution of children's health and the incidence of asthma in the long term remains to be clarified.

**Keywords:** Asthma. Children. DNA methylation. Epigenetics.

## Introduction

Asthma is the most common chronic disease in childhood and affects millions of children worldwide (FORNO *et al.*, 2019; SOLAZZO; FERRANTE; GRUTTA, 2020; CHOI *et al.*, 2021; EDRIS *et al.*, 2019). With a diverse group of conditions and broad clinical spectrum, asthma is characterized by dyspnea, airway constriction, wheezing, and mucus production, including long-term remodeling of the respiratory system (THIBEAULT; LAPRISE, 2019). An important aspect of this pathology is its significant relationship with environmental characteristics, including the intrauterine period. For example, an association between prenatal exposure to tobacco smoke and postnatal onset has been described in literature (DEVRIES *et al.*, 2022). Maternal smoking, in addition to low birth weight, is an important risk factor for childhood asthma (FORNO; CELEDÓN, 2019). Barker and his colleagues (BARKER *et al.*, 1991; BARKER *et al.*, 2002) demonstrated an association between lower birth weight and reduced lung function in adulthood. Intrauterine growth restriction and low birth weight can be correlated with an adverse intrauterine environment, leading to changes in epigenetic regulation (RIBAS WERLANG *et al.*, 2019; VON MUTIUS; SMITS, 2020) – mechanism which has received special attention in the etiopathogenesis of asthma (FORNO *et al.*, 2017; EDRIS *et al.*, 2019).

Several studies have reported an association between asthma and changes in DNA methylation (ZHANG *et al.*, 2018; CAVALLI; HEARD, 2019; POPOVIC *et al.*, 2019). Differentially methylated regions (DMRs), including the SMAD3 (SMAD family member 3) gene, have been found to be involved in this process (DEVRIES *et al.*, 2017; FORNO; CELEDÓN, 2019). The SMAD3 gene is composed of 6,464 base pairs and functions in the growth factor signaling pathway. It adjusts gene activity and cell proliferation, and presents significant molecular interactions with asthma-related genes (EDRIS *et al.*, 2019; PENG *et al.*, 2019). Furthermore, it acts in the remodeling of airways through fibroblasts, regulating the fibrosis process and immune response (LUND *et al.*, 2018; QI; XU; KOPPELMAN, 2019; ZHANG *et al.*, 2021). Hypermethylation of the gene promoter region is linked to childhood asthma, especially in patients whose mothers have the disease, suggesting that it may be inherited in part through this epigenetic mechanism (DEVRIES *et al.*, 2017; REESE *et al.*, 2019).

To the best of our knowledge, this is the first longitudinal study to evaluate variations in SMAD3 gene methylation profiles in children. These results indicate that intrauterine conditions can induce different biological responses from perinatal stressors through epigenetic mechanisms and how these variations behave over time.

## **Materials and Methods**

### **Study population**

Twenty-six children from the IVAPSA Birth Cohort were included and evaluated at birth and at preschool age up to five years (RIBAS WERLANG *et al.*, 2019). Of these, ten children were born to smoking mothers (TBC), six had idiopathic intrauterine growth restriction (IGR), and ten were in the control group (CTR).

### **Silico analysis**

Methylation-specific PCR (MSP) primer design was performed in five steps: i) gene sequence selection in the DNA database (<https://www.ncbi.nlm.nih.gov/gene/>); ii) identification of the promoter region and recovery of the gene sequence in the Eukaryotic Promoter Database (EPD) ([epd.epfl.ch/index.php](http://epd.epfl.ch/index.php)); iii) identification of transcription factors in the promoter region in the ALGGEN PROMO database (<http://algggen.lsi.upc.es/cgi-bin/promo>); iv) identification of CpG islands using Methyl Primer Express software; and v) primer selection for the methylated and unmethylated regions on the Primer3 website (<https://primer3.ut.ee/>).

### **DNA extraction and sodium bisulfite treatment**

Genomic DNA (gDNA) was obtained from epithelial mucosal cells using sterile wabs. Extraction was performed promptly after sample collection using a previously established protocol (BERNARDI *et al.*, 2012) and quantified using a NanoDrop® device (Thermo Fisher Scientific, Waltham, MA, USA). DNA (400 ng) was treated using an EZ DNA Methylation Kit following the manufacturer's recommendations.

### **Methylation-specific PCR (MSP) – high-resolution melting analysis**

The samples were subjected to MSP for SMAD3 detection. The primer sequences used are described in Supplementary Materials. As a methylated/unmethylated control, the Cells-to-CpG Methylated and Unmethylated gDNA Control Kit Zymo Research® was used. To create a range of methylated and unmethylated dilutions, the controls were mixed to obtain methylation ratios of 0%, 2.5%, 20%, 50%, and 100%. Standard curves with known methylation ratios were included in each assay and were used to deduce the methylation ratio of each sample.

The PCR mixture was prepared in a final volume of 10  $\mu$ L, containing 5  $\mu$ L of MeltDoctor (Applied Biosystems), 10 pmol of each primer, and 1  $\mu$ L (almost 10 ng/ $\mu$ L) of bisulfite-modified DNA samples. Methylation profile analysis was performed using the high-resolution melting (HRM) method on a StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Brazil) for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C with continuous acquisition at 0.2°C.

After HRM amplification, all samples of interest were purified and sequenced *using Sanger's method to confirm the results (data not shown)*. The sequences were aligned using the ClustalW algorithm in the BioEdit software and subsequently inspected manually. Curve points below 20% were considered unmethylated and those above this value were considered methylated.

## Lung function

The ISAAC (International Study of Asthma and Allergies in Childhood) questionnaire was used, and cutoff scores for asthma, rhinitis, and eczema were applied (ASHER *et al.*, 1995; SOLÉ *et al.*, 1998, VANNA *et al.*, 2001; YAMADA *et al.*, 2002). Lung function was measured only in preschoolers using spirometry, as previously described in the literature (WINCK *et al.*, 2016). The Tiffeneau-Pinelli index was used to analyze spirometry outcome data (BEEKMAN *et al.*, 2014).

## Ethical statement and consent to participate

This study was approved by the Research Ethics Committee of Porto Alegre Clinical Hospital (CEP-HCPA) under protocol number 2019-0650. Informed consent was obtained from all the patients and their parents or guardians.

## Statistical analysis

Categorical variables were described as absolute and relative frequencies. Continuous variables were expressed as means and standard deviations or medians and interquartile ranges. The Kruskal-Wallis test was used to compare groups, followed by *Dunn's test for multiple comparisons of nonparametric variables*. The correlation between the percentage of methylation and spirometry results was evaluated using the *Spearman's rank correlation coefficient*.

All statistical analyses were performed using SPSS v.29 statistical package for Windows (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant when the p value was  $< 0.05$ .

## Results

Sociodemographic characteristics of the participants are described in Table 1.

**Table 1** – Sample characterization: sociodemographic variables and anthropometric data of children at birth and at preschool age.

		Intrauterine Environment			
		TBC (n=10)	IGR (n=6)	CTR (n=10)	Total (n=26)
Family Income (monthly) <sup>1</sup> (USD)		310 [200, 600]	260 [250.8, 500]	430 [300, 572]	400 [220, 572]
Maternal Education <sup>2</sup> (years of study)		9,9 (±2,6)	10 (±3,9)	10,9 (±3,3)	10,3 (±3,1)
Paternal Study <sup>2</sup> (years of study)		10,5 (±2,9)	8,8 (±2,6)	10 (±2)	9,9 (±2,5)
Marital Status <sup>3</sup>	With partner	7 (70)	6 (100)	10 (100)	23 (88)
	Without Partner	3 (30)	0 (0)	0 (0)	3 (12)
Delivery Type <sup>3</sup>	Cesarean delivery	3 (30)	2 (33,3)	1 (10)	6 (23)
	Vaginal delivery	7 (70)	4 (66,7)	9 (90)	20 (77)
Child's Gender <sup>3</sup>	Male	6 (60)	3 (50)	4 (40)	13 (50)
	Female	4 (40)	3 (50)	6 (60)	13 (50)
Weight at birth <sup>2</sup> (kg)		3,1 (±0,31)	2,62 (±0,1)	3,13 (±0,25)	3 (±0,3)
Weight at preschool age <sup>2</sup> (kg)		22,4 (±5,42)	17,55 (±2,87)	19,88 (±3,79)	20,3 (±4,6)
Length at birth <sup>2</sup> (cm)		48,6 (±1,78)	47,5 (±1,55)	48,35 (±1,7)	48,3 (±1,7)
Height at preschool age <sup>2</sup> (m)		1,14 (±0,05)	1,06 (±0,08)	1,12 (±0,08)	1,1 (±0,1)
Age at preschool <sup>1</sup> (years)		5 [5,5]	4 [3,5]	5 [5,5]	5 [5,5]

Note: 1, median [p25, p75]; 2, mean (± standard deviation); 3, n (%). TBC, tobacco; IGR, intrauterine growth restriction; CTR, control group.

The analysis was separated by moments: at birth and preschool age (from 3 to 6 years of age). Using HRM, the data were visualized as melting curve values and methylation difference plots. There was a statistically significant difference in the SMAD3 methylation profile between the TBC and IGR groups at birth ( $p = 0.007$ ), which disappeared at preschool age ( $p = 0.174$ ) (Table 2).

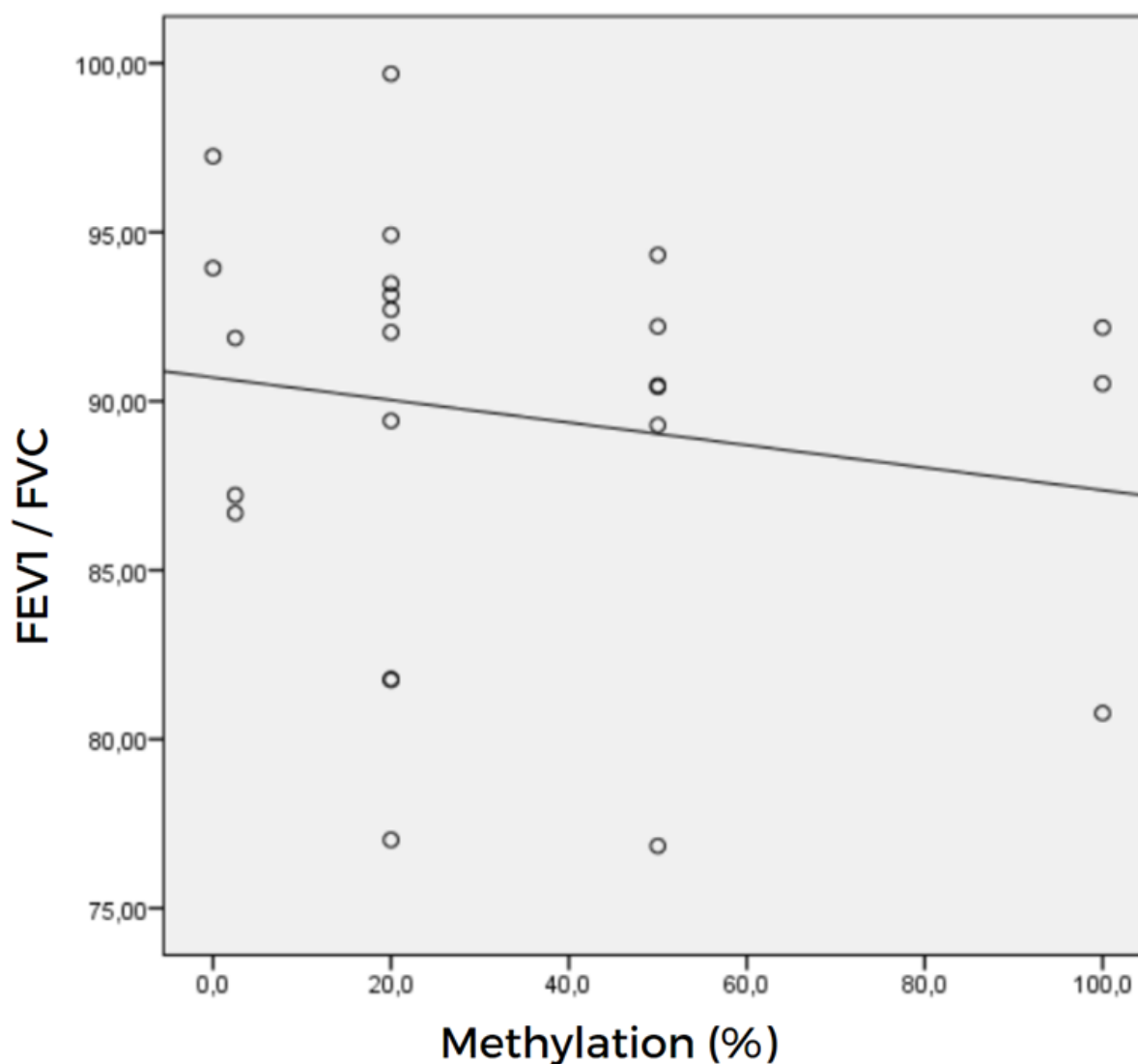
**Table 2** – Methylation profile percentage at birth and preschool age in children exposed to different intrauterine environments.

		Intrauterine Environment			
(% ) Methylation /Age		TBC (n=10)	IGR (n=6)	CTR (n=10)	
		Median [p25, p75]	Median [p25, p75]	Median [p25, p75]	P Value
<b>At Birth</b>		20 [20, 20] <sub>a</sub>	100 [100, 100] <sub>a</sub>	100 [20, 100] <sub>b</sub>	0.007
<b>Preschool</b>		20 [2.5, 50] <sub>c</sub>	50 [20, 100] <sub>c</sub>	20 [20, 50] <sub>c</sub>	0.174

Kruskal-Wallis test with Dunn's multiple comparison test [median with quartile 25;75];  $p < 0.05$ . (a) Statistical difference between the TBC and IGR groups at birth; (b) no statistical difference in CTR when compared with other groups at birth; (c) no statistical difference among groups at preschool age. TBC, tobacco; IGR, intrauterine growth restriction; CTR, control group.

No statistically significant difference was observed in the abnormal spirometry results at preschool age among the groups. Nonetheless, a trend toward a negative correlation between methylation profiles and FEV1/FVC (Figure1) ( $R = -0.22$ ;  $p = 0.29$ ) was observed.

**Figure 1** - Correlation between methylation (all groups) and spirometry (FEV1/FVC).



Independent Kruskal-Wallis test followed by Spearman's rank correlation coefficient. Legend: FEV1/FVC = forced expiratory volume at the first second/forced vital capacity;  $R = -0.22$ ;  $p = 0.29$ .



Asthma and rhinitis were defined according to the cut-off points predicted in the ISAAC questionnaire. According to these scores, there were no significant differences in the methylation profiles (Table 3).

**Table 3** - Questions from the ISAAC questionnaire related to methylation at preschool age.

ISAAC questionnaire	Cutoff*	Methylation (%)	p Value
<b>Asthma ISAAC score</b>	< 5	50 (20 - 100)	0.135
	> 5	20 (20 - 20)	
<b>Rhinitis ISAAC score</b>	< 4	20 (20 - 50)	0.838
	> 4	20 (2.5 - 50)	

Independent Kruskal-Wallis test. (\*) Low- or high-risk was defined as those below or above the cutoff point, respectively.

## Discussion

It was possible to demonstrate that children with intrauterine growth restriction had a higher percentage of SMAD3 methylation in the promoter region at birth than those whose mothers smoked during pregnancy. However, this difference disappears over time. These data show the potential reversibility of methylation as children get old, indicating how intrauterine conditions can induce different biological responses through epigenetic mechanisms and how these variations behave over time. Children who suffered intrauterine distress indeed showed a different trend of methylation compared to the other groups. Currently, follow-up is underway to determine the long-term impact of these findings at birth on the evaluated children.

The main purpose of this study was to observe the variation in the promoter region of SMAD3, which plays an important role in immune response regulation and acts with other proteins to promote fibrosis regulation (DEVRIES et al, 2017; LUND et al., 2018). Moreover, differentially methylated regions associated with asthma include the SMAD3 promoter region gene (EDRIS et al., 2019).

Hypermethylation of the SMAD3 promoter is associated with asthma, particularly in children with asthmatic mothers. In a study by DeVries and collaborators (DEVRIES et al, 2017), the methylation profile of cord blood samples was analyzed in children from three different cohorts, showing that children of asthmatic mothers had the SMAD3 gene hypermethylated at birth, and that it was highly correlated with asthma ( $\rho = 0.46$ ,  $p = 0.009$ ).

Previous studies have shown a wide range of results due to heterogeneous approaches and techniques, collection site (peripheral blood, umbilical cord blood, oral swab, and airway swab), and methods for evaluating gene expression and DNA methylation (YANG *et al.*, 2018; POPOVIC *et al.*, 2019; FORNO; CÉLEDON, 2019). However, it is important to point out that variation in the methylation profile is not necessarily simultaneous with clinical manifestations.

In this study, oral swabs were used for genomic DNA collection because it is an easily accessible, reasonable, and replicable technique, especially in newborn populations (YANG *et al.*, 2016; POPOVIC *et al.*, 2019). The search for biomarkers that allow for the understanding of the overall development of pathologies such as asthma and tracing the path from health to disease is a priority (SOLAZZO; FERRANTE; GRUTTA, 2020).

In conclusion, although this study did not show an association between SMAD3 methylation and the development of asthma, a difference could be observed in the profiles of children at birth according to perinatal stressors that disappeared with aging. Further studies on epigenetic mechanisms are needed to determine biomarkers, their relationship with environmental influences, and their impact on children's development.

## Acknowledgments

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author, Michalowski, M.B., upon reasonable request.

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**Supplementary material** Primer sequences: methylated (M) and unmethylated (U) designs of the promoter region for *SMAD3*.

Target gene	Orientation	Primer sequence *	Amplification Product	CpG's inside Amplicon
<b>SMAD3 - M</b>	Forward	5'ATATCGGTTAGTCGGTTGC--3'	138	10
	Reverse	5'-AAACTCCGCGACTTTTCTC-3'		
<b>SMAD3 - U</b>	Forward	5'-TGGTTAGTTGGTTGTGGGAG-3'	138	—
	Reverse	5'-AACTCCACAACCTTTTCTCCC-3'		

(\*) Reference: This study

## 8 CONCLUSÃO

Este estudo teve como objetivo observar a variação do perfil de metilação da região promotora do gene *SMAD3* em dois momentos diferentes em crianças expostas a ambientes intra uterinos distintos e relacioná-la com sintomas de asma na idade pré-escolar. Este é o primeiro estudo a avaliar a variação do perfil de metilação do gene *SMAD3* de forma longitudinal em crianças.

Apesar do nosso estudo apresentar algumas fragilidades como o tamanho pequeno da amostra e o fato de termos analisado somente o perfil de metilação de apenas um gene, conseguimos mostrar que crianças que tiveram restrição de crescimento intrauterino apresentaram maior percentual de metilação da região promotora do gene *SMAD3* ao nascer quando comparadas às crianças de mães tabagistas, entretanto essa diferença desapareceu na idade pré-escolar. Esses resultados podem indicar que as circunstâncias intra uterinas podem induzir diferentes respostas biológicas por meio de mecanismos epigenéticos e como essas variações se comportam ao longo do tempo.

O gene *SMAD3* desempenha um papel importante na regulação da resposta imune e atua em conjunto com outras proteínas para promover a regulação da fibrose (DEVRIES *et al*, 2017; LUND *et al.*, 2018). A hipermetilação da região promotora do gene *SMAD3* está associada à asma, particularmente em filhos de mães asmáticas. De acordo com o estudo de DeVries e colaboradores, o perfil de metilação de amostras de sangue do cordão umbilical foi analisado em crianças de três coortes diferentes, mostrando que filhos de mães asmáticas apresentavam o gene *SMAD3* hipermetilado no nascimento e que estava correlacionado com asma ( $\rho=0,46$ ,  $p=0,009$ ) (DEVRIES *et al*, 2017).

Embora nosso estudo não tenha mostrado uma associação entre a metilação da região promotora do gene *SMAD3* e o desenvolvimento da asma, observamos uma diferença no perfil de metilação das crianças ao nascer de acordo com seus estressores perinatais e que esse padrão desapareceu com o passar do tempo. Este

resultado sugere que pode haver um mecanismo de risco para asma ao nascer que não se sustenta com o tempo.



## 9 CONSIDERAÇÕES FINAIS

Com a realização deste estudo, foi possível aprimorar o conhecimento a respeito das influências de ambientes intra uterinos adversos na saúde da criança, bem como sobre os mecanismos epigenéticos que influenciam no desenvolvimento da asma.

Muitos estudos são realizados considerando variáveis diferentes, afastando o consenso sobre uma abordagem homogênea sobre o assunto. Dessa forma, procuramos uma abordagem que fosse replicável analisando um gene cuja função está intimamente ligada ao desenvolvimento da asma. Além disso, pudemos analisar o perfil de metilação do gene *SMAD3* de forma longitudinal, um marco para o estudo de genes associados à asma.

Este estudo visou identificar a variação do perfil de metilação e correlacionar com os dados clínicos de asma de amostras da nossa coorte. Ao analisar e observar os desfechos de saúde de nossa própria população, podemos trazer uma clareza do perfil epidemiológico local, sem utilizar como referência outras populações.

**ANEXO 1 - QUESTIONÁRIO ISAAC****ISAAC - Estudo internacional  
de asma e alergias em  
crianças**

**Identif**  
:

**Não Autoaplicável**

Data da entrevista:            /            /	
Entrevistador:	ISAACEN _____
Nome do responsável:	
Nome da criança:	
<p><b>Instruções: SE VOCÊ COMETER UM ERRO NAS RESPOSTAS DE ESCOLHA SIMPLES, CIRCULE OS PARÊNTESES E REMARQUE A RESPOSTA CORRETA. MARQUE SOMENTE UMA OPÇÃO, A MENOS QUE SEJA INSTRUÍDO PARA O CONTRÁRIO.</b></p>	
<p><b>Módulo 1</b></p>	
<p><b>1) Alguma vez na vida, seu filho teve sibilos (chiado no peito)? (1) Sim (0) Não</b></p> <p><b>Se você respondeu NÃO, passe para a questão número 6.</b></p>	<p>ISCHID _____</p>
<p><b>2) Nos últimos 12 (doze) meses, seu filho teve sibilos? (1) Sim (0) Não</b></p>	<p>ISSIBO _____</p>
<p><b>3) SE SIM, nos últimos 12 (doze) meses, quantas crises de sibilos seu filho teve?</b></p> <p style="text-align: center;">(1) Nenhuma crise</p> <p style="text-align: center;">(2) 1 a 3 crises</p> <p style="text-align: center;">(3) 4 a 12 crises</p> <p style="text-align: center;">(4) Mais de 12 crises</p>	<p>ISSIBQ _____</p>

<p><b>4) Nos últimos 12 (doze) meses, com que frequência seu filho teve seu sono perturbado por chiado no peito?</b></p> <p>(1) Nunca acordou com chiado (2) Menos de uma noite por semana (3) Uma ou mais noites por semana</p>	<p>ISCHIF _____</p>
<p><b>5) Nos últimos 12 (doze) meses, o chiado do seu filho foi tão forte a ponto de impedir que ele conseguisse dizer mais de duas palavras entre cada respiração?</b></p> <p>(1) Sim (0) Não</p>	<p>ISCHIP _____</p>
<p><b>6) Alguma vez na vida seu filho teve asma?</b></p> <p>(1) Sim (0) Não</p>	<p>ISASMA _____</p>
<p><b>7) Nos últimos 12 (doze) meses, seu filho teve chiado no peito após exercícios físicos?</b></p> <p>(1) Sim (0) Não</p>	<p>ISCHIE _____</p>
<p><b>8) Nos últimos 12 (doze) meses, seu filho teve tosse seca à noite, sem estar gripado ou com infecção respiratória? (1) Sim (0) Não</b></p>	<p>ISTOSS _____</p>
<p><b>Módulo 2</b></p>	
<p><b>Todas as perguntas são sobre problemas que ocorreram quando seu filho não estava gripado ou resfriado</b></p>	
<p><b>1) Alguma vez na vida seu filho teve problema com espirros ou coriza (corrimento nasal), quando não estava resfriado ou gripado? (1) Sim (0) Não</b></p>	<p>ISESPI _____</p>

<p><b>2) Nos últimos 12 (doze) meses, seu filho teve algum problema com espirros?</b></p> <p><b>(1) Sim (0) Não</b></p> <p><b>Se a resposta foi NÃO, passe para a questão número 6.</b></p>	<p>ISESPP _____</p>
<p><b>3) Nos últimos 12 (doze) meses esse problema nasal foi acompanhado de lacrimejamento ou coceira nos olhos? (1) Sim (0) Não</b></p>	<p>ISESPC _____</p>

<p><b>4) Em qual dos últimos 12 (doze) meses esse problema nasal ocorreu? (Por favor, marque em qual ou mais meses isso ocorreu)</b></p> <p>( ) Janeiro ( ) Maio ( ) Setembro ( ) Fevereiro ( ) Junho ( ) Outubro ( ) Março ( ) Julho ( ) Novembro ( ) Abril ( ) Agosto ( ) Dezembro</p>	<p>ISESPM _____</p>
<p><b>5) Nos últimos 12 (doze) meses, quantas vezes as atividades diárias do seu filho foram atrapalhadas por esse problema nasal?</b></p> <p>(1) Nada</p> <p>(2) Um pouco</p> <p>(3) Moderado</p> <p>(4) Muito</p>	<p>ISESPD _____</p>
<p><b>6) Alguma vez na vida você teve rinite? (1) Sim (0) Não</b></p>	<p>ISRINI _____</p>
<p><b>Módulo 3</b></p>	
<p><b>1) Alguma vez na vida seu filho teve manchas com coceira na pele (eczema), que apareciam e desapareciam por pelo menos 6 meses? (1) Sim (0) Não</b></p> <p><b>Se a resposta for NÃO, passe para a questão número 6.</b></p>	<p>ISECZA _____</p>

<p>2) Nos últimos 12 (doze) meses, seu filho teve essas manchas na pele (eczema)?</p> <p>(1) Sim (0) Não</p>	<p>ISECZP _____</p>
<p>3) Alguma vez essas manchas com coceira (eczema) afetaram alguns dos seguintes locais: dobras dos cotovelos, atrás dos joelhos, na frente dos tornozelos, abaixo das nádegas ou em volta do pescoço, orelhas ou olhos? (1) Sim (0) Não</p>	<p>ISECZL _____</p>
<p>4) Alguma vez essas manchas com coceira (eczema) desapareceram completamente nos últimos 12 (doze) meses? (1) Sim (0) Não</p>	<p>ISECZD _____</p>
<p>5) Nos últimos 12 (doze) meses, quantas vezes, aproximadamente, seu filho ficou acordado à noite por causa dessa coceira na pele?</p> <p>(1) Nunca nos últimos 12 meses</p> <p>(2) Menos de uma noite por semana</p> <p>(3) Uma ou mais noites por semana</p>	<p>ISECZN _____</p>
<p>6) Alguma vez seu filho teve eczema (manchas na pele)? (1) Sim (0) Não</p>	<p>ISECZM _____</p>
<p>Questões adicionais</p>	
<p>1) A mãe da criança tem ou teve asma ou rinite? (1) Sim (0) Não</p>	<p>ISASRM _____</p>
<p><i>SE SIM</i>, com quantos anos? _____</p>	<p>ISASRA _____</p>

<b>2) O pai da criança tem ou teve asma ou rinite? (1) Sim (0) Não</b>	<b>ISASRP</b> _____
<b>SE SIM, com quantos anos? _____</b>	<b>ISASPI</b> _____