UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL – UFRGS

Imunobiologia e Imunogenética de Desordens Gestacionais e Transtorno do Espectro Autista

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"Três ideias profundamente desestabilizadoras ricochetearam por todo o século XX e se dividiram em três partes desiguais: o átomo, o byte e o gene. (…) Cada uma começou a vida como um conceito científico muito abstrato, mas acabou por invadir numerosos discursos humanos e, com isso, transformou a cultura, a sociedade, a política e a linguagem. No entanto, incomparavelmente, o paralelo mais crucial entre essas ideias é conceitual: cada uma representa a unidade irredutível – o tijolo construtor, a unidade básica – de um todo maior: o átomo, da matéria; o byte (ou "bit"), da informação digitalizada; o gene, da hereditariedade e informação biológica. (...) Não podemos explicar o comportamento da matéria – por que o ouro brilha, por que o hidrogênio entra em combustão com o oxigênio – sem invocar a natureza atômica da matéria. Não podemos entender as complexidades da computação – a natureza dos algoritmos, a armazenagem ou corrupção de dados – sem compreender a anatomia estrutural da informação digitalizada. (...) De maneira análoga, (...) é impossível entender a biologia de organismos e células ou a evolução – ou ainda a patologia, o comportamento, temperamento, doença, raça, identidade ou destino dos seres humanos – sem primeiro lidar com o conceito de gene." [1]

Siddhartha Mukherjee

[1]Texto extraído do livro "O Gene: uma história íntima" (Mukherjee, Siddhartha. 1ª ed. São Paulo: Companhia das Letras, 2016, p. 20-21).

SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

Abreviaturas

ASD: *Autism Spectrum Disorder*

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

CCR5: *cysteine-cysteine chemokine receptor 5*/receptor de quimiocina cisteína-cisteína tipo 5

CCR5: gene *CCR5*

CCR5Δ32: deleção de 32 pares de bases no gene *CCR5*

CD4: *cluster of differentiation 4*/Grupamento de diferenciação 4

CD8: *cluster of differentiation 8*/Grupamento de diferenciação 8

CDC: *Centers for Disease Control and Prevention*/Centros de Controle e Prevenção de Doenças

DNA: *deoxyribonucleic acid*/ácido desoxirribonucleico

EUA: Estados Unidos da América

HLA: *human leukocyte antigen*/antígeno leucocitário humano

IL: *interleukin*/interleucina

MIA: *Maternal Immune Activation*/ativação imune materna

MICA: *MHC-I Chain Related Protein A*/Proteína A Relacionada ao MHC de Classe I

NIH: *National Institutes of Health*/ Institutos Nacionais da Saúde

NK: *Natural killer (cells)/* células *Natural Killer*

NKG2A*: Natural Killer Group 2 Member A/* Receptor de células NK Grupo 4 membro A

NKG2C*: Natural Killer Group 2 Member C/* Receptor de células NK Grupo 4 membro C

NKG2D: *Natural Killer Group 2 Member D/* Receptor de células NK Grupo 4 membro D

PE: *preeclampsia*/pré-eclâmpsia

PCR: *polymerase chain reaction*/reação em cadeia da polimerase

RNA: *ribonucleic acid*/ácido ribonucleico

SUS: Sistema Único de Saúde

TEA: Transtorno do Espectro Autista

Símbolos e unidades

Δ: delta

kb: kilobase

µm: micrômetro

nm: nanômetro

LISTA DE FIGURAS E TABELAS

RESUMO

Esta tese apresenta artigos de dados e trabalhos teóricos envolvendo imunologia da gestação, desordens gestacionais e imunogenética do Transtorno do Espectro Autista (TEA). Este trabalho está dividido em duas partes, a primeira sendo composta por quatro capítulos, e a segunda parte, por três capítulos totalizando sete artigos diferentes. Uma introdução geral precede as duas partes principais, onde são apresentados conceitos importantes abordados ao longo dos textos. A gestação humana é um processo complexo que envolve diferentes sistemas fisiológicos que sofrem influência do sistema imune materno. Complicações gestacionais e seus possíveis gatilhos ambientais são apresentados nesta tese, com destaque para desequilíbrios nos níveis de citocinas nessas condições. Além disso, é abordado o componente genético das respostas imunes aos diferentes desafios ambientais enfrentados durante a gravidez pela mãe e pelo feto em desenvolvimento. O TEA é uma condição que impacta o neurodesenvolvimento fetal, além de afetar as habilidades cognitivas e sociais dos indivíduos acometidos, geralmente manifestando-se antes dos três anos de idade. Sabe-se que o TEA possui um forte componente genético com altas taxas de herdabilidade e de concordância entre gêmeos monozigóticos. Além disso, o TEA possui um importante componente ambiental. Sabe-se, ainda, que distúrbios imunológicos são um componente do TEA. Nesta linha, são abordados polimorfismos em genes relacionados ao sistema imunológico no contexto do TEA. Um dos fatores ambientais fortemente sugeridos como risco para TEA são algumas complicações gestacionais, principalmente aquelas com fundo inflamatório. Assim, diferentes aspectos imunológicos e ambientais durante a gravidez podem ser fatores-chave para o desenvolvimento de distúrbios gestacionais e/ou para a incidência de problemas neurológicos na prole. Na “Parte I” desta tese são abordados aspectos imunológicos da gestação humana, juntamente com discussões sobre o papel das vesículas extracelulares no contexto de gravidez bem-sucedida e de complicações gestacionais e em diferentes doenças infecciosas. Ao longo da “Parte II”, são apresentados genes relacionados ao sistema imunológico, nos quais diferentes polimorfismos, especificamente variantes genéticas pró-inflamatórias, variantes genéticas do MHC e variantes genéticas imunometabólicas, já foram estudados no contexto do autismo e TEA. Diferentes gatilhos

inflamatórios durante a gravidez que já foram indicados como fatores de risco para a manifestação de TEA em crianças nascidas dessas gestações são também aqui discutidos. Nesse contexto, destacam-se as consequências da ativação imune materna (MIA) e sua influência no feto em desenvolvimento. Além disso, é proposta uma conexão mecanicista entre os principais distúrbios relacionados à inflamação na gravidez e risco para autismo considerando o “universo das vesículas extracelulares”. Por fim, são apresentados resultados de estudos imunogenéticos envolvendo a deleção do gene *NKG2C* e variantes nos genes *NKG2D* e *NKG2A* em pacientes com TEA e em seus respectivos pais biológicos.

Palavras-chave: Genética; Imunologia; Gestação; Autismo; Polimorfismo; Inflamação.

ABSTRACT

This thesis presents data and theoretical studies involving gestational immunology, gestational disorders, and immunogenetics of Autism Spectrum Disorder (ASD). This work is divided into two parts, each consisting of four chapters, totaling eight different articles. A general introduction precedes these two main parts, where important concepts covered throughout the texts are presented. Human pregnancy is a complex process that involves different physiological systems, which are influenced by the maternal immune system. Gestational complications and their possible environmental triggers are also presented in this thesis, highlighting imbalances in cytokine levels under these conditions. In addition, the genetic component of immune responses to the different environmental challenges faced during pregnancy by the mother and the developing fetus is addressed. ASD is a condition that impacts both fetal neurodevelopment and the cognitive and social abilities of affected individuals, usually manifesting before the age of three. ASD is known to have a strong genetic component with high heritability and agreement rates between monozygotic twins. In addition, it has an important environmental component. Immune disorders are also known to be a component of ASD. In this line, polymorphisms in genes related to the immune system in the context of ASD are addressed. Strongly suggested environmental risk factors for ASD are gestational complications, especially those with an inflammatory background. Thus, different immunological and environmental aspects during pregnancy may be key factors for the development of gestational disorders and/or for the incidence of neurological problems in the offspring. In “Part I” of this thesis, immunological aspects of human pregnancy are discussed, along with discussions about the role of extracellular vesicles in the contexts of both successful and complicated pregnancies and in different infectious diseases. “Part II” presentes genes related to the immune system, in which different polymorphisms have already been studied in the context of autism and ASD, specifically proinflammatory genetic variants, MHC genetic variants, and immunometabolic genetic variants. Different inflammatory triggers during pregnancy that were already suggested as risk factors for ASD in children born of these pregnancies are also discussed. In this context, the consequences of maternal immune activation (MIA) and its influence on the developing fetus are highlighted. In addition, a mechanistic connection between major inflammation-related disorders in pregnancy and risk for autism

considering the “extracellular vesicle universe” is proposed. Finally, results of immunogenetic studies involving deletion of the *NKG2C* gene and variants in the *NKG2D* and *NKG2A* genes in ASD patients and their respective biological parents are presented.

Keywords: Genetics; Immunology; Pregnancy; Autism; Polymorphism; Inflammation

APRESENTAÇÃO E ESTRUTURAÇÃO DA TESE

No tópico **Introdução Geral e Objetivos** são apresentados os temas abordados em detalhe nas **Partes I** e **II** desta tese, que trata sobre imunobiologia e imunogenética de desordens gestacionais e transtorno do espectro autista.

Na **Parte I**, encontram-se quatro capítulos, cada um correspondente a uma publicação. O papel da interface materno-fetal na tolerância imune em relação ao feto e os processos que impedem infecções nessa região, com ênfase no papel de exossomos e outras vesículas extracelulares, é abordado no **Capítulo II**. O **Capítulo III** discute o papel da molécula imunotolerogênica HLA-G e, dada a importância da mesma no período gestacional em que é altamente expressa pela placenta, é apresentada uma hipótese para o desenvolvimento de um método contraceptivo baseado em micro RNAs que afetam a sua expressão. A importância de citocinas na adequada manutenção e coordenação das respostas imunes durante a gestação é abordada no **Capítulo IV**, por meio de um artigo de dados que avaliou o perfil de citocinas no plasma sistêmico de mulheres que sofreram aborto espontâneo idiopático e em mulheres gestantes sem apresentação de intercorrências. Aspectos imunogenéticos da gestação humana são discutidos no **Capítulo V**, o qual consiste em um artigo de dados que avaliou o impacto da deleção completa do gene *NKG2C* e da variante CCR5Δ32 sobre o desenvolvimento da pré-enclâmpsia. Estes genes foram escolhidos como alvo do estudo dado o potencial dessas moléculas em modificar o comportamento de células inflamatórias. O estudo foi realizado no contexto de uma intercorrência gestacional de caráter complexo e multifatorial, a pré-eclâmpsia, em que abordagens imunogenéticas têm potencial para contribuir no entendimento de suas causas, agravantes e/ou gatilhos.

A **Parte II** desta tese é baseada em aspectos imunogenéticos do Transtorno do Espectro Autista (TEA), apresentando quatro capítulos correspondentes a dois artigos de revisão e outros dois artigos de dados, todos em fase final de preparação para publicação. Além disso, este eixo da tese conecta-se com a **Parte I** através da discussão acerca dos gatilhos inflamatórios gestacionais que são potenciais fatores de risco para o desenvolvimento de autismo. O **Capítulo VI** apresenta um trabalho de revisão sobre polimorfismos em genes relacionados ao sistema imune já investigados no contexto do

TEA. O impacto de processos inflamatórios durante a gestação e o risco de desenvolvimento de autismo nas crianças nascidas dessas gestações é discutido no **Capítulo VII**, em que vesículas extracelulares são indicadas como um possível elo mecanístico negligenciado nos processos inflamatórios abordados. O **Capítulo VIII** é composto por um estudo em que a deleção completa do gene *NKG2C* foi avaliada em indivíduos autistas e em seus respectivos pais biológicos, juntamente com o papel de diferentes SNPs dos genes *NKG2D* e *NKG2A*. Por fim, o **Capítulo IX** de uma discussão geral e conclusão, conectando brevemente os temas abordados nesta tese, além de apresentar um fechamento deste trabalho.

Capítulo I

Introdução e Objetivos

INTRODUÇÃO

1. *Imunogenética*

Imunogenética é a área do conhecimento que trata da base genética das respostas imunes. Além disso, ela inclui o estudo das vias imunológicas consideradas “normais” e o estudo de variações genéticas que resultam em respostas imunes defectivas ou ineficientes. Além de serem importantes *per se*, os estudos no ramo da imunogenética têm um grande potencial para a descoberta de novos alvos terapêuticos para diversas doenças relacionadas ao sistema imunológico (Nature, 2019).

1.1. *Imunobiologia e Imunogenética da Gestação Humana*

A gestação humana é um processo complexo e finamente regulado pelo sistema imune materno mesmo antes da implantação do embrião até após o momento do parto. Após a fecundação, a fusão das membranas do ovócito e do espermatozóide induz alterações químicas que impedem a penetração e fecundação por outros espermatozóides. Os cromossomos parentais são pareados e as primeiras divisões celulares ocorrem cerca de 24h após a fecundação. Essas primeiras divisões celulares são controladas por componentes citoplasmáticos do óvulo, pois até esse momento não há síntese de mRNA. O desafio imunológico da gestação inicia-se mais efetivamente de dois a três dias após a fertilização, com a chamada “ativação gênica do zigoto”, quando os antígenos de origem paterna começam a ser expressos. A partir desse momento, o sistema imune da mãe é posto em contato com antígenos não-próprios e, no decorrer de uma gestação de sucesso, o ambiente uterino deve configurar-se de forma a evitar a rejeição do feto em desenvolvimento. Nesse contexto, a placenta começa a se desenvolver, com a expressão de diferentes moléculas, recrutamento de diversas células imunes e uma intensa produção de exossomos e outras vesículas celulares. Todos esses componentes atuam de forma conjunta e, em última instância, possibilitam o adequado desenvolvimento do feto (Braude et al., 1988; Capmany et al., 1996; Hedlund et al., 2009; Stenqvist et al., 2013).

A placenta, órgão temporário derivado do feto, além de mediar as trocas gasosas e de nutrientes, atua como um importante regulador imune. Além disso, nesse órgão são observadas

estratégias que evitam a passagem de patógenos e o estabelecimento de infecções na interface materno-fetal (Gude et al., 2004; Kaminski et al., 2019a). A placenta torna-se um hemocorial após o remodelamento dos vasos sanguíneos maternos e o desenvolvimento das artérias espirais. Nesse contexto, o reconhecimento de antígenos paternos expressos pela placenta envolve tanto respostas imunes locais, na interface materno-fetal, quanto sistêmicas. O contato íntimo entre células fetais e células e tecidos imunes maternos representa um substancial desafio imunológico para o feto em desenvolvimento. Assim, diferentes células e moléculas atuam em sincronia culminando em um ambiente altamente imunomodulado, de forma a tolerar o desenvolvimento do feto, que pode ser comparado a um enxerto semi-alogênico (Trowsdale e Betz, 2006; Vianna et al., 2011; Svensson-Arvelund et al., 2015; Kaminski et al., 2019a). Diferentes estratégias para evitar um eventual "ataque" do sistema imune materno à placenta em desenvolvimento podem ser observadas na interface materno-fetal.

É importante salientar que, embora o ambiente central das reações imunológicas durante a gestação seja a interface materno-fetal, é possível que padrões de respostas imunes sejam também detectados na circulação sistêmica de gestantes. Ainda, os primeiros estudos acerca da imunologia da gestação e o histórico das descobertas ao longo das décadas foram realizados no contexto de caracterização do perfil de citocinas na circulação periférica de gestantes. A Figura 1 apresenta um histórico resumido dos estudos sobre a imunologia da gestação humana. Detalhes dos aspectos históricos das descobertas estão descritos na Parte I desta tese, principalmente no Capítulo I.

As primeiras inferências sobre a importância da imunologia na gestação foram realizadas pelo grupo de pesquisa do Sir Peter Medawar, nos anos 1950, quando a gestação humana era ainda vista como um fenômeno possível devido à separação anatômica entre a mãe e o feto, à imaturidade antigênica do feto e à inércia do sistema imune da mãe em relação ao feto (Billingham et al., 1953). No entanto, pesquisas subsequentes investigando o processo de reprodução humana postularam que as propostas de Medawar não eram compatíveis com a realidade e a totalidade do período gestacional de mamíferos, ou não eram suficientes para explicar o sucesso do processo gestacional (revisado em Kaminski et al., 2019a).

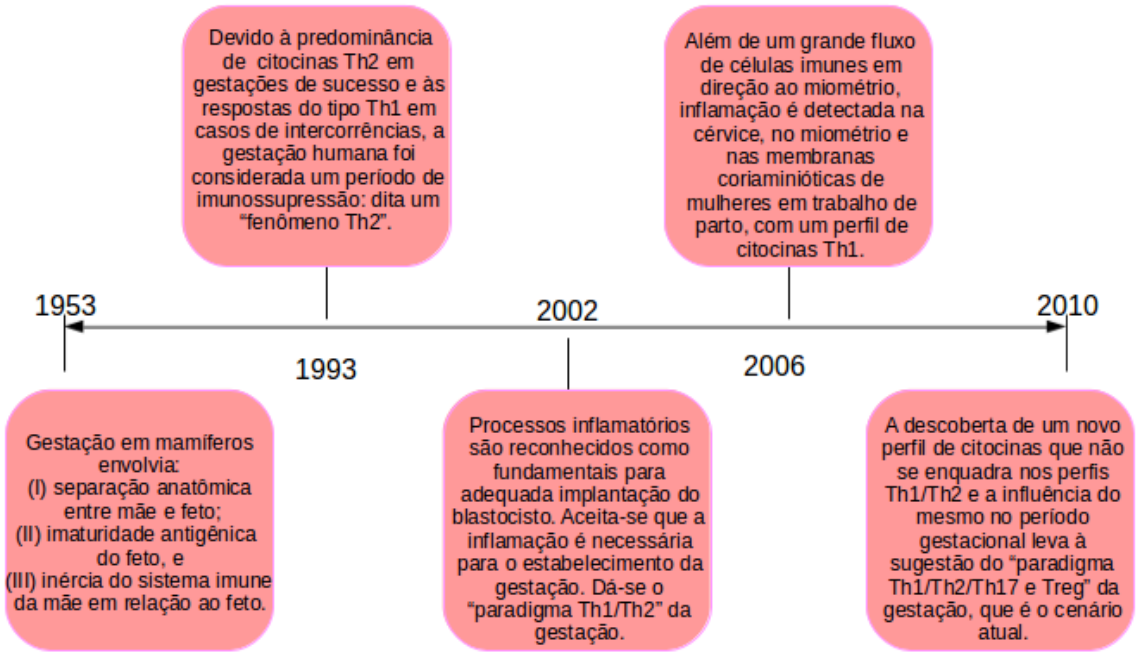

Figura 1. Histórico resumido dos estudos sobre a imunologia da gestação humana. As distâncias entre os anos das descobertas plotadas na figura não representam escala temporal correspondente.

Citocinas são pequenas proteínas secretadas pelas células que participam das interações e comunicações celulares (Zhang e An, 2007). Considerando o predomínio de citocinas antiinflamatórias na circulação sistêmica de gestantes, nos anos 1990 a gestação humana era vista como um "fenômeno Th2" (Wegmann et al., 1993). Na época, essa ideia foi reforçada devido à predominância de citocinas do tipo Th1, pró-inflamatórias, na circulação de gestantes que passavam por intercorrências gestacionais, como o aborto e a pré-eclâmpsia (Piccini et al., 1998; Raghupathy et al., 2000).

No entanto, após as descobertas de que tanto o processo de implantação do blastocisto quanto o parto são eventos fisiológicos em que respostas pró-inflamatórias são fundamentais, estabeleceu-se o "paradigma Th1/Th2 da gestação" (Chaoaut et al., 2002; Hill et al., 1995; Mor et al., 2011; Piccinni, 2002; Raghupathy et al., 2000; Wegmann et al., 1993). Assim, a complexidade da regulação imunológica durante a gestação começou a ser melhor compreendida. Porém, a descoberta de outras citocinas que não se enquadravam nem no perfil Th1 nem no perfil Th2 e a demonstração da importância de tais citocinas na gestação mexeu,

novamente, com os conceitos até então estabelecidos na imunologia gestacional (Chaoaut et al., 2002; Zenclussen, 2013).

O papel de células T regulatórias (Tregs) e Th17 e das citocinas produzidas por essas células na gestação (Wu et al., 2014) levou ao estabelecimento do chamado "Paradigma Th1/Th2/Th17 e Treg" da gestação, sendo este o cenário atualmente aceito (Saito et al., 2010). Discutido em maiores detalhes no Capítulo III, o papel de células (e citocinas) Th17 está relacionado à manutenção de períodos gestacionais prolongados (Pongcharoen et al., 2007; Martínez-García et al., 2011; Chavan et al., 2017; Kaminski et al., 2018).

Considerando o contexto da regulação imunológica para uma gestação de sucesso, além das citocinas abordadas acima, quimiocinas e receptores de quimiocinas são também importantes. Quimiocinas são citocinas quimiotáticas que direcionam a migração celular em diferentes contextos imunológicos. Por meio da ligação às quimiocinas via receptores de quimiocinas presentes em suas membranas, as células do sistema imune migram da corrente sanguínea em direção aos sítios de inflamação. Esse processo de migração se dá em resposta ao gradiente de quimiocinas estabelecido, cuja concentração aumenta em direção ao local em que o processo inflamatório está localizado (Ellwanger et al., 2019). Polimorfismos nos genes das quimiocinas e seus receptores são abordados em estudos imunogenéticos. Considerando a imunogenética de desordens gestacionais, diferentes trabalhos já evidenciaram o impacto de variantes em genes do sistema imune sobre os desfechos da gestação humana (Michita et al., 2016; Michita et al., 2018; Kaminski et al., 2019b).

Além das citocinas e seus receptores, diferentes células e moléculas residentes na decídua, ou derivadas da placenta, atuam ao longo do período gestacional. Tais componentes agem controlando diferentes aspectos e etapas da gestação, desde a implantação do embrião até o desenvolvimento completo do feto e o trabalho de parto. A decídua corresponde ao tecido interno de um útero gravídico. Esse tecido é formado por glândulas endometriais, vasos sanguíneos e pelo estroma. De todas as células que compõem a decídua, em número, os leucócitos representam de 15 a 30%, podendo estar distribuídos em grupos (*cell clusters*), em regiões subepiteliais ou distribuídos de maneira aleatória. Desses leucócitos, a maioria são células *natural killer* (NK) uterinas, células T do tipo αβ e γδ, células dendríticas (DCs) e macrófagos; células B são raras ou ausentes (Mincheva-Nilson et al., 1994; Moffet-King, 2002; Lash et al., 2010). A **Tabela 1** apresenta uma visão geral das moléculas envolvidas no controle das respostas imunes durante a gestação abordadas na presente tese.

Tabela 1. Visão geral das moléculas envolvidas no controle das respostas imunes durante a gestação.

Moléculas	Funções	Referências
Estradiol, progesterona e gonadotrofina coriônica humana	No contexto da receptividade uterina ao blastocisto a ser implantado, regulam fatores de transcrição, produção de citocinas e a expressão de moléculas de adesão.	Gude et al., 2004; Makrigiannakis et al., 2017
HLA-C, HLA-E, HLA-F e HLA-G	A expressão de HLA-E por células do trofoblasto extraviloso as permite evadir do ataque de células NK. HLA-G atua na modulação de células NK e APCs, também induzindo-as a secretarem citocinas e fatores pró-angiogênicos.	King et al., 2000; Moffett-King, 2002; Hackmon et al.,2017; van der Meer et al., 2004; Li et al., 2009; LeMaoult et al., 2004
Indoleamina - 2,3 dioxigenase (IDO)	Detectada ao longo da gestação e, no blastocisto, a partir do dia 6, IDO é uma enzima que cataliza a síntese do triptofano. Dessa maneira, induz células T à inanição, impedindo sua proliferação.	Kudo et al., 2004; Shayda et al., 2009
Fas e FasL	Receptor e ligante, respectivamente. Moléculas envolvidas em vias apoptóticas, podem ser detectadas no citotrofoblasto viloso e no sinciciotrofoblasto. Junto de outras moléculas, atuam na regulação das respostas imunes maternas na interface materno-fetal.	Uckam et al., 1997; Pongcharoen et al., 2004; Abrahams et al., 2004; Frängsmyr et al., 2005; Stenqvist et al., 2013
Ligante Indutor de Apoptose Relacionado a TNF (TRAIL)	TRAIL é expresso pela placenta de forma constitutiva. Em cooperação com o sistema Fas/FasL, TRAIL induz apoptose em linfócitos ativados na interface materno-fetal. São, assim, importantes durante a invasão e diferenciação do trofoblasto, promovendo um ambiente de imunotolerância.	Huppertz et al., 1998; Mor et al., 2002; Wiley et al., 1995; Bai et al., 2009

Early pregnancy factor (EPF)	Molécula imunossupressora, é detectada no soro de gestantes.	Fan and Zheng, 1997
Fator Indutor de Leucemia (LIF)	LIF é uma glicoproteína da família da Interleucina-6, com efeitos pleiotróficos, entre eles a indução de proliferação, diferenciação e sobrevivência de células do trofoblasto.	Aghajanova, 2004
CD59, Proteína Cofator de Membrana (MCP) e Fator de Aceleração de Decaimento (DAF)	São proteínas regulatórias do Complemento. São expressas pela placenta a partir da 6ª semana da gestação, evitando ou minimizando a ativação do complemento. A presença delas na interface materno-fetal é fundamental para a manutenção da gestação, evitando respostas imunes mediadas pelo Complemento.	Holmes et al., 1992
Receptor de células NK Grupo 2 membro D (NKG2D) e seus ligantes: Proteínas Relacionadas ao MHC-I (MIC) e Proteínas ligantes de UL16 (ULBP)1-6	NKG2D é um receptor ativatório expresso por células NK, NKT, Tαβ e Tγδ CD8+. O sistema receptor-ligante é um forte indutor de citotoxicidade, direcionado à eliminação de células estressadas, estrangeiras, invasoras ou infectadas. Os ligantes MICA/B e ULBP são expressos pela placenta em suas formas solúveis, associados à exossomos, resultando na regulação negativa do receptor cognato, suprimindo potenciais eventos de citotoxicidade na interface materno-fetal, promovendo a tolerância das células maternas em relação aos tecidos fetais/placentários.	Bauer et al., 1999; Stern-Ginossar e Mandelboim, 2009; Hedlund et al., 2009

Além das células e moléculas do sistema imune, vesículas extracelulares são extremamente abundantes na interface materno-fetal e evidências têm demonstrado que são de fundamental importância nos processos envolvendo uma gestação de sucesso. Dentre essas vesículas, destacam-se os exossomos, que são nanovesículas formadas por uma bicamada lipídica, com capacidade de transportar diferentes moléculas tanto em seu interior quanto na própria membrana, constituindo um importante mecanismo de comunicação celular à longa distância (Théry et al., 2002). A placenta ativamente produz e secreta exossomos, o que leva à formação de uma "nuvem" dessas nanovesículas na interface materno-fetal, cuja concentração diminui conforme a distância dessa região. Por meio de estudos de caracterização do perfil de moléculas associadas a esses exossomos, é sabido que os mesmos atuam na promoção de um ambiente imunossupressor, controlando respostas imunes maternas que podem ser nocivas ao feto e à placenta (Hedlund et al., 2009; Mincheva-Nilson, 2010; Stenqvist at al., 2013).

Exossomos derivados da placenta podem ser detectados na circulação periférica de gestantes, e sabe-se que aumentam em número ao longo do período gestacional (Salomon et al., 2014). Além disso, em casos de pré-eclâmpsia, por exemplo, o número dessas nanovesículas é muito maior em comparação ao encontrado em uma gestação normotensa, sendo a avaliação do perfil de exossomos uma potencial estratégia para monitorar o risco de intercorrências gestacionais (Kshirsagar et al., 2012; Tannetta et al., 2017a; 2017b).

1.2. *Imunogenética do Transtorno do Espectro Autista*

O autismo é caracterizado como uma desordem do neurodesenvolvimento humano, composta por um conjunto de condições clínicas de início precoce que se manifestam na infância. Atualmente esta condição está inserida em um conjunto de características neurocomportamentais referidas como "Transtorno do Espectro Autista" (TEA). Mencionado pela primeira vez em 1911 pelo psiquiatra alemão Eugen Bleuler, somente algumas décadas mais tarde Leo Kanner definiu o termo autismo, após a observação de um grupo de crianças com comportamento estereotipado (Kanner e Eisenberg, 1957).

Dentre as manifestações típicas de indivíduos com TEA encontram-se déficit de interação social e da comunicação verbal e não verbal, presença de comportamento repetitivo e estereotipado e interesses e atividades restritos. A manifestação deste quadro

clínico ocorre antes dos três anos de idade, afetando mais meninos do que meninas em uma relação de 4:1, o que remete à conhecida relação entre autismo e muitas doenças ligadas ao cromossomo X. Indivíduos diagnosticados com TEA podem apresentar diversos sintomas e comorbidades, caracterizando uma grande heterogeneidade de manifestações clínicas, com fenótipos que variam de leve a extremamente severos (O'Hare, 2009).

Os critérios diagnósticos do TEA são identificados através do Manual Diagnóstico e Estatístico dos Transtornos Mentais, que se encontra em sua 5ª edição revisada (DSM-V-TR). O TEA é um novo transtorno do DSM-V que engloba o transtorno autista do DSM-IV (autismo), transtorno de Asperger, transtorno desintegrativo da infância, transtorno de Rett e transtorno invasivo do desenvolvimento sem outra especificação (PDD-NOS) (Baio et al., 2018).

Devido à heterogeneidade na manifestação destes transtornos, o diagnóstico do TEA requer manifestação de déficits em dois domínios centrais: 1) déficits na comunicação social e interação social e 2) padrões repetitivos de comportamento, com interesses e atividades restritos. Ambos os domínios independem da manifestação de prejuízos intelectuais e de linguagem, sendo classificados em níveis de gravidade/severidade da manifestação (APA, 2013).

Em razão de se apresentar como uma doença com alta herdabilidade, um dos principais focos de pesquisa envolvendo o TEA é o seu provável fundo genético (Bai et al., 2019). Considerando que outras doenças genéticas se mostram associadas ao TEA, atualmente é amplamente aceito que o transtorno seja o resultado de interações entre fatores genéticos, epigenéticos e ambientais, incluindo os fatores associados ao sistema imunológico (Ivanov et al., 2015). Especificamente, idade paterna avançada (Wu et al., 2017), mudanças epigenéticas (Loke et al., 2015; Duffney et al., 2018), intercorrências gestacionais (Meltzer e Van der Water, 2017), uso de medicamentos como ácido valpróico (Nicolini e Fahnestock, 2018) e exposição a alergenos (Singer et al., 2016) durante a gestação, assim como desbalanços do microbioma (Kraneveld et al., 2016) são potenciais fatores contribuintes para a manifestação do TEA.

Como anteriormente citado, é atualmente consenso a contribuição de fatores genéticos para o desenvolvimento do TEA (Muhle et al., 2004; Michaelsonn et al., 2012; Vorstman et al., 2017). Segundo um trabalho recente envolvendo cinco países e mais de dois milhões de indivíduos diagnosticados com TEA, a contribuição de fatores genéticos

para essa condição é de aproximadamente 80% (Bai et al., 2019). Embora muitos estudos evidenciem a contribuição de muitos genes e variantes genéticas nos casos de autismo, até o momento nenhum padrão genético que, isoladamente, explique o quadro clínico foi identificado. Nesse contexto, abordagens envolvendo estudos genéticos de associação em larga escala (GWAS – *Genome Wide Association Studies*) têm sido realizados na tentativa de encontrar variantes em genes que podem atuar como fatores de risco para o TEA (Jiang et al., 2013). Salienta-se, ainda, que mutações genéticas únicas contribuem para 1-2% dos casos de TEA (Abrahams e Geschwind, 2008). Dessa forma, estudos genéticos em trios de famílias de diferentes populações abordando genes candidatos são, também, uma excelente ferramenta para avaliar a influência de polimorfismos nessa condição clínica complexa e multifatorial.

Considerando as alterações imunes presentes no TEA, o estudo de variantes em genes relacionados ao sistema imune contribui para o entendimento tanto da susceptibilidade quanto das diferentes manifestações clínicas da doença. Nesse contexto, diversos estudos abordando imunogenética e o TEA já foram realizados e estão apresentados detalhadamente no Capítulo V desta tese.

1.2.1. *Alterações Imunológicas na Gestação e Transtorno do Espectro Autista*

Originalmente, o TEA é referenciado como um transtorno neurocomportamental, porém, cada vez mais evidências indicam uma forte participação do sistema imune nesta condição (Masi et al. 2017; Meltzer e Van de Water, 2017). Assim, as interações entre o sistema imune e o ambiente parecem ser importantes não apenas após o nascimento do indivíduo, mas também ao longo do desenvolvimento fetal, situação já descrita como MIA (do inglês *maternal immune activation*, ativação imune materna), a qual já foi testada em modelos experimentais com camundongos e macacos rhesus (Meltzer e Van de Water, 2017).

Alterações nos níveis de citocinas no sangue de mães de crianças com TEA durante a gestação destacam-se entre os componentes imunes já descritos no contexto do TEA. Além disso, intercorrências gestacionais com fundo imunológico já foram associadas com a manifestação aumentada de TEA nos filhos de gestantes afetadas por tais intercorrências (Zerbo et al., 2017; Meltzer e Van de Water, 2017; Maher et al., 2018). Nesse contexto, já

foram relatados níveis aumentados de IFN-γ, IL-4 e IL-5 durante a gestação (Goines et al., 2011), além de um maior histórico de doenças auto-imunes em mães de filhos com TEA (Croen et al., 2005). Além disso, auto-anticorpos maternos contra proteínas cerebrais da criança já foram encontrados durante o período de gravidez. Experimentos com esses auto-anticorpos já foram conduzidos em camundongos. Fêmeas gestantes foram injetadas com auto-anticorpos provenientes de fêmeas que haviam gerado prole com características autistas. Os resultados mostraram que tal exposição provoca alterações no comportamento exploratório e motor na prole das fêmeas teste (Dalton et al., 2003). No mesmo modelo utilizando camundongos, a exposição pré-natal a auto-anticorpos humanos provenientes de mães de filhos autistas provocou alterações de ansiedade, reflexos de sobressalto e alterações de sociabilidade nos filhotes nascidos das fêmeas expostas (Singer et al., 2009). Estudos posteriores, avaliando moléculas IgG de camundongos que tiveram prole com traços autistas, demonstraram a especificidade desses auto-anticorpos em relação a proteínas cerebrais de 37 e 73 kDa (Braunschweig et al., 2012). Atualmente, sabe-se que auto-anticorpos com este padrão de reconhecimento são encontrados em 12% das mulheres que tiveram filhos autistas. Corroborando esta observação, um estudo utilizando macacos rhesus como modelo demonstrou alterações no comportamento social e no tamanho cerebral da prole de fêmeas que foram expostas a essa classe de IgGs contra as proteínas cerebrais do feto (Bauman et al., 2013).

Também é importante salientar que a inflamação, seja ela subclínica, crônica, aguda ou patológica, é cada vez mais observada como envolvida na patogênese de transtornos psiquiátricos. Embora não seja reconhecido como uma doença autoimune, o TEA compartilha diversas características com essa classe de doenças, como a predisposição genética a anormalidades imunes e grandes disparidades em relação ao sexo dos afetados (Lyall et al., 2017). Por fim, considerando que vesículas extracelulares já foram implicadas no contexto do autismo e de outras doenças psiquiátricas (Tsilioni e Theoharides, 2018; Saeedi et al., 2019) e considerando o papel dessas vesículas nas respostas inflamatórias e modulações do sistema imune, é possível que as vesículas extracelulares desempenhem um papel chave na susceptibilidade e/ou nas diferentes manifestações clínicas do TEA.

Considerando que alterações em respostas inflamatórias faz parte tanto (I) do quadro clínico de problemas gestacionais, (II) das possíveis causas para o desenvolvimento de TEA e (III) do quadro clínico do TEA, justifica-se o desenvolvimento da presente tese.

OBJETIVOS

Objetivo geral

Abordar de forma integrada os principais fatores imunogenéticos e ambientais que contribuem para o desenvolvimento de uma gestação de sucesso e/ou de intercorrências gestacionais, além de discutir como fatores inflamatórios e imunogenéticos podem ser fatores de risco para o desenvolvimento do Transtorno do Espectro Autista.

Objetivos específicos

- Correlacionar e resumir o papel da interface materno-fetal na tolerância do sistema imune materno em relação ao feto e os processos que modulam o trânsito de patógenos na interface materno-fetal, com ênfase na participação de exossomos em ambos processos.

- Abordar a molécula HLA-G e sua importância no estabelecimento e manutenção da gestação através da proposta de uma prova de conceito de um método contraceptivo baseado na regulação negativa dessa molécula no ambiente uterino.

- Comparar os níveis de citocinas Th1/Th2/Th17 presentes na circulação periférica de mulheres que sofreram aborto com os níveis dessas moléculas em um grupo de mulheres grávidas sem intercorrências gestacionais.

- Avaliar o impacto da deleção completa do gene *NKG2C* em mulheres que sofreram pré-eclâmpsia, em comparação com mulheres que tiveram gestação normotensiva.

- Avaliar o impacto da variante genética CCR5Δ32 em mulheres que sofreram pré-eclâmpsia, comparado com mulheres que tiveram gestação normotensiva.

- Revisar, apresentar e discutir o papel de variantes de genes relacionados ao sistema imune no contexto do Transtorno do Espectro Autista.

- Descrever e relacionar os fatores imunológicos envolvidos na inflamação durante a gestação que são considerados fatores de risco para o desenvolvimento do Transtorno do Espectro Autista.

- Avaliar o impacto da deleção completa do gene *NKG2C* em indivíduos diagnosticados com Transtorno do Espectro Autista e seus respectivos pais biológicos, abordando a sintomatologia dos pacientes e a transmissão dessa deleção dos pais para os filhos.

- Avaliar o impacto de polimorfismos nos genes *NKG2D* e *NKG2A* em indivíduos diagnosticados com Transtorno do Espectro Autista e seus respectivos pais biológicos, abordando a sintomatologia dos pacientes e a transmissão dessa deleção dos pais para os filhos.

Parte I

Capítulos I, II, III e IV

Capítulo II

Extracellular vesicles in host-pathogen interactions and immune regulation—exosomes as emerging actors in the immunological theater of pregnancy

Valéria de Lima Kaminski, Joel Henrique Ellwanger, José Artur Bogo Chies

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Review Article

Extracellular vesicles in host-pathogen interactions and immune regulation — exosomes as emerging actors in the immunological theater of pregnancy

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ABSTRACT

This review correlates and summarizes the role of the maternal-fetal interface in the immune tolerance of the fetus and the processes that lead to infection avoidance, emphasizing the participation of exosomes and other extracellular vesicles in both situations. Exosomes are released into the extracellular medium by several cell types and are excellent carriers of biomolecules. Host-derived exosomes and the transport of pathogen-derived molecules by exosomes impact infections in different ways. The interactions of exosomes with the maternal immune system are pivotal to a favorable gestational outcome. In this review, we highlight the potential role of exosomes in the establishment of an adequate milieu that enables embryo implantation and discuss the participation of exosomes released at the maternal-fetal interface during the establishment of an immune-privileged compartment for fetal development. The placenta is a component where important strategies are used to minimize the risk of infection. To present a contrast, we also discuss possible mechanisms used by pathogens to cross the maternal-fetal interface. We review the processes, mechanisms, and potential consequences of dysregulation in all of the above-mentioned phenomena. Basic information about exosomes and their roles in viral immune evasion is also presented. The interactions between extracellular vesicles and bacteria, fungi, parasites and proteinaceous infectious agents are addressed. The discovery of the placental microbiota and the implications of this new microbiota are also discussed, and current proposals that explain fetal/placental colonization by both pathogenic and commensal microbes are addressed. The comprehension of such interactions will help us to understand the immune dynamics of human pregnancy and the mechanisms of immune evasion used by different pathogens.

1. Introduction

In humans, recognition of self and nonself antigens, tissues or even whole organisms encompasses both local and systemic immune reactions. In the context of pregnancy, the intimate contact of fetal cells and maternal immune cells and tissues represents a substantial immune challenge. The maternal immune system must be shaped to tolerate the developing fetus, which can be compared to a semiallogeneic graft (Trowsdale and Betz, 2006; Vianna et al., 2011; Svensson-Arvelund et al., 2015). The search for factors involved in such immune adaptation has led many researchers in the field of reproductive immunology to examine the new universe of extracellular vesicles.

Extracellular vesicles (EVs) are secreted by cells from all eukaryotes and by prokaryotic organisms through shedding mechanisms (Colombo et al., 2014). Various biological fluids contain EVs, which can cross physical and physiological barriers and perform essential roles in cell-to-cell communication. Thus, EVs are critical modulators of the immune response under normal and pathological conditions (Nair and Salomon, 2018). EVs are usually classified according to their size and tissue or cell of origin (Colombo et al., 2014). However, it is difficult to assume the origin of a specific EV unless it is captured at the time of shedding by adequate imaging techniques. Therefore, it is now strongly recommended that operational terms encompassing size, shape, and biochemical composition be used for identifying EV subtypes (reviewed in Théry et al., 2018).

The term "EVs" encompasses microparticles, microvesicles (MVs), nanovesicles, nanoparticles, ectosomes, exosomes, exovesicles, and exosome-like vesicles (Colombo et al., 2014). The diversity of EVs, in terms of origin and function, makes an individual classification difficult for each type, and EVs have usually been differentiated based on their size, cargo, and origin (Nair and Salomon, 2018). Although we are in agreement with the recommendation of MISEV2018 (Minimal

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information for studies of extracellular vesicles 2018) that "terms such as exosome and microvesicle that are historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis" (Théry et al., 2018), the purpose of this review is to present an overview of the role of exosomes in host-pathogen interactions and immune regulation in human pregnancy; therefore, the nomenclature for the EVs will be identical to that used by the authors of the original articles.

One specific group of EVs, exosomes, has received special attention, mainly due to its reported roles in both normal pregnancies as well as in pregnancy-related disorders (Mitchell et al., 2015). Exosomes are released into the extracellular space by virtually all types of viable cells and have a prominent function in intercellular and intracellular communication and as biomolecule carriers (Théry et al., 2002a). Different cell types release distinct types of exosomes under healthy and pathological conditions (Corrado et al., 2013). Exosomes in healthy pregnancy are known for their ability to induce and maintain, at least partially, a local immunosuppressive environment at the maternal-fetal interface (Hedlund et al., 2009). This ability is fundamental to the control of the maternal immune responses that would otherwise be harmful to the "semiallogeneic" fetus (Mincheva-Nilsson and Baranov, 2010; Mitchell et al., 2015).

It seems plausible that such intimate contact between mother and fetus, allied to local immune modulation, creates a scenario that favors infection of both individuals; however, with some exceptions, viral infections during pregnancy have been considered low-risk conditions until recently (Silasi et al., 2015). Even with placental control of both the mother's immune reactions and pathogen infections, microbes have found ways to bypass the placenta (Silasi et al., 2015; Coyne and Lazear, 2016). In addition, the findings revealing a healthy, normal microbiota in the placenta (Aagaard et al., 2014; Parnell et al., 2017; Seferovic et al., 2019) led to the following question: how do these organisms or their genetic material get in contact with the fetus before birth? The mechanisms that modulate the immune system and the universe of extracellular vesicles will probably offer some hints to the answer.

It is no wonder that an intensely immunomodulated environment at the maternal-fetal interface can open a window to relatively easy development of viral or bacterial infections. Nevertheless, placental tissues have physiological characteristics that hinder the entry of pathogens (Arora et al., 2017). Such strategies, whether physical or molecular in nature, make the placenta a complex organ where nutrient and gas exchanges occur while immune responses must be carefully regulated. In this review, we address important aspects of the local immune adjustments that enable embryo implantation and acceptance, minimizing the risk of abortion/rejection, and call attention to the placental microbiota and its implications during pregnancy. In addition, some aspects of exosomes produced by cells from the male genital tract and specific facts about sexually transmitted infections during pregnancy will be discussed.

2. Main text

Immunomodulation – is there relevant systemic immune suppression in pregnancy?

That the maternal immune system is largely suppressed in pregnancy is an oversimplified concept. Several studies indicate that immune system activation is a crucial step for healthy pregnancy development. Thus, it is inappropriate to think that human pregnancy could develop better without immune responses even in the absence of pathogens (Mor et al., 2011). The first studies on the immunology of pregnancy were conducted in the 1950s when Sir Peter Medawar first questioned that the fetus was accepted by the maternal immune system. His group postulated that fetal acceptance by the maternal immune system was due to an anatomical separation of the fetus from the mother, antigenic immaturity of the fetus, and immunological indolence/inertness of the mother toward the fetus (Billingham et al., 1953). Since then, various publications have addressed immune functions and dynamics during the gestational period. Nevertheless, in the 1990s, human pregnancy was still viewed as a period of immune inertness. Since T helper type 2 (Th2) cells are classically considered to be involved in anti-inflammatory responses, it seemed plausible to classify, at that time, human pregnancy as a "Th2 phenomenon" (Wegmann et al., 1993). In this line, as T helper type 1 (Th1) cells and cytokines were predominantly linked to inflammatory situations, Th1 cell responses were classically related to pregnancy complications (Raghupathy et al., 2000; Piccinni, 2002), and inadequate physiological outcomes of pregnancy or unsuccessful pregnancies were considered the result of a "Th1 response."

A subtle inflammatory process that involves the presence of numerous immune cells is essential for successful implantation. However, inflammation at the implantation site, more than a response against the fetus, promotes tissue remodeling and enables embryo implantation (Mor et al., 2011). Additionally, it is important to emphasize that the blastocyst is highly adhesive and travels throughout the fallopian tube to the implantation site. The endometrium is extensively covered by molecules that avoid blastocyst implantation, and it has been hypothesized that cytokines and chemokines produced by macrophages and dendritic cells (DCs) promote the degradation of these molecules covering the implantation site (Mor et al., 2011).

Considering that inflammation is a process characterized by the presence of a large number of Th1 cells and molecules derived from these cells, parturition was considered to be a pro-inflammatory phenomenon (Romero et al., 2006). Supporting this idea, inflammation has been detected in the cervix, myometrium, chorioamniotic membranes, and amniotic cavity of women in labor. Prior to parturition, there is a large influx of immune cells to the myometrium, creating an inflammatory profile and culminating in uterine contractions and delivery (Romero et al., 2006). Initially, it was believed that if such an inflammatory profile happened early in the gestational period, preterm delivery, miscarriage or other pregnancy complications would follow as a logical consequence (Wegmann et al., 1993; Raghupathy et al., 2000; Piccinni, 2002), but later, the need for a more complex Th1/Th2 balance was observed (Chaouat et al., 2002). These observations led to a new paradigm suggesting that slight inflammation at the beginning of pregnancy is followed by a longer period featured mainly by anti-inflammatory characteristics. Finally, signaling created by high levels of inflammation was expected near and at delivery. Since the description of this theory, several studies have supported it, but others have also contradicted the described dichotomy (Wegmann et al., 1993; Hill et al., 1995; Piccinni, 2002; Raghupathy et al., 2000; Chaouat et al., 2002; Mor et al., 2011).

Given such a dynamic balance at all phases of pregnancy, systemic immune suppression in pregnancy was doubted. How could an immune-suppressed organism control the dynamic fluctuations of cytokines and the migration, differentiation, and proliferation of so many immune cell types? The answer to this question is based on the absence of strong systemic immune suppression in pregnancy. Instead, tight immune regulation involves numerous cell types and molecules. Currently, human pregnancy is considered a very complex and meticulously regulated immune process. The description of a new set of cytokine-producing cells that did not fit the Th1/Th2 profile was essential to changing this pre-established paradigm (Chaouat et al., 2002; Zenclussen et al., 2002). Studies revealing the role of T regulatory (Treg) and Th17 cells and the molecules they produce during the gestational period are examples of recent discoveries that challenged the dichotomous Th1/Th2 view of human gestation (Saito et al., 2010). In this context, we highlight Interleukin-17 (IL-17), a pro-inflammatory cytokine that induces the expression of several inflammatory mediators (Witowski et al., 2004) and is produced mostly by T cells (Th17 cells) (Fu et al., 2014). Importantly, IL-17 has been shown to induce the production of proangiogenic molecules and to favor neovascularization (Numasaki et al., 2003). Concerning the human maternal-fetal interface, it was already been demonstrated that decidual cells recruit peripheral Th17 cells into the decidua by secreting CCL2 (Wu et al., 2014). At this location, Th17 cells

promote the proliferation and invasion of human trophoblast cells through the secretion of IL-17, which also inhibits apoptosis during the first trimester of pregnancy (Wu et al., 2014). Moreover, it had already been observed that IL-17 levels continuously increase throughout pregnancy (Martínez-García et al., 2011; Kaminski et al., 2018). In the context of mammalian pregnancy evolution, IL-17 can be assumed to be one of the molecules responsible for the maintenance of prolonged periods of gestation (Fu et al., 2014). The absence of IL-17A in marsupials suggests that it is an essential signaling molecule for the maintenance of the prolonged pregnancy observed in eutherian mammals, which differs from that of marsupials (Chavan et al., 2017). Of note, a new "Th1/Th2/Th17 and Treg" cell paradigm of pregnancy has been suggested and is the current trend. In this context, Th17 cells favor implantation and induce a protective immune response against microbes by the induction of inflammation, and Tregs, in contrast, are important for the immunoregulation and induction of tolerance (Saito et al., 2010).

Exosomes

As previously described, the term EV refers to exosomes (30–150 nm in diameter), microvesicles (0.1–1 μm) and apoptotic bodies (0.5–5 μm) released during both pathologic and healthy physiological situations (De Toro et al., 2015; Lo Cicero et al., 2015; Yáñez-Mó et al., 2015). Exosomes originate from multivesicular bodies (MVBs) and are formed by a lipid bilayer derived from their cells of origin. Various biomolecules, such as proteins and nucleic acids, are found attached to the lipid bilayer and/or inside the exosomes (Théry et al., 2002a). The secretion of proteins and nucleic acids through exosomes confers interesting features and advantages to this process: (I) the three-dimensional structure and biological role of the cargo molecules are preserved; (II) the delivery of molecular signals can occur independently of direct cell-cell contact; (III) the concentration of specific proteins inside exosomes can be maintained at high levels; (IV) the accurate delivery of biomolecules to the target (due to specific surface markers) is assured and can be achieved with long distances between the cells; and (V) *de novo* secretion in the target cell is not necessary (Mincheva-Nilsson and Baranov, 2010).

Due to the diversity of the cargos and target cells, exosomes can interfere with distinct pathways and affect different body systems. Taking into account the interests specific to the present review, it is important to emphasize that exosomes can act as modulators of immune responses. In this sense, exosomes derived from antigen-presenting cells have immune-activating properties (Théry et al., 2002b; Hwang et al., 2003). Additionally, syncytiotrophoblast-derived exosomes from non-pathological human placenta seem to participate in pathogen infection resistance pathways, although they can be immune suppressive or tolerogenic, such as exosomes from the majority of tumors and epithelial cells (Karlsson et al., 2001; Andreola et al., 2002; Mincheva-Nilsson and Baranov, 2010).

There is great debate over the most appropriate methods for isolating and characterizing exosomes. Diverse EV isolation techniques can be found in the original articles cited throughout this review. These methods are mainly based on differential and/or density gradient ultracentrifugation, size-based isolation techniques, coprecipitation, and immunoaffinity enrichment.

The most widely used exosome isolation technique is ultracentrifugation, which is considered the gold standard method. Ultracentrifugation isolation is based on the weight and size of the exosomes, and its low cost presents a major advantage over the other available methods; however, the exosome recovery is low. Size-based methodologies (which also consider molecular weight) produce a high yield through rapid processing; however, they lack specificity and require specific equipment, which are disadvantages. Based on the surface proteins present in the exosomes, the fastest and easiest method to isolate them is coprecipitation, which is characterized by high cost, low recovery, and a relative lack of specificity. At high cost and with low recovery capacity, the method of immunoaffinity enrichment recovers many exosomes of high purity (Bu et al., 2019).

Such techniques vary in adequacy depending on the sample of interest and are in continuous need of improvement (Bu et al., 2019). Considering these variables, we highlight the importance of following the latest proposals from the International Society for Extracellular Vesicles that are featured in MISEV2018 (Théry et al., 2018), the gold standard reference that presents the latest scientific advances for better handling of samples, from collection to storage, and are quite suitable for use with cell culture, biological fluids, or tissues.

An overview of exosome isolation methods is shown in the studies addressing placental exosomes from maternal circulation. For example, enriched fractions of these specific nanovesicles with minimal "contamination" from other EVs can be obtained through methods based on the proposed use of buoyant density centrifugation (Salomon et al., 2014; Sarker et al., 2014) and immunoaffinity capture using antibody-conjugated agarose beads (Lai et al., 2018). Alternatively, some studies have obtained exosomes from the supernatant of placental explant cultures using sequential centrifugation and ultracentrifugation, followed by identification and characterization by Western blotting, immune electron microscopy, and immuno-flow cytometry based on the proteins expressed on the surface of the isolated placental exosomes (Hedlund et al., 2009; Stenqvist et al., 2013). It is also important to consider the following limitation: most isolation methods cannot ensure the complete purity of the obtained vesicles, and it is possible to coisolate other nontargeted EVs and viral particles with the desired exosomes (Ellwanger et al., 2017).

To date, the most studied exosome markers are ALIX, TSG101, CD9, CD63, and CD81 (Ellwanger et al., 2017). However, the list of exosome markers is continuously revised, with new markers being incorporated at the same time that previously established markers are considered not sufficiently specific for exosomes. The direct consequence of such a dynamic research field is that different studies use different markers to identify exosomes. Thus, it is always a challenge to know when the authors actually worked with exosomes or with another type of extracellular vesicle (Ellwanger et al., 2017). Taking this into consideration, although we use the term "exosomes" throughout this review, it is important to emphasize that the data discussed here can possibly extend to the other types of EVs described in the literature collectively as "exosomes." In an attempt to clarify this situation, web portals have been organized through which researchers are working together to better classify the different subsets of extracellular vesicles, including exosomes (Kim et al., 2015).

The maternal-fetal interface: a site of intense immune regulation accounting for exosomes and other EVs

Just after fertilization of the oocyte by the spermatozoon, the binding and fusion of the sperm cell and oocyte membranes promote oocyte changes that block polyspermic fertilization and drive the resumption of oocyte meiosis (Capmany et al., 1996). At 24h post fertilization, parental chromosomes have intermixed, and the first cellular division occurs. Messenger RNA (mRNA) synthesis is absent as the initial cells divide, apparently driven exclusively by the maternal cytoplasmic signals, an event designated as the 'maternal legacy' (Braude et al., 1988). Such maternal signals could originate from maternal mitochondrial DNA, which replicates during early embryonic cell division. The point at which the paternal genome is activated and undergoes transcription is called zygotic gene activation and is first detected in embryos 2–3 days after fertilization (Braude et al., 1988). From this moment, the mother's immune system addresses the emergence of nonself antigens (those of paternal origin) to enable adequate fetal development. During pregnancy, the uterine environment promotes tolerance in relation to the developing fetus, avoiding maternal rejection of the fetal allograft, and it has been suggested that exosomes may be pivotal in the establishment of such an immune-privileged environment (Hedlund et al., 2009; Stenqvist et al., 2013).

The uterine receptiveness to blastocyst implantation is modulated by cyclic secretion of estradiol, progesterone, and human chorionic gonadotropin — the first known hormonal signals of the conceptus. These hormones regulate growth factors, cytokine production and the expression of the adhesion molecules that alter the endometrial surface, openning an implantation window (Gude et al., 2004; Makrigiannakis et al., 2017). The blastocyst is composed of two cell types with an inner and an outer cell mass. The inner cell mass develops into the fetus. The outer cell mass consists of undifferentiated trophoblast stem cells that form the cytotrophoblast (CTB) and the syncytiotrophoblast (STB). Before attachment, the zona pellucida is lost, and the trophoblast cell layer rapidly proliferates and differentiates into an inner layer, the CTB, and in an outer multinucleated mass, the STB (Gude et al., 2004). Subsequently, the STB extends into the endometrial epithelium and invades the connective tissue, breaking through the endometrial surface, provoking the natural tissue damage that ultimately enables implantation. Thus, the uterine endothelium and vascular smooth muscles of the mother's blood vessels are gradually replaced by trophoblast cells, creating the optimal conditions for initiating and developing the placental-fetal blood supply (Gude et al., 2004; Mor et al., 2011).

After the development of spiral arteries due to remodeling of the mother's blood vessels, the human placenta becomes a hemochorial villous organ. Such proximity enables the maternal blood to come into direct contact with the placental trophoblast cells (Gude et al., 2004). The functions of the placenta range from the exchange of nutrients, gases and metabolic residues to the production of regulatory molecules, which reveals its role as an immunomodulatory organ. The formation of the placenta starts at the implantation of the blastocyst into the uterine mucosa within 5–6 days post fertilization (Cross et al., 1994). The placenta is composed of two types of villi, the floating villi, which comprise an inner layer of CTB covered by the STB, which is bathed in maternal blood at the intervillous space, and the anchoring villi, which attach to the decidual tissue by highly invasive CTB cells referred to as extravillous trophoblasts (EVTs) (Hamilton and Boyd, 1960). The placental villous surface is in direct contact with maternal blood through the STB in a compartment that enables nutrient and oxygen supplementation and metabolic residue and cell debris removal and that, at the same time, hinders the passage of potential pathogens from the maternal circulation to the fetus (Gude et al., 2004; Delorme-Axford et al., 2013; Arora et al., 2017). Of note, in the STB, it is possible to find physical and molecular functions that ultimately block immune activation at the maternal-fetal interface (Robbins and Bakardjiev, 2012).

In this environment of intimate contact between the uterine region and the placenta, the exosomes secreted in the maternal blood are possibly the most abundant in the intervillous space of the chorionic villi. Moreover, the continuous release of exosomes by the STB creates an exosomal concentration gradient, accounting for stronger protection against an exacerbated maternal immune response at the maternal-fetal interface. It is said that the fetus, together with the placenta, is surrounded by a “cloud of exosomes” (Mincheva-Nilsson, 2010). Importantly, the concentration of the placenta-derived exosomes in the maternal blood increases during a healthy pregnancy (Salomon et al., 2014). In this context, it has been suggested that the concentration of placenta-derived exosomes in maternal blood could also be a potential marker of abnormal placentation (Kshirsagar et al., 2012). Trophoblast-derived exosomes could regulate the recruitment and differentiation of monocytes into tissue macrophages by inducing them to secrete the cytokines and chemokines required for trophoblast growth and survival (Atay et al., 2011). Placenta-derived exosomes lack the classical major histocompatibility complex (MHC) expression, presenting with the same characteristics as their tissue of origin. Instead, they express the nonclassical molecules MICA/B and RAE-T1/ULBP1–5, ligands of the activated Natural Killer (NK) cell receptor NKG2D (Mincheva--Nilsson et al., 2006; Hedlund et al., 2009).

The decidua is the mucosal layer of the resulting pregnant uterus. It comprises the endometrial/decidual glands, blood vessels, and the decidual stroma. Furthermore, leukocytes represent approximately 15–30% of all the cells in the early pregnant decidua of humans (Mincheva-Nilsson et al., 1994). The organization of decidual lymphoid tissue is unique and includes lymphoid cell clusters, subepithelial lymphoid cells, and individual immune cells that are randomly distributed. B cells are absent or rare; instead, there are abundant uterine NK (uNK) cells, αβ T and γδ T cells, DCs, and macrophages. Interestingly, uNK cells represent up to 70% of decidual leukocytes in the first trimester (Moffett-King, 2002), while there is no consensus regarding the distribution and number of uNK cells in later stages of gestation (Lash et al., 2010).

Immunomodulation in human pregnancy: tolerance is enhanced by exosomes and other EVs

Early studies on human preimplantation embryos reported the absence of expression of MHC class I or II genes (Roberts et al., 1992). The villous trophoblast, exposed to maternal blood, seems to lack expression of both MHC class I and class II proteins. However, the EVTs, which invade the uterus, express a particular combination of four MHC class I molecules: the classical HLA-C and the nonclassical class I molecules, HLA-E, HLA-F, and HLA-G (Moffett-King, 2002; Hackmon et al., 2017). The expression of HLA-E in EVTs enables them to evade NK cell-mediated cytotoxicity (King et al., 2000). Regarding HLA-G, it was first proposed that its expression in trophoblast cells also protected the fetus from maternal NK cell cytotoxicity, but this proposal is being debated, as HLA-G can induce secretion of cytokines and proangiogenic factors from decidual NK cells and human decidual antigen-presenting cells (APCs) *in vitro* (van der Meer et al., 2004; Li et al., 2009). Thus, it seems that HLA-G contributes to fetal tolerance by modulating decidual NK cell and APC responses (LeMaoult et al., 2004). Such nonclassical MHC molecules are of key importance not only in the establishment of the pregnancy but also in its maintenance, as revealed by studies in both healthy and pathological pregnancies (Tripathi et al., 2006; Michita et al., 2016; Persson et al., 2017; Meuleman et al., 2018). Notably, it has been shown that immunomodulatory molecules from the B7 family and the soluble HLA-G isoform HLA-G5 are secreted from the placenta during the first trimester and at term via exosomes (Kshirsagar et al., 2012).

As previously described, blastocyst implantation requires an inflammatory environment (Fest et al., 2007). The implantation process is achieved via the proper interaction of the innate uterine immune cells with the invading trophoblast (Huppertz et al., 1998; von Rango et al., 2003; Shih et al., 2006). During implantation, the uNK cells are pivotal for trophoblast invasion, and their absence is a predictor of poor vascularization of the placenta and pregnancy interruption (Hanna et al., 2006). Additionally, depletion of DCs leads to blastocyst implantation failure and prevents decidual development, likely due to failure in uterine receptivity; in healthy pregnancy, DCs orchestrate uterine receptivity through the regulation of tissue remodeling and angiogenesis (Placks et al., 2008).

In addition, equal to the importance of the role played by decidual leukocytes, cellular receptors and other molecules are important for fetal tolerance. For example, indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the degradation of tryptophan, leading to cell starvation and inhibition of T cell proliferation. Interestingly, in the human blastocyst, IDO is detected from day 6 (Kudo et al., 2004). Furthermore, immunohistochemical analysis of mice revealed the presence of IDO throughout pregnancy (Shayda et al., 2009).

Genetic variants have also been reported as important in the likelihood of different pregnancy outcomes. For example, different gene polymorphisms can impact human gestation in such ways that can enhance or attenuate inflammation-related pregnancy disorders (Michita et al., 2016, 2018; Kaminski et al., 2019).

Membrane-associated and secreted immune regulatory factors are widely produced and secreted by placental tissues and are also detected in the maternal serum during the gestation period. For example, expression of Fas and FasL can be detected in the villous CTB and in the

STB (Pongcharoen et al., 2004; Frängsmyr et al., 2005); early pregnancy factor (EPF) activity was detected in sera from pregnant women (Fan and Zheng, 1997); progesterone-induced blocking factor (PIBF), which blocks lytic NK cell activity, was observed in term placentas (Anderle et al., 2008); and a variety of cytokines are expressed and tightly regulated both locally and systemically throughout pregnancy (von Rango et al., 2003). An important role for the Leukemia Inducing Factor (LIF) in implantation was shown in *LIF*-knockout mice, in which embryos failed to implant (Stewart et al., 1992). Notably, LIF is a secreted glycoprotein belonging to the interleukin 6 (IL-6) family, with pleiotropic effects that include the induction of the proliferation, differentiation, and survival of trophoblast cells (Aghajanova, 2004).

Complement activation at the maternal-fetal interface is avoided, or at least minimized, by the expression of the complement regulatory proteins CD59, MCP, and DAF in the placenta. These proteins are expressed on the trophoblast from at least 6 weeks of gestation, and their presence at the maternal-fetal interface (Holmes et al., 1992) implies a pivotal role in the maintenance of pregnancy, probably by protecting the developing fetus against maternal complement-mediated immune responses.

As previously stated, the human placenta constitutively expresses Fas and Fas-ligand (FasL) (Abrahams et al., 2004; Stenqvist et al., 2013). These molecules participate with other factors that regulate the maternal immune system. Fas is a type I membrane protein that is a member of the tumor necrosis factor (TNF) receptor family and is expressed by a wide variety of cells. FasL is a type II transmembrane protein expressed by activated T cells, some tumor cells and epithelial and other cells at immune-privileged sites (Ferguson and Griffith, 2006). Cross-linking of Fas expressed on the cell surface with its natural ligand FasL (also called CD95L) induces apoptosis (Nagata and Golstein, 1995). The FasL protein and mRNA transcripts were detected in human term placenta, with higher expression being detected in the STB layer (Uckan et al., 1997). It has been suggested that Fas determinants are expressed in lymphocytes when maternal lymphocytes are activated by fetal antigens. In this case, the interaction of the activated Fas-expressing T lymphocytes with the Fas-expressing STB cells would lead to apoptosis of the activated maternal lymphocytes, thus mitigating or preventing inflammatory responses towards the fetus (Uckan et al., 1997). Subsequent to this proposal, more studies confirmed the role of Fas and FasL in the human placenta. Apoptotic leukocytes, mainly T lymphocytes, can be seen at the maternal-fetal interface, strongly suggesting that these cells are negatively affected by immune suppression (Mor et al., 1998; Hammer and Dohr, 2000). In this context, it was proposed that clonal deletion of the activated immune cells through the Fas/FasL apoptotic pathway is involved in the establishment of the immune-privileged maternal-fetal interface (Runic et al., 1996; Uckan et al., 1997; Ohshima et al., 2001). In summary, the activated maternal lymphocytes that express the Fas receptor will undergo apoptosis when they interact with the FasL-expressing trophoblast. Experimental data demonstrate the importance of such molecules in human pregnancy. Abrahams et al. (2004) showed that, despite the absence of membrane-associated FasL in isolated first-trimester trophoblast cells, a cytoplasmic form of FasL is expressed in association with a specialized secretory lysosomal pathway (Abrahams et al., 2004). Later, this association was further investigated, and the abovementioned FasL association was found to be related to placenta-derived exosomes (Stenqvist et al., 2013).

TNF-Related Apoptosis-Inducing Ligand (TRAIL) is a type II membrane protein detected in a variety of tissues. TRAIL is constitutively expressed by the placenta, and its receptors DcR1 and DcR2 are located predominantly in the STB, and DR4 and DR5 are preferentially found in the CTB (Bai et al., 2009). DcR1 and DcR2 act as decoy receptors, while DR4 and DR5 are death receptors that are responsible for activating the apoptotic pathway (Truneh et al., 2000). TRAIL activates intracellular apoptotic pathways in a way similar to that of FasL, indicating a potential functional redundancy between these two ligands. Thus, TRAIL has been proposed, together with FasL, to be a cooperating factor for inducing apoptosis in activated lymphocytes (Wiley et al., 1995; Bai et al., 2009). These two molecules represent the most important apoptosis pathways and can be observed in the human placenta throughout pregnancy, where they participate in important processes such as trophoblast invasion and differentiation (Huppertz et al., 1998; Mor et al., 2002) and in the development of maternal immune tolerance towards the fetus (Phillips et al., 1999; Mincheva-Nilsson et al., 2000; Clark, 2005).

Importantly, the intracellular localization of FasL and TRAIL in the human placenta is intimately connected to the biogenesis of exosomes. These molecules, in their membrane form, are associated with induction of apoptosis. The observation of a constitutive release of FasL- and TRAIL-expressing exosomes from the apical microvillous surface of the STB suggests an important role of such structures in the protection of the fetus from maternal lymphocytes (Stenqvist et al., 2013). This finding is in accordance with the first demonstrations showing that FasL is targeted to the MVB of the secretory lysosomes and is expressed on exosome-like microvesicles (Martínez-Lorenzo et al., 1999; Jodo et al., 2000; Mincheva-Nilsson et al., 2000; Monleón et al., 2001; Andreola et al., 2002; Smith et al., 2003; Frängsmyr et al., 2005).

It is also important to consider the regulation of NK cell activity during pregnancy. In this context, both activating and inhibitory receptors should be taken into account. For example, the receptor Natural Killer Group 2 Member D (NKG2D) is a type II transmembrane protein belonging to the C-type lectin-like family and is expressed on the surface of NK, NKT, αβ T, and CD8+ γδ T cells. In these cells, NKG2D acts as an activating receptor (Bauer et al., 1999). NKG2D ligands are divided into two families: the MHC chain-related proteins A and B (MICA and MICB, respectively) and the UL16-binding protein (ULBP) 1–6, which is also known as retinoic acid early transcript 1 (RAET1). These ligands are distantly related to MHC class I molecules and are themselves signals of cellular stress instead of antigen-presenting molecules (Stern-Ginossar and Mandelboim, 2009). NKG2D stands out as a major activating NK cell receptor, and its ligand/receptor system is a potent inducer of cytotoxicity through a mechanism directed to the elimination of stressed, foreign, transformed or infected cells.

NKG2D ligands are expressed at low levels in normal cells. However, NKG2D ligands are upregulated or expressed *de novo* in response to a great variety of biological stress signals, such as those triggered by DNA damage, irradiation, oxidative stress, and inflammation, as a strategy to display stress, danger or pathological conditions in the cell (Raulet, 2003). Soluble NKG2D ligands downregulate the cognate receptor, suppress cytotoxicity and, upon release from tumors, protect tumor cells from host immune attack through an evasion strategy (Groh et al., 2002; Song et al., 2006). Interestingly, the release of soluble NKG2D ligands has been associated with exosomes in the context of cancer (Clayton et al., 2008) and pregnancy (Mincheva-Nilsson et al., 2006). MIC proteins A and B, the human ligands of the receptor NKG2D, are expressed by the placenta, delivered to the MVB of the STB and released via MIC-bearing exosomes into the circulating blood. In sera from pregnant women, a constitutive MIC is produced and released in its soluble form by the STB. It was suggested that this MIC release is associated with placenta-derived exosomes. Notably, the soluble MIC is able to downregulate the NKG2D receptor on peripheral blood NK cells and T cells, impairing NKG2D-mediated cytotoxicity (Mincheva-Nilsson et al., 2006). The second family of human NKG2D ligands, ULBP, is also expressed by the placenta (Hedlund et al., 2009). Immunoelectron microscopy revealed that ULBP1–5 are produced and retained in the MVB of the STB on microvesicles/exosomes. In addition, it has been confirmed that exosomes bearing NKG2D ligands are released by the human placenta. The isolation of placental exosomes indicated their ability to carry ULBP1–5 and MIC on their surface and to induce the downregulation of NKG2D on NK, CD8+ and γδ T cells, which culminated in the reduction of their cytotoxic effects without affecting the perforin-mediated lytic apoptosis pathway *in vitro* (Hedlund et al., 2009). Placental delivery of NKG2D ligands via exosomes suggests a bioactive role for the soluble forms of these ligands (Hedlund et al., 2009). Such discoveries emphasize a role

for NKG2D ligand-bearing placental exosomes in the evasion of the fetus from the maternal immune responses and reinforce the view of the placenta as an important temporary immune organ.

It is noteworthy that placental EVs can also be pro-inflammatory (Holder et al., 2016; Tannetta et al., 2017a, 2017b). In agreement with the slight inflammation required in early pregnancy, the syncytiotrophoblast-derived EVs from the initial gestational periods have more inflammation-inducing characteristics than has been observed for EVs secreted by the term placenta (Tannetta et al., 2017a). During normal healthy pregnancy, the exosome concentration in plasma can be as much as 50-fold greater in pregnant women than in nonpregnant women, with levels increasing significantly with gestational age. Such an increase is observed for both placenta- and nonplacenta-derived exosomes (Salomon et al., 2014). Since the characteristics of EVs resemble the cell type from which they were derived, Tannetta et al. (2017b) reviewed and called attention to the potential use of STB-derived EVs from the maternal circulation in pregnancy monitoring. According to this suggestion, alterations in cellular responses would likely alter the EV content, thus enabling the identification of potential imbalances in tissues located in regions of the body where an optimal biopsy cannot be performed. Moreover, EV levels could be measured throughout gestation and in a personalized manner.

With regard to other important features worth noting, EVs are very transitory in the maternal circulation and do not accumulate such that the analysis of a sample would represent an up-to-date picture of "placental well-being" in terms of EV levels and molecular fingerprints. One great example of the applicability of this proposed monitoring tool involves cases of preeclampsia, which is an important heterogeneous pregnancy disorder with symptoms, which include systemic inflammation, that are triggered by the placenta because of its impaired functioning. Notably, it has been shown that release of microvesicles and nanovesicles from the placenta is greatly augmented in preeclampsia, and all fractions of such EVs from preeclamptic placenta can induce activation of endothelial cells, likely via sequestration of Vascular Endothelial Growth Factor (VEGF) by fms-kinase 1, a vasoactive factor (Tong et al., 2017); VEGF is a component of the EVs isolated from normal gestation (Tong et al., 2016) and is also found in high levels in the circulation of women with preeclampsia (Tannetta et al., 2017b).

Avoiding the vertical transmission of pathogens at the maternal-fetal interface

The defense mechanisms by which the placenta limits microbial access to the fetus are still unknown. Notably, the intervillous space could contain as much as 500 mL of maternal blood, exposing the villous surfaces to microbes present in the mother (Arora et al., 2017). In addition to the place where the placenta implants, the decidua basalis is also the location where the semiallogeneic fetal trophoblast is in direct contact with these maternal cells and acts on immune tolerance. It is believed that the decidua keeps its immune privileged condition because of its immune cell components. This composition limits lymphocyte access, and precise regulation of chemokine expression is responsible for controlling cell traffic (Red-Horse et al., 2004; Nancy et al., 2012). This regulated immune environment is maintained due to constant maternal-fetal cross-talk between the invading fetal trophoblast cells and various maternal immune cell subsets (Mor and Cardenas, 2010; Arck and Hecher, 2013; Erlebacher, 2013; Zenclussen, 2013).

The syncytial surface of the human placenta acts as a first line of protection with unique physical properties, such as the presence of dense, branched microvilli at the apical surface and a complex cortical actin network that might limit microbial invasion (Cantle et al., 1987; Fisher et al., 2000; Koi et al., 2001; McDonagh et al., 2004; Maidji et al., 2010; Robbins et al., 2010; Zeldovich et al., 2011, 2013). In this context, it was demonstrated that disruption of the actin cytoskeleton subtly facilitates the invasion of *Listeria monocytogenes* (Zeldovich et al., 2013), indicating the existence of direct physical barriers that restrict pathogen infections (Arora et al., 2017). In a healthy pregnancy, the STB layer is greatly resistant to infection by viruses such as human cytomegalovirus (HCMV), herpes simplex virus-1 (HSV1), and Zika virus (ZIKV), and other pathogens such as *L. monocytogenes* and *Toxoplasma gondii* (Fisher et al., 2000; Koi et al., 2001; Maidji et al., 2006, 2010; Robbins et al., 2010; Delorme-Axford et al., 2013; Bayer et al., 2015, 2016). In addition to relying on physical barriers, resistance could be acquired by transfer from the STB of a full-term placenta to the nonplacental cells in a paracrine manner. Experiments have shown that this transfer involves placenta-specific microRNAs (miRNAs) and type III interferons that are both packaged within exosomes (Delorme-Axford et al., 2013; Bayer et al., 2015, 2016; Ouyang et al., 2016).

In an interesting experiment, primary human trophoblast cells were infected with different RNA and DNA viruses — coxsackievirus B3 (CVB), poliovirus (PV), vesicular stomatitis virus (VSV), vaccinia virus (VV), HSV-1, and HCMV. The cells showed high resistance to these infections. Additionally, when nonplacental cells, normally permissive to these viruses, were cultured with a medium containing material isolated from naïve primary human trophoblast cells, the nonplacental cells also presented some degree of resistance to the infection. In fact, the authors showed that this antiviral profile was due to exosomes released by primary human trophoblast cells (Delorme-Axford et al., 2013). These exosomes contain miRNA members of the chromosome 19 miRNA cluster (C19MC) that are almost exclusively expressed in the human placenta (Noguer-Dance et al., 2010; Donker et al., 2012). The trophoblast-derived exosomes packing these miRNAs from C19MC are capable of attenuating viral replication in target cells by inducing autophagy, thus representing a striking evolutionary adaptation that enhances protection of the fetus against viral infections (Delorme-Axford et al., 2013). Another study demonstrated this attenuated infection by the human immunodeficiency virus (HIV)-1, varicella zoster, rubella and other togaviruses in nonplacental cells previously exposed to the same abovementioned trophoblast-conditioned medium, emphasizing that human trophoblast cells can confer resistance to viruses implicated in perinatal infection (Bayer et al., 2015).

Other molecules important to the immune response to pathogens should also be cited here. Interferons (IFNs) are pro-inflammatory cytokines that enhance adaptive immunity and antiviral responses (Schneider et al., 2014). According to Bayer et al. (2016), primary human trophoblast cells isolated from full-term placenta are resistant to infection caused by two strains of ZIKV. Exposure to the conditioned medium isolated from these cells conferred resistance against these same ZIKV strains to nontrophoblast cells, likely due to the release of IFNλ1; as a result, the ZIKV must evade this strong antiviral response or bypass these cells and use another mechanism to access the fetal compartment *in vivo* (Bayer et al., 2016).

Defensins are part of a large family of antimicrobial peptides (Ganz, 2003) directed against specific gram-negative and gram-positive bacteria, yeasts, filamentous-phase fungi, and enveloped viruses (Svinarich et al., 1997). At the transcriptional level, defensins are also present in the human placenta, amnion, and chorion, suggesting their participation in the protection of the fetus against pathogen infection (Svinarich et al., 1997).

Pathogen-associated molecular patterns (PAMPs) are microbe-derived molecules which act as critical regulators of the innate immune response (Medzhitov and Janeway, 1997) and can be a threat to the development of a healthy pregnancy. In this context, Koh et al. (2014) addressed the release of pro-inflammatory cytokines and the expression of *NF-kβ* gene by JEG-3 and BeWo human choriocarcinoma cell lines under the influence of lipopolysaccharide (LPS), a common PAMP recognized by the immune system. Interestingly, an elevated inflammatory response was observed in JEG-3 cells in comparison to the BeWo cell line, indicating that LPS influence trophoblast cells in different ways. Moreover, despite the lack of NF-kβ response in BeWo cells, this study corroborates that bacterial products such as LPS can trigger an inflammatory response in trophoblast cells, thus representing a risk factor for

pregnancy disorders like preterm labor. Toll-like receptors (TLRs) are, in humans, a family of ten molecules that recognize and respond to PAMPs. Both TLR-2 and TLR-4 are expressed by amniotic epithelial cells (Kim et al., 2004; Adams et al., 2007), and TLR-2 expression is limited to the basolateral side of these cells (Kim et al., 2004). In situations where inflammation occurs, this expression pattern is lost, and both TLR-2 and TLR-4 are upregulated. Decidual cells, decidual macrophages, and neutrophils also express TLR-2 and TLR-4 (Kim et al., 2004). Decidual cells from the first and second trimester express TLR-2 and TLR-4 (Krikun et al., 2007), and at term, these cells express TLR-1 and TLR-6 (Canavan and Simhan, 2007). Regarding mRNA expression, all ten TLRs have been identified in term placentas (Zarember and Godowski, 2002; Abrahams, 2008). In the first trimester, EVTs and villous CTB cells highly express TLR-2 and TLR-4. The STB lacks expression of TLRs; however, in the third trimester, expression of TLR-2 and TLR-4 can be found in the outer STB layer and in intermediate and EVT cells (Holmlund et al., 2002; Kumazaki et al., 2004; Ma et al., 2007; Rindsjö et al., 2007). This change in the TLR expression pattern shows the ability of the placental villi to promptly respond to an infection at the placental surface. Additionally, this shift in TLR expression could reflect changes in placental function throughout the gestational period and might suggest how infection can impact pregnancy at each trimester (Abrahams, 2008).

The trophoblast also expresses cytoplasmic-based Nod-like receptors (NLRs) (Costello et al., 2007). Nucleotide-binding Oligomerization Domain (NOD) proteins recognize peptides derived from the degradation of bacterial peptidoglycans during normal bacterial growth or destruction (Girardin et al., 2003). NOD proteins are thought to be a second line of defense in cases where TLR signaling is defective, reduced, absent or has been evaded (Abrahams, 2008). In the first trimester of pregnancy, NOD1 and NOD2 proteins are detected in the CTB and STB (Costello et al., 2007); in term placentas, only NOD1 expression has been observed (Abrahams, 2011). In the decidual stroma and glandular epithelium, NOD1 and NOD2 are also expressed (King et al., 2000).

Interestingly, transplacental trafficking of EVs from the mother to the fetus was also demonstrated. Holder et al. (2016) showed that macrophage-derived exosomes are internalized by the human placenta. This process likely occurs in a time- and dose-dependent manner via clathrin-dependent endocytosis. Such internalized exosomes have the ability to prompt the secretion of proinflammatory cytokines, thus potentially enhancing the responses to maternal inflammation and infection and thereby thwarting harm to the developing fetus. This is an important finding that indicates the existence of a highly controlled and bidirectional extracellular vesicle-mediated transfer of protein and nucleic acids that accounts for the balance of immune responses at the maternal-fetal interface (Holder et al., 2016).

The different pathways discussed here highlight the diverse immune mechanisms that protect the developing fetus from pathogen infections without invoking harmful immune responses. This process results in an immune uterine environment that must undertake controlled responsiveness such that a slight inflammatory state is created for embryo implantation. In a second, concomitant task of the uterine immune environment, immune responses are developed towards potential infections in such a careful manner that the first task is not disrupted.

Pathogens bypassing maternal-fetal immune defenses: an arms race in the biological world

The STB has been shown to be refractory to several infections. However, this feature seems to be almost exclusive to this cell type, since neither the amniotic epithelium and CTB cells of the chorionic villi isolated from mid- and late-gestation placentas nor explants from the first trimester showed such resistance (Tabata et al., 2016). Interestingly, experiments with ZIKV showed that early trophoblasts are quite susceptible to infection, but this susceptibility is lost as the STB is formed, with the trophoblast cells becoming increasingly resistant to ZIKV infection (Sheridan et al., 2017).

The classic ways of vertical infection of a developing fetus by pathogens are (I) infection of endothelial cells in the maternal microvasculature that spread to invasive extravillous trophoblasts (EVTs); (II) trafficking of infected maternal immune cells across the placental barrier; (III) paracellular or transcellular transport from maternal blood across the villous trees and into the fetal capillaries; (IV) damage to the villous tree and breaks in the STB layer; and (V) transvaginal ascending infection (Coyne and Lazear, 2016). However, except for the infections caused by some specific pathogens included in the TORCH group (toxoplasmosis, "other," Rubella, CMV, and HSV), viral infections during pregnancy are often considered of little concern from a clinical point of view. Women are frequently infected by viruses during pregnancy without severe consequences to their developing progeny (Silasi et al., 2015). Nonetheless, the recent ZIKV epidemic and the developmental problems related to ZIKV infection in newborns reveal the possible consequences of neglecting pathogens in pregnant women, including undesirable gestational outcomes and a concomitant high cost in terms of health services (Schuler-Faccini et al., 2016; Ellwanger and Chies, 2018).

When pathogens breach the STB and reach the underlying villous core, inflammation of the placental villous is the result. Such inflammation eventually induces monocyte binding to the syncytial surface through ICAM-1 (Juliano et al., 2006). This reaction can cause an immune-mediated breakdown of the STB, which creates damage that could predispose the individual to infections mediated by other pathogens (Mor and Cardenas, 2010).

As already discussed, there are two anatomical interfaces between maternal cells and fetal cells — the trophoblasts, which constitute the villous region where maternal blood bathes the STB for nutrient exchange, and the maternal decidua, where the EVT anchors the villous region to the uterus. Using first-trimester human placental explants, it was shown that the interface composed of the EVT is significantly more vulnerable to infections, despite having a much smaller surface area (Koi et al., 2001; McDonagh et al., 2004; Robbins et al., 2012). Furthermore, it has been shown that EVT cells are not as resistant to infections as STB cells, and distinct studies suggested EVTs as favorite targets of some pathogens (Robbins et al., 2010, 2012; Tabata et al., 2016). In this context, an experiment demonstrated the preference of *L. monocytogenes* for infecting EVTs by penetrating the intrauterine space (Robbins et al., 2010). This is probably due to the lack of E-cadherin expression by the STB, which is the receptor for internalin, a surface protein required for the entry of *L. monocytogenes* into epithelial cells (Mengaud et al., 1996). Additionally, a study used cultures from first trimester placentas to define where and how *L. monocytogenes* breaches the maternal-fetal barrier and demonstrated that the EVT is the preferred site for the initial placental infection (Robbins et al., 2010). A cell culture model system of primary human EVT was used to study the intracellular life cycle of *L. monocytogenes* inside EVTs. Isolated EVTs were able to restrict intracellular bacterial growth and spread, preventing vacuolar escape, and were also capable of guiding vacuolated bacteria towards lysosomes for degradation. This finding suggested that the EVT has effective defense mechanisms against intracellular pathogens and is a significant bottleneck to transplacental infections (Zeldovich et al., 2011).

Among the different strategies of immune system evasion, an interesting example comes from *T. gondii*. It was suggested that a fetal infection from *T. gondii* starts with maternal immune cells of the decidua acting as "Trojan horses" (Oz, 2017). Subsequently, the pathogen is transferred from the infected leukocytes to susceptible EVT cells or even to other cell types. Once successful in those two steps, *T. gondii* bypasses the villous core and infects fetal vascular tissues such that it reaches the central nervous system (Arora et al., 2017). The use of immune cells as Trojan horses by *T. gondii* facilitates parasite entry into immune-privileged sites. Additionally, *T. gondii*-derived exosomes have the ability to change the cytokine profile of the macrophages to modulate their activation *in vitro*. A positive aspect of this mode of infection is that *T. gondii*-derived exosomes are excellent therapeutic candidates since they have been shown to trigger humoral and cellular immune responses

and induce partial protection against acute parasitic infection in mice (Li et al., 2018).

The HCMV replicates in the underlying CTB of the floating and anchored villi, which is a place where STB is sparse. Therefore, this virus must first breach the STB and its defenses. As suggested, HCMV may bypass the STB through transcytosis of the virions in an antibody-mediated manner throughout the neonatal Fc receptor that serves as an IgG transporter instead of through direct infection (Arora et al., 2017). In addition, studies using decidual tissue cultures with clinically derived and laboratory-derived viral strains *ex vivo* showed that the HCMV could also target the EVT as well as the microvasculature and leukocytes to reach the CTB (Weisblum et al., 2011).

Immune evasion of pathogens mediated by exosomes and other EVs: a double-edged sword

The constant clash described as "pathogens *versus* immune system" involves a multifaced and very complex process. Several immune evasion mechanisms were selected during viral evolution, and many viruses usurp both exosomal trafficking and budding pathways with distinct consequences in terms of infectivity and viral spread (Gould et al., 2003; Anderson et al., 2016; Raab-Traub and Dittmer, 2017; Sadeghipour and Mathias, 2017). For instance, exosomes packing viral particles can promote viral persistence, increasing the potential for viral infection, since the viral material is "masked." Viruses, namely those that enter cells by endocytosis, can usurp endosomal/exosomal pathways to advance their infectivity and spread (Nour and Modis, 2014; Anderson et al., 2016). Dengue virus (DENV), West Nile virus, hepatitis C virus (HCV), and ZIKV are examples of pathogens that enter cells with mechanisms related to endosome formation, and exosomes also have an endosomal origin such that their proximity may facilitate the accumulation of viral antigens in exosomes, thus increasing their spread and infection capacities (Smit et al., 2011; Anderson et al., 2016). When secreted as exosomes, intraluminal vesicles (ILVs) containing viral genomes can target uninfected cells and then penetrate them via endocytic pathways. Evidence of this process has already been observed in cases of HCV, the genome of which can be secreted in ILVs as infectious particles (Liu et al., 2014). Thus, viruses such as HCV can hijack components of the vesicular trafficking machinery and thereby integrate viral components into exosomes (Liu et al., 2014; Raab-Traub and Dittmer, 2017). Therefore, it is likely that other viruses with genomes that can be found in endosomal ILVs are also trafficked between cells via exosomes (Raab-Traub and Dittmer, 2017; Sadeghipour and Mathias, 2017). Recently, many studies have addressed the interaction of exosomes with different viruses. Some viruses reportedly interact with exosomes, such as bunyavirus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), hepatitis A virus (HAV),

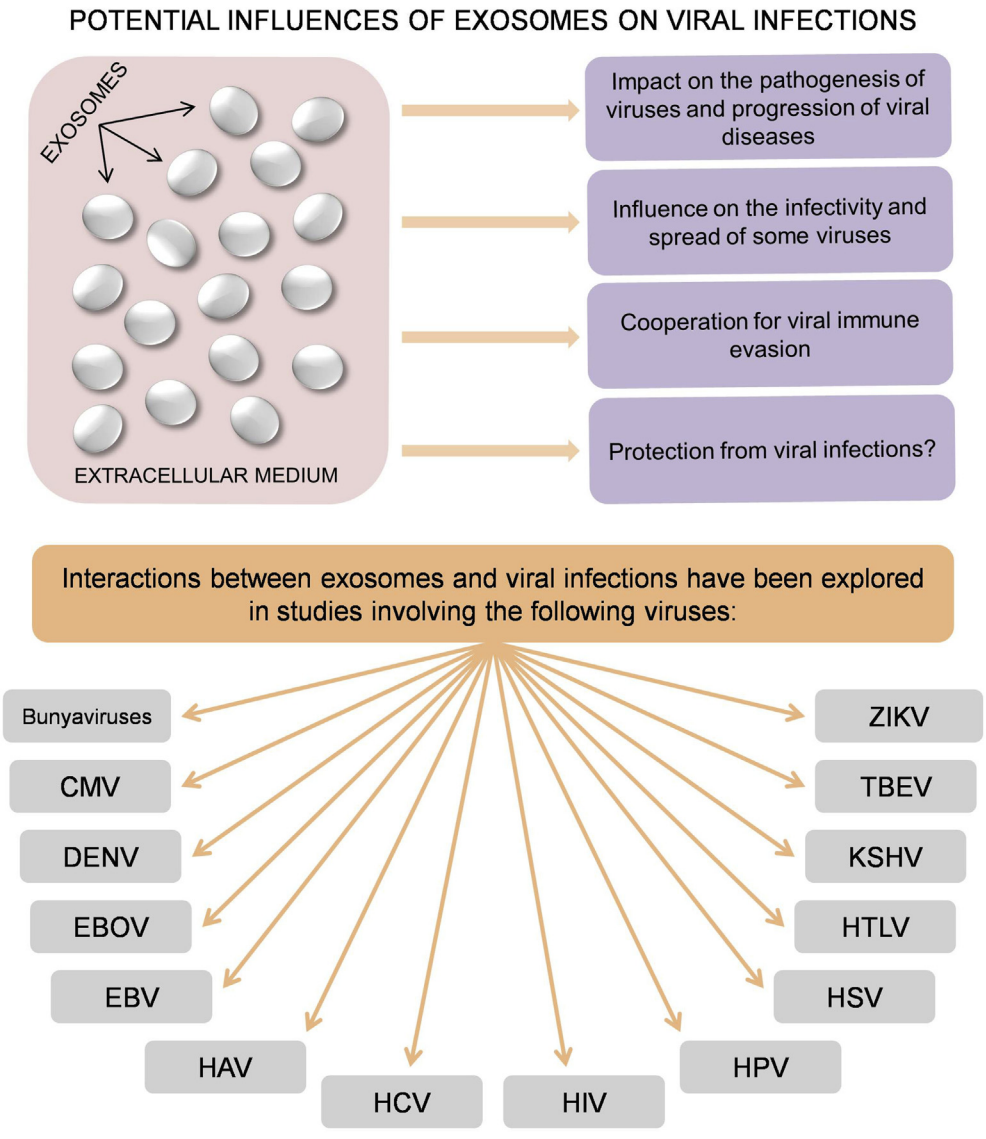

Fig. 1. Potential influences of exosomes on viral infections and examples of viruses for which their interaction with exosomes have been investigated using different methodological approaches. CMV: Cytomegalovirus; DENV: Dengue virus; EBOV: Ebola virus; EBV: Epstein-Barr virus; HAV: Hepatitis A virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HPV: Human papillomavirus; HSV: Herpes simplex virus; HTLV: Human T cell lymphotropic virus; KSHV: Kaposi's sarcoma-associated herpesvirus; TBEV: Tick-borne encephalitis virus; ZIKV: Zika virus. References are cited throughout the text.

herpes simplex virus 1 (HSV-1), HIV, human papillomavirus (HPV), human T cell lymphotropic virus (HTLV), DENV, ZIKV, tick-borne encephalitis virus (TBEV), and Kaposi's sarcoma-associated herpesvirus (KSHV) (Anderson et al., 2016; Ellwanger et al., 2017; Raab-Traub and Dittmer, 2017; Sadeghipour and Mathias, 2017; Martins et al., 2018; Zhou et al., 2019; Ellwanger and Chies, 2019; Reyes-Ruiz et al., 2019). In addition to the viruses covered in these studies, Ebola virus (EBOV) also appears to be associated with exosomes, in particular, those that package the viral protein VP40, causing immune cell dysfunction (Pleet et al., 2016). Furthermore, a prominent research field to be explored stems from the antiviral action of exosomes (Li et al., 2013; Madison et al., 2014). Fig. 1 presents a summary of some interactions between exosomes and viral infections that have been suggested to date.

As stated in previous sections, EVs can influence other pathogens besides viruses. The interaction between exosomes and pathogens can modify the outcomes of the infections. Such modifications caused by the action of EVs can enhance the infectivity of a particular microorganism via the delivery of infective particles and pathogen-derived molecules to distant sites from the primary focus of infection. Alternatively, EVs can favor the immune evasion of the pathogens from the host (Zhang et al., 2018). For example, *Staphylococcus aureus*-derived exosomes can transfer virulence factors, such as α-toxin, to cells found in distant physiological sites from the original location of the bacteria (Husmann et al., 2009). Also, cells infected by *Bacillus anthracis* secrete exosomes containing pathogen-derived toxins, thus enabling the action of virulence factors at long-distances (Abrami et al., 2013). In the context of *Helicobacter pylori* infection, exosomes have been associated to the secretion of cytotoxins by the host gastric epithelial cells, promoting extragastric complications (Shimoda et al., 2016).

Intrauterine infections by bacteria also represent a major threat to pregnancy when they gain access to gestational tissues. Bacterial infections can take place via the maternal circulation, in the peritoneal cavity, or ascend into the uterus from the lower tract (Espinoza et al., 2006). Regarding *L. monocytogenes,* a successful infection in the EVT could rely on the capacity of this bacterium to produce extracellular vesicles, called bacterial membrane vesicles (MVs). Studies have shown that MVs help bacteria survive inside mouse embryonic fibroblasts *in vitro* (Vdovikova et al., 2017). Considering opportunistic infections of the genitourinary tract, Group B *Streptococcus*-derived MVs loaded with virulence factors led to up-regulation of pro-inflammatory cytokines and inflammation related-symptoms of chorio-amnionitis in mice. These observations suggested that these bacterial-derived MVs are capable of triggering events at the maternal-fetal interface associated with preterm birth or even fetal death (Surve et al., 2016).

Intracellular pathogens such as *Mycobacterium tuberculosis*, which primarily infect macrophages in the lungs, also interact with microvesicles. It is known that infection of macrophages with mycobacteria leads to the release of EVs by the infected host cells. These released EVs contain numerous mycobacterial lipoglycans, lipoproteins, and antigens that modulate the immune response of the host (Bhatnagar et al., 2007). Moreover, electron microscopy images of *M. bovis* BCG-infected macrophages suggested that these EVs are indeed exosomes (Giri and Schorey, 2008). However, a study brought important evidence regarding the origin of EVs in the context of *M. tuberculosis* infection. It was demonstrated that some immune modulations related to TLR2 signaling provoked by this pathogen in the host are derived from the bacterial membrane vesicles rather than exosomes derived from the infected macrophages. In summary, the study suggested that the impairments on immune effector functions are primarily driven by the exportation of *M. tuberculosis* lipoglycans and lipoproteins released from the pathogen cell membrane (Athman et al., 2015).

Other infectious agents besides viruses and bacteria present interesting links with EVs. Prions are proteinaceous infectious particles that spread in the host cells due to the ability of these proteins to interact and shape the quaternary structure of nascent proteins. The resulting quaternary structure is an exact copy of the proteinaceous infectious proteins, which can also modify other proteins, thus further spreading the infection. Prions are responsible for transmissible spongiform encephalopathies in humans and other mammals. These infections are fatal and commonly known as prion diseases (Prusiner, 1982; Watts et al., 2006). Interestingly, exosomes can contain and transport prion proteins, contributing to the spread of these proteins in the infected organism (Robertson et al., 2006; Fevrier et al., 2004; Hartmann et al., 2017). Moreover, a study demonstrated that mouse neuroblastoma cells transmit cytosolic prions in association with membrane-bound vesicles besides the classical transmission by direct cell contact. The presence of flotillin, Alix-1, and Tsg101 and cup-shaped appearance of the EVs indicated that these vesicles indeed represented exosomes (Liu et al., 2016).

Regarding parasites, interesting features involving the pathogenesis of malaria and EVs secretion by host cells were addressed. Red blood cells infected by *Plasmodium falciparum* can secrete EVs containing molecules involved in the silencing of gene expression in endothelial cells. Also, these EVs were efficiently internalized by the endothelial cells and can disrupt the mechanisms responsible for hindering the entry of pathogens, thus enhancing the infection of the parasite in the host target cells (Mantel et al., 2016).

Besides the previously mentioned aspects regarding EVs and *T. gondii* infection in pregnancy, the pathogenesis of this parasite can be further affected by exosomes through the transference of molecules that alter the host cell cycle. Such alterations eventually decrease host cell proliferation, favoring the parasite because it invades cells in the S stage more easily than in other phases of the host cell cycle (Kim et al., 2016).

The bloodstream form of *Trypanosoma brucei* secrete EVs that interact with the cell membrane of host erythrocytes. Thus, it was postulated that fusogenic EVs derived from the trypanosome may act as vehicles for pathogen-to-host cell transfer of membrane proteins. Of note, this fusion between EVs from the pathogen and host cells results in the transfer of lipids and antigens derived from the parasite to the host cells. Such traffic of molecules has the potential to cause host anemia, a clinical outcome due to modifications in the structure of the host erythrocytes probably related to the incorporation of lipids from the parasite via EV fusion (Szempruch et al., 2016).

The first discovery regarding the role of EVs in fungal infection was made addressing *Cryptococcus neoformans* (Rodrigues et al., 2007). It was demonstrated that the *Cryptococcus*-derived virulence factor glucuronoxylomannan was produced inside the cell and then released in the extracellular environment inside EVs. Of note, EVs released by *C. neoformans* facilitate the pathogen passage through the blood-brain barrier and modulate the host immune responses, enhancing *C. neoformans* pathogenesis (Huang et al., 2012; Bielska and May, 2019).

Subsequently, various studies addressing associations between fungi and EVs have emerged (Rodrigues et al., 2014; Coakley et al., 2015; Peres da Silva et al., 2015; Joffe et al., 2016; Bielska and May, 2019). Like EVs derived from other species, fungal EVs transport proteins, lipids, pigments, polysaccharides, and genetic cargoes (Joffe et al., 2016). Thus, fungal EVs can induce strong and different impacts on host immunity, including stimulation of pro- and anti-inflammatory cytokine production (Bielska and May, 2019). Therefore, it can be speculated that fungi-derived EVs may have some impact on the host's inflammatory status, triggering other health problems not directly related to the fungal infection, but due to unbalanced inflammation. Importantly, fungi EVs can also interact with other pathogenic microorganisms in co-infected hosts, generating an even more complex immune landscape. Also, considering that approximately three hundred species of fungi are pathogenic for humans and only eleven species have their EVs addressed, more studies in this field are necessary (Bielska and May, 2019).

Parasitic helminths are metazoan organisms that also produce and secrete exosomes. In this context, studies addressing trematodes demonstrated intact exosomes in the parasites' teguments, indicating that these vesicles could also reach the host environment. Initially, it was speculated that exosomes derived from these parasites participated in the

down-regulation of the host immune responses, a common feature of helminth infections. The immune manipulation of the host immune responses by these parasites eventually ensures the survival of the parasites, mainly by exporting a range of immuno-modulatory mediators that interact with host cells and tissues. Evidence for the role of exosomes in the host immune modulation by helminths was demonstrated by the internalization of helminth-derived exosomes by host intestinal epithelial cells (Coakley et al., 2016).

Open questions and emerging topics

Seminal exosomes — friends or foes in sexually transmitted infections?

The role of semen in sexually transmitted viral infections is another interesting topic that connects exosomes, infectious diseases, and reproduction. Semen is a complex fluid composed of cells and seminal plasma. Human semen contains immunosuppressive components with the ability to drive tolerance towards paternal antigens, consequently maximizing the chances of successful fertilization. This immune suppression is likely derived from the low incidence of antibodies against sperm and the soluble components of semen in the woman body (Johansson et al., 2004). However, the immunosuppressive properties of semen could also contribute to the evolutionary success of sexually transmitted viruses; that is, they may take advantage of the immune suppressed environment that follows from exposure to semen (Sabatté et al., 2011). Seminal plasma has a high concentration of subcellular lipid-bound microparticles that are morphologically and molecularly consistent with exosomes that originate from multiple cellular sources of the male genital tract (Renneberg et al., 1997). These microparticles are, in general terms, called "seminal exosomes" (Vojtech et al., 2014). In summary, the immunosuppressive properties of seminal plasma seem to be related to its exosome fraction, and therefore, exposure to seminal exosomes could facilitate the establishment of viral infections (Vojtech et al., 2014).

The wide variety of cells that secrete exosomes also dictates the spectrum of biological fluids from which they can be isolated: amniotic fluid, breast milk, bronchoalveolar lavage fluid, cerebrospinal fluid, malignant ascites, plasma, saliva, synovial fluid, urine, vaginal fluid, and semen, among others (Ellwanger et al., 2017). Considering semen, each ejaculate contains trillions of exosomes with an average of 2.2×10^{13} particles (Vojtech et al., 2014, 2016). Seminal exosomes are considered immunosuppressive particles due to their inhibitory action during lymphoproliferative responses (Kelly et al., 1991), phagocytic cells (Skibinski et al., 1992), and NK cell function (Tarazona et al., 2011). Seminal exosomes are efficiently and rapidly captured by peripheral and vaginal DCs, whereas seminal exosomes are captured by T cells in the vaginal environment at a lower rate and efficacy (Vojtech et al., 2016). The immunosuppressive properties of semen are predominantly restricted to the seminal plasma since isolated sperm cells can induce immune responses and alterations in the uterine environment, and seminal plasma alone induces tolerance to paternal antigens and confers benefits to the offspring mainly in early pregnancy (Robertson et al., 2009; Bromfield, 2014).

Although there is some evidence suggesting that semen-derived exosomes have anti-HIV activity, exosomes apparently do not have an effect on the replication of other viruses (Madison et al., 2014). Recently, it was shown that Herpesviruses hijack host exosomes, which contributes to their viral pathogenesis (Sadeghipour and Mathias, 2017). Thus, once viruses take advantage of the local altered/suppressed immune responses induced by exposure to semen, semen immunosuppressive properties can contribute to the prevalence of sexually transmitted viral infections. In addition, as described above, the tremendous number of exosomes present in semen could facilitate viral spread.

Do exosomes facilitate transplacental and sexually transmitted viral infections?

The role of exosomes as facilitators of (I) transplacental and (II) sexually transmitted viral infections (Fig. 2) should be considered based on the following four premises:

1st) Trojan exosomes: Gould et al. (2003) hypothesized that retroviruses such as HIV and HTLV could usurp the machinery that causes the budding and trafficking of exosomes to infect new cells without being recognized by the immune system. Although the Trojan exosome hypothesis is still debated, some experimental evidence supporting the cellular mechanisms consistent with this theory has been published (Nguyen et al., 2003; Booth et al., 2006; Gan and Gould, 2012; Kadiu et al., 2012). Interestingly, the presence of HCV particles in exosomes has already been demonstrated (Liu et al., 2014). Moreover, the detailed cellular mechanisms of the budding/trafficking of exosomes that could be used by HCV, HAV, HIV, EBV, and KSHV to spread from cell to cell were recently revised (Raab-Traub and Dittmer, 2017). Thus, the mechanism that leads to the budding/trafficking of exosomes may also be employed by viruses to cross the maternal-fetal barrier.

2nd) Immunomodulation in pregnancy: Pregnancy is considered a challenge to the woman's immune system. In fact, fifty percent of the fetal genome, and consequently the antigens and other immune molecules present, are of paternal origin. Thus, the immune system of a pregnant woman must be readjusted during pregnancy to avoid perturbing the developing fetus (Mincheva-Nilsson, 2010; Mor et al., 2011; Stenqvist et al., 2013). When immune adaptation fails, abortion is a likely consequence (Trowsdale and Betz, 2006). The local downregulation of the maternal immune system, especially in the first trimester of pregnancy, could favor transplacental viral infection.

3rd) The cloud of exosomes at the maternal-fetal interface: Taking into consideration the immune system adjustments during pregnancy that promote a tolerogenic environment for the fetus, the general suppression of the immune system during the entire gestational period would be expected. However, as previously discussed, systemic immunosuppression would not be desirable because the blastocyst would not implant, and the pregnant woman would be highly susceptible to a variety of infections. Here, we present a series of studies showing that placenta-derived exosomes play important roles in this tolerogenic process by carrying immunomodulatory molecules through the maternal-fetal interface (Hedlund et al., 2009; Mincheva-Nilsson, 2010; Stenqvist et al., 2013). It is believed that exosomes contribute to this process by forming a "cloud of exosomes", which would protect the fetus from exacerbated maternal immune responses (Mincheva-Nilsson, 2010). Of note, this process would not compromise the woman's immune defenses as a whole.

4th) Seminal exosomes: Semen has trillions of exosomes (Karlsson et al., 2001; Vojtech et al., 2014). Furthermore, although semen is an immune privileged biological fluid, some viruses have been detected in this fluid months after host infection (Madison et al., 2014; Abbate et al., 2016; Anderson et al., 2016; D'Ortenzio et al., 2016; Uyeki et al., 2016). Exosomes present in semen could facilitate sexual transmission of viruses through semen-derived immunosuppression and hide, in some cases, viral components from the host immunological system.

The placental/fetal microbiota

Aagaard et al. (2014) reported that placental and amniotic fluid from healthy human placenta are not sterile. Historically, the uterus was considered a sterile environment, but it is currently viewed by some researchers as a compartment where the microbiota starts to be established (Stinson et al., 2017). Lactic acid bacteria and other commensal bacteria were isolated from meconium obtained from healthy neonates born either by labor or cesarean section, indicating that mother-to-child efflux of commensal bacteria may exist through the placenta (Martín et al., 2004; Jiménez et al., 2008). In 2005, Jiménez et al. (2005) also found commensal bacteria in the umbilical cord blood of healthy neonates born by cesarean section. In addition, a study using placental tissues from low-gestational-age neonates showed that almost one-half of second-trimester placentas harbor organisms within the chorionic plate

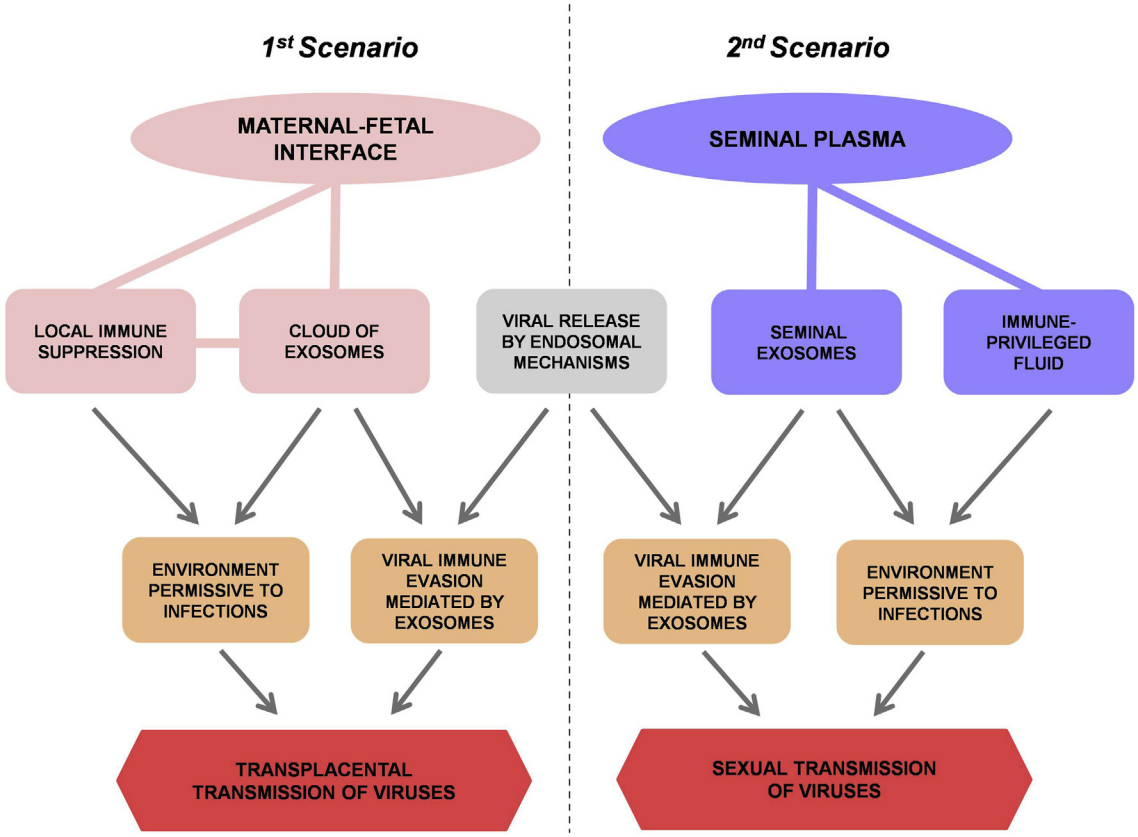

Fig. 2. Potential roles of exosomes in transplacental (1st scenario) and sexually transmitted (2nd scenario) viral infections. References are cited throughout the text.

(Onderdonk et al., 2008a). Another study showed that the chorion of placentas from preterm labor pregnancies (not related to preeclampsia) had a much higher rate of microorganism recovery than that of placentas from increasingly severe preeclampsia pregnancies (Onderdonk et al., 2008b). The presence of microorganisms in the placental parenchyma was associated with the presence of neutrophils in the fetal stem vessels of the chorion and umbilical cord, strongly indicating that the presence of microorganisms within the placental parenchyma is biologically important (Onderdonk et al., 2008b). The presence of microorganisms may correlate with the high number of neutrophils recruited to the maternal-fetal interface, as they may create an inflammatory environment similar to that necessary for the labor process but at the wrong time.

The following main question arises from these observations: how do microorganisms, or their genetic material, make contact with the fetus before birth? To date, three different origins for the fetal/placental microbiota have been proposed: maternal gut microbiota, vaginal microbiota, and oral microbiota (Stinson et al., 2017). Regarding the transference of microorganisms from the maternal gut, it is possible that the microorganisms are translocated into the maternal bloodstream from the gut epithelium, and the DCs could be quite important for this process. The intestinal epithelial barrier prevents bacteria from entering into the bloodstream. However, DCs can take up bacteria from the intestinal lumen by penetrating the gut epithelium. DCs packing bacteria could traffic to mesenteric lymph nodes via intestinal lymphatics and thus spread the bacteria to other body compartments (Stinson et al., 2017). Of note, maternal intestinal bacteria can also be found in breast milk, reportedly through the same DC-based dissemination pathway hypothesized for the delivery to the fetal tissues (Fernández et al., 2013).

The vaginal pathway by which microbes would reach the placenta is not well known, but it has been well established that microbes may ascend from the vagina and reach the amniotic cavity. One suggested mechanism involves the microbial colonization of the decidua, through mechanisms previously discussed, from which the microorganisms spread to fetal membranes and invade the amniotic fluid. Another suggested pathway involves direct microbial invasion of the amniotic fluid by penetration of a discontinuous section of fetal membranes (Stinson et al., 2017). Whatever the pathways of infection, DNA from vaginal microbes in the amniotic fluid (DiGiulio, 2012), fetal membranes (Steel et al., 2005) and the placenta (Aagaard et al., 2014) have already been found in both normal and complicated pregnancies. For example, pathogenic oral species of bacteria have been found in the placenta and amniotic fluid of pregnant women with periodontal disease (Barak et al., 2007; Katz et al., 2009). This finding indicates an opportunistic migration of bacteria from the oral cavity to the uterine environment and has been extensively correlated with preterm birth. Comparing the placental microbiome with the microbiome derived from different body compartments, the oral cavity showed the greatest similarity in terms of bacterial composition (Aagaard et al., 2014). Despite this finding, these studies were performed with the microbiota of healthy nonpregnant individuals, which makes it difficult to infer routes of transmission (Stinson et al., 2017). The effect of sexual practices in the transfer of oral and gut bacteria to the intrauterine cavity also needs further investigation. In this context, oral or anal sex preceding vaginal sex may present a mechanism of microbial transfer. The resolution of these tangled issues could not only create the possibility for studying, measuring, and mapping healthy placental/fetal microbiota from its precise beginning but could also provide an additional basis for the establishment of new public health strategies and improvement of clinical practices for complicated pregnancies with the aim of reducing the cases of newborns with severe sequelae.

However, a recent study addressing hundreds of placental samples stated that healthy placentas do not display a microbiome. This study represents the largest sample number in this field of research and was composed of 537 placental samples. de Goffau et al. (2019) performed such elegant experiments that allowed the identification of even possible contaminants from DNA extraction kits, and the results showed the

presence of only one type of microorganism in 5% of placentas: *Streptoccocus agalactiae*. Of note, this microorganism is one of the main concerns regarding the risk of neonatal sepsis, and is probably transmitted from the mother's genital tract. Besides revealing possible routes of contamination during the experimental procedure in previous related studies, these findings revealed a possible way of early detection of potential harmful agents during pregnancy. In summary, this study presented convincing evidence to support that healthy placentas lack a microbiome. The study reinforces that, despite the lack of a placental microbiome, pathogens may eventually be found in the placenta, although bacterial infection of this transient organ is not a common cause of gestational complications (de Goffau et al., 2019).

Taking together, we believe that future discussions and experimentation should consider the role of exosomes and other EVs as potential vehicles used by microorganisms in the establishment of the newborn microbiome. Finally, it is possible that the observed first microbiome in meconium samples is a result of EV-mediated traffic of bacteria from the vaginal tract, placenta or uterine cavity towards the fetus during the first signals of labor or even during delivery, thus representing the early seeds of the neonatal microbiome. Considering the emergence of studies addressing the establishment of the neonate microbiome, these hypotheses should be investigated.

3. Conclusion

The immune system of a pregnant woman, far from being in a resting state, undergoes several changes throughout the entire gestation period, encompassing distinct organs, tissues, cellular, and molecular profiles. Over the years, studies in the reproductive biology field have elegantly approached the multiple interactions at the maternal-fetal interface, which can result in normal or pathological pregnancies. Firstly considered a threat to a successful pregnancy, inflammation is currently recognized as an essential step to pregnancy establishment and maintenance, although such an immune response should be regulated. Exacerbated inflammation can cause abortion and other pregnancy complications, but the absence of inflammation precludes effective implantation due to inadequate tissue remodeling. A shift to a less inflammatory environment occurs during pregnancy, enabling fetal development. Finally, by the end of the third trimester, near parturition, a range of physiological alterations occurs, and a pro-inflammatory milieu is again predominant.

Additionally, when implantation takes place, the paternal antigens are expressed, and the maternal immune system meets two challenges: avoiding immune activation and rejection of the developing fetus while simultaneously inducing immune activation to avoid pathogen infection. Fetal tolerization is a complex process that transpires during the entire gestation period and involves modulation of local immune responses towards an anti-inflammatory profile. Interestingly, the placenta is a vigorous producer of exosomes, extracellular vesicles that have been described as key players in the regulation of maternal immune responses. The syncytiotrophoblast has important physical and molecular mechanisms that prevent microbes from bypassing the placenta and reaching the fetus, and these features range from dense, branched microvilli at the apical surface to soluble receptors carried by exosomes. Recent studies have revealed the importance of exosomes to a successful pregnancy, namely, as partners of the immune system at the maternal-fetal interface.

The trafficking of molecules, cells and even pathogens between mother and fetus during pregnancy is currently seen as a natural phenomenon. In this context, exosomes can be important mediators of transplacental infections. Additionally, the immunosuppression induced by seminal exosomes can help explain the persistence of the many viruses found in semen. In addition, this review revisited the discussion about the processes that enable viruses (and possibly other pathogens) to overcome the maternal-fetal barrier through sexual transmission. Taking into consideration the particularities of each cell type and virus *per se,* we call urgent attention to the role of exosomes and other microvesicles in viral infectivity and spread. Finally, the traditional view that establishes serious potential complications to the fetus should a microorganism succeed in crossing the placenta has been revised. In this regard, bacteria found in the normal gut, oral cavity, and vagina were detected in the amniotic cavity and in the placenta of normal pregnancies. Despite the recent emergence of controversial findings regarding this aspect, such discoveries raised important discussions about potential routes for the establishment of the newborn microbiota. Current studies are now trying to elucidate the distinct pathways used by microbes to colonize the developing fetus. Thus, we expect this review to provide insights for future investigations and new studies on all the topics addressed, since the universe of extracellular vesicles is similar to an iceberg from which, at the present moment, we are able to see only the portion that lies above the water. In this sense, a submerged “EV world” awaits our discovery, and with new methods, approaches and the establishment of connections among the several scientific fields involved, we will be able to uncover the immersed portions that will help us comprehend the immunology of gestation, fetal microbiome, and even the transplacental and sexual transmission of pathogens.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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Capítulo III

Down-regulation of *HLA-G* gene expression as an immunogenetic contraceptive therapy

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Down-regulation of *HLA-G* gene expression as an immunogenetic contraceptive therapy

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ABSTRACT

HLA-G is a nonclassical HLA immunotolerogenic molecule expressed in different human cell types. Successful embryo implantation is a consequence of information exchange between the uterus and the blastocyst. It is widely accepted that HLA-G expression by the fetus promotes the establishment of several mechanisms that, ultimately, would protect the developing embryo from maternal immune rejection and seems to be essential to both an adequate implantation and a healthy pregnancy. MicroRNAs miR-148a and miR-152 down-regulate HLA-G expression. The levels of both microRNAs in the placenta are very low. Although various contraceptive methods are available in the market, several of the most popular are based on hormone administration, an approach that have been causing concerns regarding their adverse effects. This scenario has led the research and development of new contraceptive methods meant to induce low disturbances in women body. Based on this context, we hypothesize that the delivery of miR-148a and miR-152 microRNAs, carried by liposomes, into the uterus, would locally induce a down-regulation of the immunotolerogenic HLA-G molecule. In this sense, a local concentration increase of both miR-148a and miR-152 would counteract HLA-G expression and therefore prevent pregnancy development, being a potential tool for the development of a new contraceptive therapy.

Introduction

HLA-G is a non-classical Human Leukocyte Antigen (HLA) class I molecule from the Major Histocompatibility Complex (MHC) that possesses unique features, such as low polymorphism and restricted tissue expression [1]. Under normal physiologic conditions, HLA-G expression is observed in embryonic tissues directly involved in maternal-fetal tolerance, namely the cytotrophoblast and placenta [2]; In adults, HLA-G expression was already described in the cornea [3], epithelial thymic cells [4], and in some specific subpopulations of monocytes [5], bone marrow cells [6], and CD4+ and CD8+ T cells [7].

Successful embryo implantation depends on an intimate 'cross-talk' between the blastocyst and uterus in both a temporal and cellular specific manner [8]. The expression of HLA-G protects the fetal extravillous trophoblast cells from maternal immune mediated rejection and therefore seems to be essential to a healthy pregnancy [9]. In order to a successful pregnancy, the uterus should be in a receptive state, defined as the limited time during which the uterine environment is conducive to blastocyst acceptance and implantation [8].

Several mechanisms seem to be involved on the establishment of such a receptive state. The reduced, or lack of expression of classical MHC molecules on the trophoblast cells surface and the HLA-G expression on these same cells, constitute examples of tolerogenic mechanisms occurring at the maternal-fetal interface. Playing a key role in implantation by modulating the secretion of cytokines, HLA-G may act controlling trophoblast cell invasion. Importantly, the HLA-G molecule is recognized by receptors present at the surface of Natural Killer (NK) cells, not only providing protection against deciduous NK cell mediated cytotoxicity, but also activating such cells in order to induce the release of angiogenic molecules important to neovascularization and embryo implantation [9]. Thus, HLA-G contributes to trophoblast invasiveness, decidual cell differentiation and vascular remodeling, helping the establishment of a local immunosuppressive environment [10].

It was possible, through bioinformatic approaches, to identify several microRNAs that target the *HLA-G* gene [11]. MicroRNAs are non-coding RNA molecules, approximately 23 nucleotides long, that usually post-transcriptionally regulate gene expression, mainly by binding to the 3′UTR (untranslated region) of mRNAs.

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Such binding results in either translational inhibition or in mRNA degradation [12]. Both microRNAs miR-148a and miR-152 down-regulate HLA-G expression. These microRNAs are found at very low levels in the placenta when compared to other healthy tissues. In the placenta, *HLA-G* mRNA presents the highest ratio relative to its targeting microRNAs, which potentially explains the almost exclusive expression of HLA-G in such environment [13]. Synthetic microRNAs regulate gene expression when transfected into cells. In this context, liposomes are frequently used as delivery vehicles of microRNAs in the cell environment and can be used in strategies for molecular therapy both *in vitro* as well as *in vivo* [14].

Since its introduction in the 1960s, contraception based on hormonal approaches has been highly accessible and widely used. However, hormonal contraceptives are associated to important adverse effects on the woman body metabolism, including alterations in hemostatic variables, disturbances in the metabolism of lipid and carbohydrates, and cardiovascular disorders. Considering this scenario, the development of non-hormonal contraceptive methods focused on safety is a very interesting field. In addition, the current contraceptive methods are not widely available or are acceptable to all people interested in use them [15,16]. Thus, we hypothesize about a new contraceptive therapy targeting the

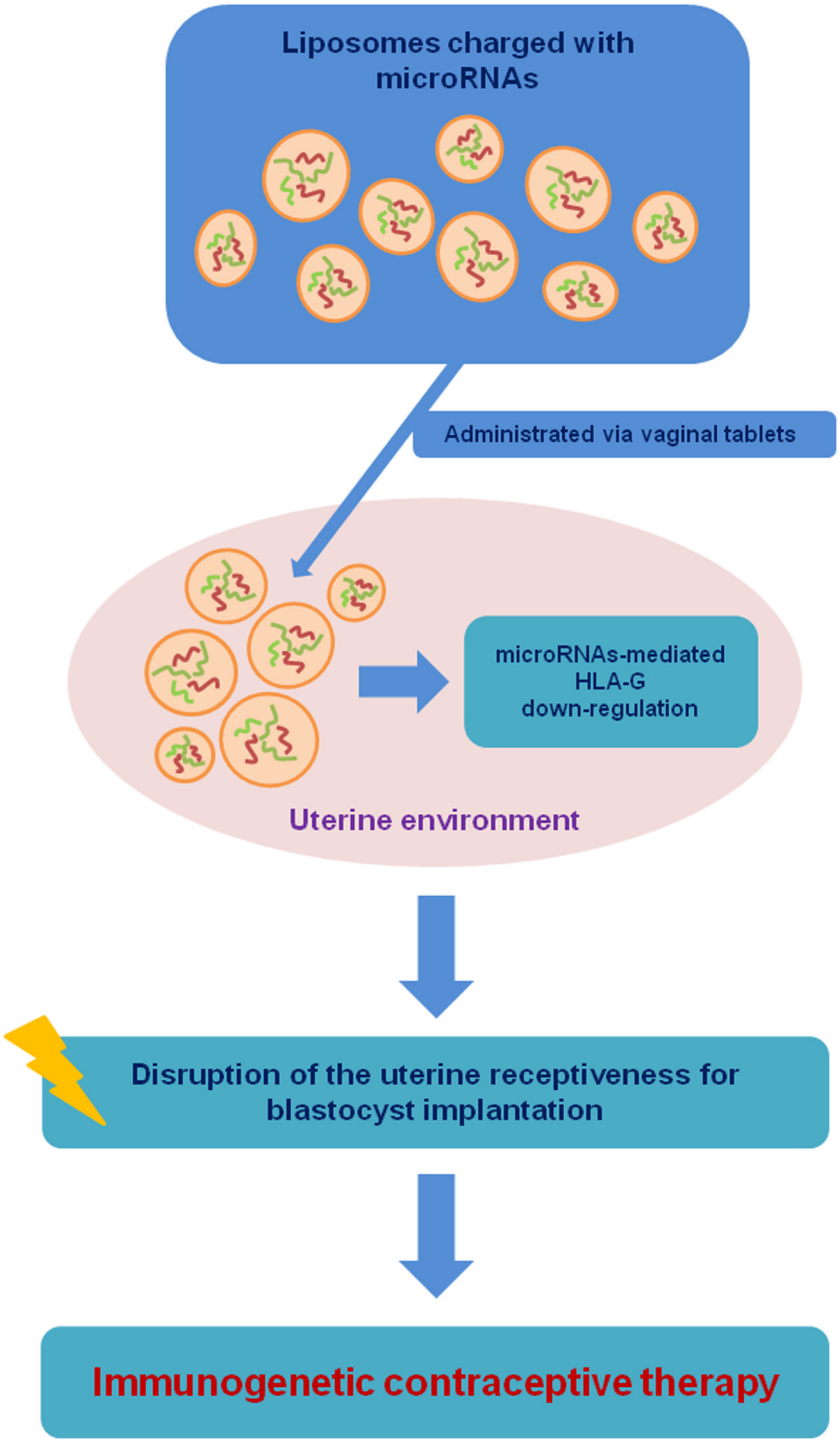

Fig. 1. Schematic representation of our hypothesis.

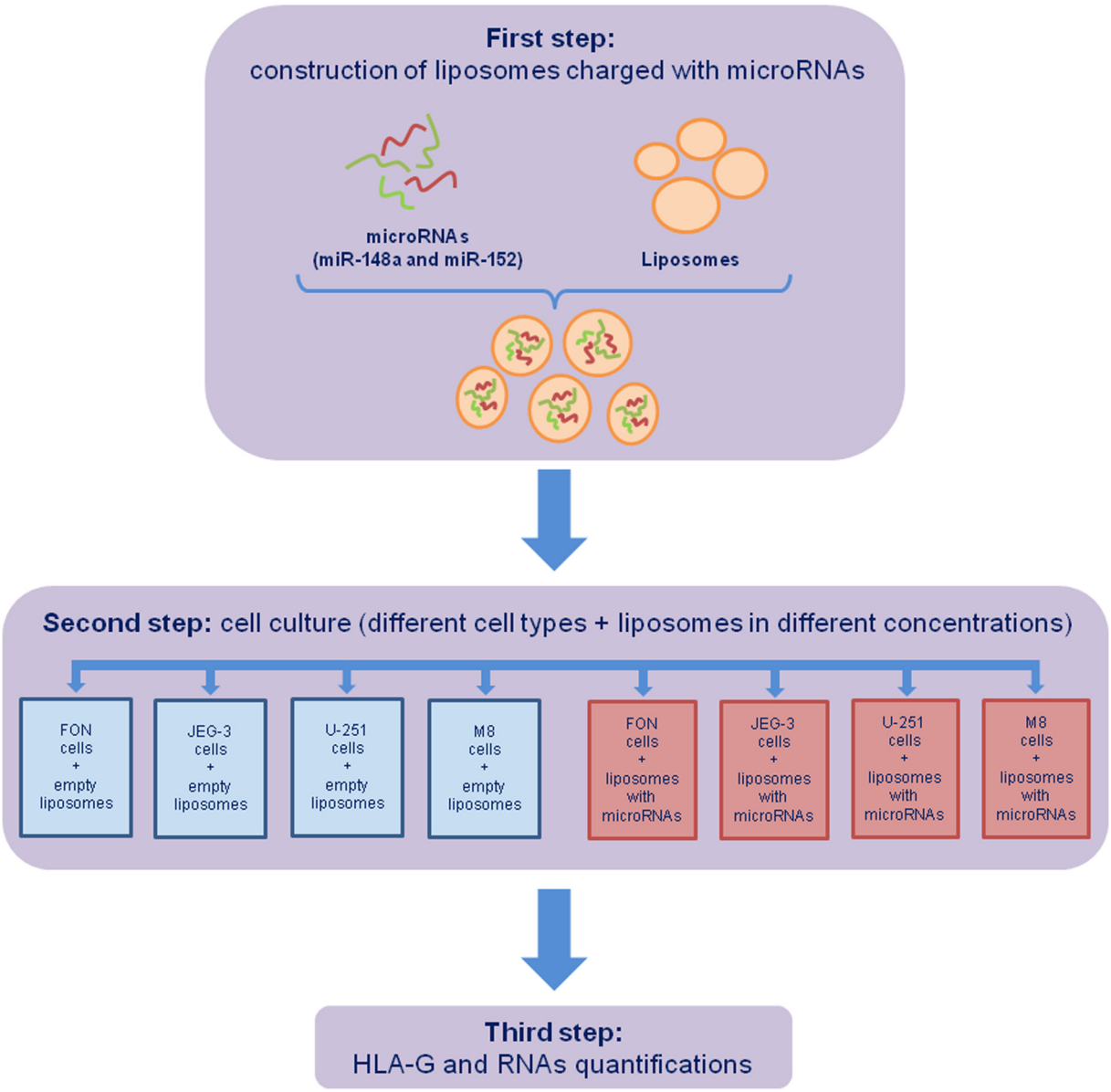

Fig. 2. Suggested experimental design to test the proof-of-concept of our hypothesis.

implantation period, potentially without disturbing other physiological functions of the woman body.

The hypothesis

We suggest the use of liposome-mediated delivery to direct miR-148a and miR-152 to the uterus, in order to locally induce a down-regulation of HLA-G expression. We hypothesize that this local HLA-G down-regulation will disrupt uterine receptiveness for blastocyst implantation, as the result of interference on the NK cells function during the preimplantation period.

Our hypothesis is based on two main points: (I) the crucial role of the HLA-G molecule on the establishment and maintenance of pregnancy [1,2,17], and (II) the extremely regulated expression of the HLA-G, both in normal adult tissues and in different steps of human development during pregnancy [1–7]. Thus, due to its very controlled local expression and its importance during the blastocyst implantation period [1], HLA-G is an ideal molecule to be target in a new contraceptive therapy, without disturbing other physiological functions of the woman body. Fig. 1 schematically represents our hypothesis.

How to test our hypothesis

A first approach to test our hypothesis consists of a 'proof-of-concept' and would involve the following steps:

- Development of liposomes loaded with miR-148a and miR-152 that will target the *HLA-G* mRNA.
- Down-regulation of the HLA-G expression in different cell lineages (both HLA-G+ and HLA-G− cell lines) using these liposomes charged with microRNAs.
- Evaluation to confirm that the down-regulation of the HLA-G expression is specific and mediated by the microRNAs delivered by the liposomes.

Suggested experimental design

MicroRNAs targeting *HLA-G* mRNA and control microRNA oligonucleotides would be synthesized. Thus, these microRNAs

should be charged into liposomes. Different vesicles can be developed to contain either miR-148a or miR-152 separately as well as both miRNAs together. Four cell lines should be used to start the experiments: two with high HLA-G expression and two cell lines that do not express HLA-G. We suggest the use of the following cell lines:

- melanoma cell line (FON), established from an HLA-G-positive melanoma biopsy (high HLA-G expression);
- JEG-3 cell line from placenta choriocarcinoma (high HLA-G expression);
- U-251 cell line, derived from a malignant glioblastoma tumor by explant technique (no HLA-G expression);
- M8, a melanoma cell line (no HLA-G expression).

Each cell line will be exposed to different concentrations of (I) empty liposomes, (II) liposomes charged with miR148a, (III) liposomes charged with miR-152, (IV) liposomes charged with both miR148a and miR-152, and (V) liposomes containing a control unrelated microRNA. After 24h of cell culture (cells + liposomes), the level of microRNAs, HLA-G protein, and *HLA-G* mRNA should be accessed and measured.

All tests should be performed with three increasing concentrations of empty liposomes and liposomes plus microRNAs. Transfection of empty liposomes would be used as a negative control of the experiments to check if liposomes alone induce immune gene up-regulation and if so, the importance and degree of this up-regulation. Liposomes containing an unrelated microRNA will also be used as a control.

Total RNA would be isolated and quantified by spectrophotometry. cDNA will be prepared from each sample, and quantitative reverse-transcription PCR (qRT-PCR) will be performed to measure *HLA-G* mRNA expression before and after the transfection step. HLA-G protein expression (both considering soluble HLA-G levels as well as surface HLA-G molecules) will be accessed from samples of the different cell lines used in the experiments. Our suggested experimental design is divided in three basic steps and is schematically shown in Fig. 2.

Perspectives

After this proof-of-concept step, and considering results that confirm our expectations about HLA-G down-regulation mediated by the proposed microRNAs, it will be essential to evaluate this liposome-mediated microRNA delivery system in experimental animal models and hereafter to test the delivery feasibility of liposomes via vaginal tablets. For these tests, proper cytotoxicity and genotoxicity assays to ensure the safety of the proposed therapy must be performed. Once the results are positive for all the previous steps, the next goal would be testing the properties of exosomes for delivery of RNA molecules into the target cells. Such way of delivery via exosomes could replace the use of liposomes, since exosomes are potential highly efficient vesicles to be used in the delivery of microRNAs [18–20].

Conclusion

The possibility of a new contraceptive therapy which does not directly target women's hormonal cycles is an important step towards the control of the adverse effects that often come along with this type of treatments. We believe that the need for such alternative therapies is highlighted by the growing search of a better life quality combined with the interest in safer and more effective contraceptive methods associated to low adverse effects. Taking together, these aspects point to a good opportunity for new targets in the field of reproductive medicine focused on innovative contraceptive approaches from research areas such as genetics and molecular biology. We believe that testing the HLA-G down-regulation as a new contraceptive therapy could contribute for a new era where birth control would be associated with minor disturbances in the female body.

Conflict of interest statement

The authors declare no conflicts of interest.

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Capítulo IV

IL-17 blood levels increase in healthy pregnancy but not in spontaneous abortion

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SHORT COMMUNICATION

IL-17 blood levels increase in healthy pregnancy but not in spontaneous abortion

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Abstract

Cytokines are essential to maintain and coordinate the correct activity of immune cells during human pregnancy. IL-17 is a pro-inflammatory cytokine that induces the expression of many inflammatory mediators. The aim of this study was to compare the levels of Th1, Th2 and Th17 cytokines of women ongoing normal pregnancy with those found in women who suffered spontaneous abortion. IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ peripheral blood levels were measured in women who suffered spontaneous abortion ($n = 13$, blood collected up to 24 h after abortion), and were compared with healthy successful pregnancies ($n = 16$). Cytokine levels were measured using a cytometric bead array (CBA analysis). Similar cytokine levels were observed between spontaneous abortion and healthy pregnant women excepted to IL-17, which levels were increased in the healthy pregnant women ($p = 0.0232$). Our results show high IL-17 levels in the peripheral blood of women at late stages of healthy pregnancy, although low IL-17 levels were detected in the peripheral blood of women just after spontaneous abortion. In line with recent studies, this finding highlights IL-17 as a regulatory cytokine essential to the maintenance of a successful pregnancy.

Keywords Pregnancy · IL-17 · Th17 cells · Spontaneous abortion

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Introduction

Mammalian pregnancy was first questioned and hypothesized in the context of immunology by Medawar and his research group in the 1950s. They suggested that a successful pregnancy (meaning maternal-fetal tolerance and absence of rejection) is a consequence of (I) anatomical separation of the fetus from the mother, (II) antigenic immaturity of the fetus, and (III) immunological indolence/inertness of the mother towards the fetus [1]. However, several researchers investigated the process of human reproduction since then and realized that Medawar proposals could not completely explain the reality of a mammalian gestational period [2].

In the context of immune regulation during human successful pregnancy, resident decidual immune cells control different aspects and steps of the embryo implantation and of the fetal development [2]. Presently, it is well accepted that a mild pro-inflammatory environment is quite necessary for local tissue remodeling and neovascularization, which is important for embryo implantation, allowing the establishment of successful embryo attachments and enabling healthy development of the fetus [3]. Cytokines produced

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by immune cells are key factors to maintain and coordinate the correct immune response of the mother, mainly due to their role on the activation or down-regulation of Natural Killer cells, their influence on adhesion molecules, and their regulatory effects on the vascularization process [3].

Initial proposals approaching the cytokine balance in pregnancy relied on the existence of a hypothetical Th1/Th2 equilibrium controlling pregnancy outcome, where a Th2-type cytokine response would predominates [4]. Nowadays it is known that inflammation is tightly controlled during all stages of pregnancy [2]. The first stage of pregnancy involves blastocyst implantation into the uterus, characterizing a pro-inflammatory phase in which the mother's immune system have to deal with the damage caused by the invading trophoblast. This stage is characterized by a localized activation of inflammatory mediators [5]. The second phase of pregnancy seems to be predominantly anti-inflammatory, with increased Th2 cytokine levels locally, at the feto-maternal interface, or even systemically [5]. The last phase would involve parturition, including a range of physiological alterations, as uterus contractions and delivery *per se*, with the return of a pro-inflammatory milieu [5].

Interleukin 17 (IL-17) is a pro-inflammatory cytokine which induces the expression of several inflammatory mediators [6]. Although IL-17 is largely produced by T cells (Th17 cells) [7], it can derives from other cells [6, 8, 9]. Importantly, IL-17 has been shown to induce neovascularization as well as the production of proangiogenic molecules [10]. Concerning the human maternal-fetal interface, decidual cells attract Th17 cells by secreting CCL2 and, through the secretion of IL-17, these recruited cells inhibit apoptosis of human trophoblast cells as well as induce them to proliferate and invade the decidua [11]. Considering the role of pro-inflammatory cytokines in healthy pregnancy and spontaneous abortion, the aim of this study was to compare the levels of Th1, Th2 and Th17 cytokines of women ongoing normal pregnancy with those found in women who suffered spontaneous abortion.

Materials and methods

Sixteen healthy pregnant women (age mean of 29.9 ± 9.1 years) and 13 women (age mean of 27.9 ± 4.5 years) who suffered spontaneous abortion were selected for this study. All women from the spontaneous abortion group were in the first trimester of pregnancy. Women from the healthy pregnant group were in the second or third trimester of pregnancy. Approximately 8 mL of peripheral blood was collected from each participant of the study. For the spontaneous abortion group, women were recruited for the study at the emergency room of the *Hospital de Clínicas de Porto Alegre* (HCPA, Porto Alegre, Rio Grande do Sul State, Brazil), and blood was collected until 24 h after the occurrence of abortion. Blood samples were diluted in PBS (1:1), and plasma was collected after density gradient centrifugation. Plasma samples were stored at − 80 °C until the execution of the Cytometric Bead Array experiments. All participants signed a consent form and this study was approved by the research ethics committees of HCPA and *Universidade Federal do Rio Grande do Sul*.

Th1/Th2/Th17 cytokine profile

Cytokine analyses were performed using the Human Th1/Th2/Th17 Cytometric Bead Array kit (CBA; BD Biosciences, San Jose, CA, USA; Catalog No. 560484), which allowed the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A by flow cytometry. Aliquots of plasma were diluted with assay diluent, and CBA analysis was performed according to the manufacturer's instructions. Briefly, 300 µL of each sample were plated on PRO-BIND™ 96-well assay plates and analyzed on the FACS Array Bioanalyzer, using the FCAP FCS Filter and FCAP Array software (BD Biosciences). Debris were filtered from the data, the bead populations were identified and mean fluorescence intensities (MFIs) were assessed. Posteriorly, using GraphPad Prism 5.01 software (GraphPad Software, Inc., San Diego, CA, USA), cytokine levels (in pg/mL) were compared between the groups through the non-parametric Mann–Whitney test. p-values < 0.05 were set as statistically significant.

Results and discussion

The cytokine levels in each group are shown in Table 1. IL-17 levels were increased in the group of healthy pregnant women when compared to the spontaneous abortion group ($p = 0.0232$). IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ levels were not statistically different between the two groups ($p > 0.05$).

IL-17 expression was subject to evaluation in distinct situations related to pregnancy, although controversial data comes out from such studies. For instance, Cai et al. [12] described higher IL-17 levels in patients with unexplained recurrent spontaneous abortion (URSA) as compared to women with normal early pregnancies. Importantly, in this study, samples were obtained before artificial miscarriage [12]. Conversely, Hosseine et al. [13] detected high IL-17 levels in menstrual blood of healthy fertile women but not in URSA patients, suggesting that the presence of high IL-17 levels would be part of a unique milieu, which ultimately will represent optimal conditions towards a successful embryo implantation [13]. Besides, the invasion of maternal tissues by the fetus can be compared to an allograft

Table 1 Cytokine levels in health pregnant and spontaneous abortion patients

Cytokine	Health pregnant pg/mL, median (IQR) ($n=16$)[a]	Spontaneous abortion pg/mL, median (IQR) ($n=13$)	p value (Mann–Whitney test)
IL-2	0.06887 (0.06161–0.07165)	0.06737 (0.06466–0.06887)	0.7087
IL-4	0.01719 (0.01488–0.02169)	0.01935 (0.01640–0.03034)	0.0652
IL-6	0.03094 (0.02891–0.03413)	0.03133 (0.02690–0.03452)	0.8434
IL-10	0.03169 (0.02650–0.03641)	0.03004 (0.02842–0.03515)	0.9301
IL-17	0.2059 (0.1223–0.6755)	0.1238 (0.1091–0.1274)	**0.0232**
TNF	0.03209 (0.02863–0.03699)	0.03221 (0.02811–0.03295)	0.3677
IFN-γ	0.02620 (0.02043–0.02840)	0.02923 (0.02349–0.03320)	0.0906

Significant p-value is showed in bold

IQR interquartile range

[a]For IL-17, in the health pregnant group the sample number was 10

[2], and Th17 cells have already been reported as important in allograft rejection [14]. The proposal of a Th1/Th2 balance to a favorable pregnancy outcome, in which a Th2-type cytokine response is predominant, was reinforced by a Th1 prevalence in various pregnancy complications [15]. However, this dichotomy has been challenged by recent findings regarding the role of Th17 cytokines both in normal and pathological pregnancies [16, 17]. Our findings point to higher levels of IL-17 in the peripheral blood of healthy pregnant women when compared to women who suffered spontaneous abortion. In agreement, it was already observed that IL-17 levels continuously increase throughout the gestation period [17]. Moreover, the importance of Th17 cells to a successful pregnancy was highlighted by studies in mice models, since a high-dose of IFN-γ promoted abortion in mice by suppressing T regulatory (Treg) and Th17 polarization [18].

What makes the levels of IL-17 higher in normal pregnancy as compared to spontaneous abortion? First, there is a massive attraction of peripheral Th17 cells to the decidua in the first trimester of pregnancy [11]. Also, Th17 and Treg cells have some level of plasticity [19–21]. In particular situations, this plasticity can allow Treg cells to transdifferentiate into Th17 cells [19] and vice versa [20, 21]. Second, an exacerbated inflammatory status of abortion could be accentuated at the maternal-fetal interface due to the recruitment of Th17 cells by decidual resident cells [11]. In the decidua, Th17 cells could be subsequently regulated by Treg cells, potentially in the light of the above-mentioned plasticity, what ends up by affecting the peripheral blood level of IL-17. Recalling the inflammatory nature of parturition [5], the samples from healthy pregnant women used in our study corresponds to late gestational periods (second and third trimesters), and high levels of IL-17 have already been seen in healthy women with term parturition [17] as well as in different tissues from placenta [22]. Taking into account these observations, the gestational stage approached in a give study seems to be an important point to be considered. Recently, Chavan et al. [23], comparing eutherian mammalian pregnancy with the equivalent phenomena in marsupials, suggested that IL-17A would be an essential signaling molecule, which would prevent neutrophils to enter the endometrium, thus allowing the maintenance of the prolonged pregnancy seen in these animals.

In line with this suggestion, the present study highlights IL-17A as a possible biomarker for miscarriage risk in the monitoring of early pregnancies. In this context, IL-17A absence or low levels would be associated to gestational loss risk, which is in accordance with our results. In addition, further research using samples from the same gestational period and a larger sample number would reinforce the findings concerning the importance of this cytokine in healthy and complicated pregnancies.

Finally, we would like to highlight that the flow cytometry kit used to quantify the cytokines in our study detects specifically IL-17A. Once the majority of authors did not specify the subtype of IL-17 measured in their studies it is difficult to ensure that the comparisons of our findings with previous researches are accurate. Besides, since measurements were performed using plasma directly obtained from patients, it provides us with an actual picture of cytokine levels in the peripheral blood, just after spontaneous abortion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the research ethics committees of HCPA and Universidade Federal do Rio Grande do Sul under the register CAAE: 11390313.7.0000.5347 and all participants signed an informed consent form.

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Capítulo V

Influence of *NKG2C* gene deletion and CCR5Δ32 in Pre-eclampsia—Approaching the effect of innate immune gene variants in pregnancy

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Influence of NKG2C gene deletion and CCR5Δ32 in Pre-eclampsia—Approaching the effect of innate immune gene variants in pregnancy

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Abstract

Pre-eclampsia (PE) is a hypertensive disorder that affects an important number of pregnant women worldwide. The exact causes of PE remain poorly understood. However, inflammation and deregulation of innate immune cells, such as natural killer (NK) cells, contribute to PE pathogenesis. Besides, the mother's genetic background also impacts on PE susceptibility. Thus, genetic variants that potentially modify the behaviour of inflammatory cells may help us to understand the causes of PE. Variants of genes encoding NKG2C (expressed in NK cells) and C–C chemokine receptor type 5 (CCR5) (expressed mainly in leucocytes) are important targets in the study of gestational disorders. In this context, we evaluated the impact of both *NKGC2* gene deletion and CCR5Δ32 gene variant on PE susceptibility in a population sample from central-southeast Brazil composed by 369 women (156 with PE and 213 healthy pregnant women). No statistically significant association between the *NKG2C* gene deletion and susceptibility to PE was observed. However, taking into consideration the important role of NK cells in pregnancy, the influence of *NKG2C* gene deletion on PE pathogenesis should not be ruled out and deserves further studies in populations with different genetic/ethnic backgrounds. In addition, our results regarding CCR5Δ32 corroborate previous data from our group approaching a distinct cohort and reinforce CCR5Δ32 as a protective factor against PE development ($p < 0.05$).

KEYWORDS

CCR5, CCR5Δ32, inflammation, innate immunity, NK cells, *NKG2C*, Pre-eclampsia

1 | INTRODUCTION

Pre-eclampsia (PE) is a hypertensive disorder that affects 2%–8% of all pregnant women (Duley, 2009). PE is a polygenic disorder resulting from both foetal/placental and maternal genetic contributions and manifests as a complex phenotype (Michita, Kaminski, & Chies, 2018; Triche et al., 2014). In pathophysiological terms, PE is characterized by de novo hypertension after 20 weeks of gestation combined with proteinuria (Duley, 2009; Mol et al., 2016). One or more of the following disorders can also be found in women with PE: renal insufficiency, liver involvement, neurological/haematological complications and uteroplacental dysfunction (Mol et al., 2016). In addition, foetal growth restriction can be associated with PE (Mol et al., 2016).

The exact causes of PE are still poorly understood. However, it is known that chronic inflammation and inflammation-related complications are pivotal in PE pathogenesis (Borzychowski, Sargent, & Redman, 2006; Harmon et al., 2016). Besides, it is well established the influence of maternal coagulation unbalances in PE development, in which a major event is the non-adequate blood supply

to the placenta, resulting in high oxidative stress in placental cells (Borzychowski et al., 2006). The physiological processes involved in the haemostatic dynamics necessary for adequate placentation are closely related to immune processes required for maternal tolerance towards the foetus (Li & Huang, 2009). Interestingly, innate immune responses are involved in both the processes of coagulation and inflammation in pregnancy. In these circumstances, macrophages, dendritic cells and Natural killer (NK) cells are the major innate immune cells that have been demonstrated to play essential roles in early gestation periods. More specifically, immune cells can trigger coagulation cascades, and as a counterpart, coagulation proteases exhibit substantial immuno-modulatory effects (Li & Huang, 2009). Upon exogenous challenges, the immune and coagulation systems can potentiate each other, thus leading to a vicious cycle (Li & Huang, 2009), in which alterations could bring unwanted outcomes for the mother and/or the foetus.

Natural killer cells are cytotoxic lymphocytes which secrete cytokines and modulate the function of antigen-presenting cells and the adaptive response of T cells. Thus, NK cells can be viewed as a connection between the innate and adaptive immunity and are important cells to an adequate development of immune responses (Long, Kim, Liu, Peterson, & Rajagopalan, 2013).

Healthy pregnancy and gestational disorders are also affected by NK cells (Dosiou & Giudice, 2005). Notably, deregulated NK cells function contributes to PE development (Sargent, Borzychowski, & Redman, 2007). NK cells express a family of receptors called CD94/NKG2, whose members could induce either a suppressive or an activating activity (Borrego, Masilamani, Marusina, Tang, & Coligan, 2006). In humans, the NKG2 receptors family comprises the following members: NKG2A, NKG2B, NKG2C, NKG2D, NKG2E, NKG2F and NKG2H (Brostjan et al., 2000). Genes encoding CD94/NKG2 receptors are clustered in the NK gene complex, at chromosome 12, in the 12p12-13 region (Hikami, Tsuchiya, Yabe, & Tokunaga, 2003). Several polymorphisms have already been described in the NKG2 gene family (Hikami et al., 2003), including an *NKG2C* gene deletion (Hikami et al., 2003; Moraru et al., 2012). NKG2C is an activating receptor (Muntasell, Vilches, Angulo, & López-Botet, 2013), and NKG2C gene deletion could impair NK cells activity. Moreover, HLA-E, a specific ligand of the NKG2C receptor, is expressed in the context of human pregnancy in trophoblast cells (Hackmon et al., 2017). Thus, the role of the *NKG2C* gene deletion in healthy and pathological situations could contribute to unraveling important immune aspects of PE.

Increased systemic production of pro-inflammatory chemokines is another key finding in women with PE (Szarka, Rigó, Lázár, Bekő, & Molvarec, 2010). Of note, factors related to the mother's genetic background also contribute to PE development (Williams & Pipkin, 2011). Looking at the above-mentioned scenario, an approach to elucidate some of the potential genetic factors involved in innate immune-related causes of PE encompasses the investigation of genetic variants related to molecules involved in different inflammation pathways.

Cysteine–cysteine chemokine receptor type 5 (CCR5) is a protein encoded by the *CCR5* gene, which is localized on chromosome 3, at 3p21.3 region (Maho, Bensimon, Vassart, & Parmentier, 1999; Samson, Soularue, Vassart, & Parmentier, 1996). CCR5 is expressed in leucocytes and some other cell types and is an important receptor in inflammatory reactions (Barmania & Pepper, 2013). CCL3/MIP-1α, CCL4/MIP-1β and RANTES/CCL5 are the main CCR5 agonists (Blanpain et al., 1999; Jones, Maguire, & Davenport, 2011). In a recent study, Salazar Garcia et al. (2018) described the occurrence of increased CCL3/MIP-1α levels in women with PE. Moreover, increased CCL5/RANTES levels were also observed in both plasma and placental tissues of preeclamptic women compared to healthy pregnant ones (Hentschke et al., 2012), data which corroborate the upregulation of the *RANTES* gene expression in women with PE reported by Heikkilä et al. (2005). Taking together, these findings suggest that a CCR5-mediated inflammation during pregnancy could be involved in PE development. On the other hand, at least partially, some data do not support the hypothesis that high levels of CCR5 ligands are involved in PE pathogenesis (Adela et al., 2017; Jonsson et al., 2006; Mosimann, Wagner, Poon, Bansal, & Nicolaides, 2013). These conflicting findings highlight the need to study in greater detail the involvement of the CCR5 molecule in PE pathogenesis. An interesting *CCR5* gene variant, the so-called CCR5Δ32 allele, presents a 32-base pair deletion and is found mainly in individuals having a Caucasian origin (Lucotte, 2001). Homozygous individuals for the CCR5Δ32 allele lack the expression of a functional CCR5 on the cell surface, while heterozygous individuals for this variant express lower levels of functional CCR5 as compared to wild-type homozygous individuals (Venkatesan et al., 2002; Wu et al., 1997). Remarkably, there is some evidence showing a protective effect of CCR5Δ32 on PE development (Gurdol, Yurdum, Ozturk, Isbilen, & Cakmakoglu, 2012; Telini, Veit, Chies, & Vianna, 2014). However, such evidence is still scarce and must be explored in studies involving different human populations.

Considering the NKG2C molecule in the context of innate immunity, the action of NK cells in pregnancy and the importance of the CCR5 molecule on inflammatory processes, this study aimed to explore the frequencies of the *NKG2C* gene deletion and of the CCR5Δ32 variant in a cohort of Brazilian women who developed PE, in comparison to a group of women with healthy pregnancy.

2 | MATERIALS AND METHODS

Regarding the subjects' enrolment, 213 healthy pregnant women with uncomplicated pregnancies (Healthy Pregnancy group) and one hundred and fifty-six pregnant women with primary PE (PE group) were recruited at Hospital Sofia Feldman in Belo Horizonte (Central-Southern Brazil). All participants signed a written informed consent form for blood sample collection. Importantly, this study was approved by the Hospital Ethics committee (CAAE: 01822312.0.1111.5132). The diagnosis for PE was made according to the presence of hypertension (blood pressure >140 mm Hg [systolic] and/or 90 mm Hg [diastolic]) and proteinuria (>300 mg of protein every 24 hr). In this study, women were classified as Caucasians or non-Caucasians according to phenotypic

characteristics and ethnicity data from parents/grandparents reported by the participants in an appropriate questionnaire.

The frequencies of both *NKG2C* gene deletion and CCR5Δ32 variant were evaluated in the above-mentioned cohort of Brazilian women who developed PE, compared with the group of healthy pregnant women. Table 1 shows the clinical and demographic characteristics of women included in our analysis. Of note, part of women studied here was previously included in a study performed by Pontillo et al. (2015). The *NKG2C* gene deletion and the CCR5Δ32 variant were genotyped using conventional PCR according to the methods described by Moraru et al. (2012) and Chies and Hutz (2003), respectively. As a technical control, 10% of the DNA samples used in genotyping of *NKG2C* gene deletion were genotyped twice to confirm the reliability of the results. For the statistical analysis of both genetic variants, the numbers of carriers and non-carriers of the deletion allele were compared between the groups using chi-square test with Yates's correction. Odds ratio and Wald 95% confidence interval were also considered. *p* Values<0.05 were set as statistically significant. The statistical analyses were performed with WINPEPI (version 11.65) software (Abramson, 2011). All groups were tested for the Hardy–Weinberg equilibrium applying chi-square test.

3 | RESULTS AND DISCUSSION

The genetic profiles (allele and genotype frequencies) of the women included in this study and comparisons between the groups are shown in Table 2. All genotype frequencies were in agreement with the expectations to Hardy–Weinberg equilibrium ($p > 0.05$). The CCR5Δ32 allele frequency observed in the control group (0.045) was slightly lower than that found in a previous study evaluating a Caucasian southern Brazilian population (0.066) (Ellwanger et al., 2018). In this study, no statistically significant difference was found between the groups regarding the frequency of the *NKG2C* deletion allele. On the other hand, healthy pregnant women showed a higher frequency of Δ32 allele when compared to women with PE ($p = 0.047$), suggesting a protective effect of CCR5Δ32 on PE development.

In the context of pregnancy, the consequences of the *NKG2C* gene deletion are potentially important due to the role of NK cells in pregnancy development. It was estimated that ~70% of the maternal immune cells recruited during decidualization correspond to NK cells (Cartwright, James-Allan, Buckley, & Wallace, 2017). To the best of our knowledge, this is the first study evaluating the potential influence of the *NKG2C* gene deletion on PE development. According to Bachmayer et al. (2009), women with PE had significantly higher levels of NKG2A and NKG2C in peripheral NK cells when compared to healthy pregnant women. In accordance, Bueno-Sánchez et al. (2013) found an increased percentage of $NKG2C^+$ NK cells in women with PE. Such high NKG2C expression in PE could reflect an innate adapting mechanism of NK cells to face the immune challenges found in women undergoing PE (Bachmayer et al., 2009). In this sense, the *NKG2C* gene deletion could modify the influence of NK cells in PE development, a hypothesis which is not corroborated by our data.

In a previous study, our group had already verified a potential protective effect of the CCR5Δ32 allele on PE development in a southern Brazilian population. Importantly, this effect was independent of the ethnic background of the studied population (Telini et al., 2014). Thus, the present study corroborates our previous findings and indicates that the potential influence of CCR5Δ32 on PE susceptibility is shared by different Brazilian populations. These findings are also in agreement with data from a Turkish cohort (Gurdol et al., 2012). Therefore, we propose that the presence of the CCR5Δ32 allele, which is associated to lower CCR5 expression levels, could prevent an exacerbated CCR5-mediated inflammatory response during pregnancy, thus affecting the inflammatory component in PE susceptibility and development. The potential impact of CCR5Δ32 on the inflammatory component of PE is schematically presented in Figure 1, taking into account the role of inflammation in PE development (Borzychowski et al., 2006; Harmon et al., 2016), as well as the evidence showing that different genetic profiles of CCR5Δ32 are implicated in distinct levels

TABLE 1 Characteristics of the women included in each group

Characteristic	Healthy pregnancy group (*n* = 213)	Pre-eclampsia group (*n* = 156)	*p*-value
Maternal age, median (IQR)	25 (21–30)[a]	26 (21–32)[b]	>0.05[i]
Pre-pregnancy BMI, median (IQR)	25.59 (24.05–28.41)[c]	26.45 (23.3–28.09)[d]	>0.05[i]
SBP, median mm Hg (IQR)	120 (110–130)[e]	155 (140–164)[f]	**<0.0001**[i]
DBP, median mm Hg (IQR)	64 (60–70)[g]	100 (86–100)[h]	**<0.0001**[i]
Caucasians, *n* (%)	33/212 (15.6%)	34/150 (22.7%)	>0.05[j]
Non-Caucasians, *n* (%)	179/212 (84.4%)	116/150 (77.3%)	

Note. BMI: body mass index; DBP: diastolic blood pressure; IQR: interquartile range; *n*: sample number; SBP: systolic blood pressure.
[a]Based on *n* = 211. [b]Based on *n* = 154. [c]Based on *n* = 33. [d]Based on *n* = 150. [e]Based on *n* = 212. [f]Based on *n* = 154. [g]Based on *n* = 212. [h]Based on *n* = 154. [i]Based on non-parametric Mann–Whitney test. [j]Based on Pearson's chi-square with Yates's correction.
Statistically significant values are shown in bold.

TABLE 2 Genetic profiles of the study subjects and comparisons between the groups

Genetic variant	Genetic profile	Healthy pregnancy group	Pre-eclampsia group	O.R. (C.I. 95%)	*p*-value*
CCR5Δ32 (rs333)	Total *n* genotyped	213	156		
	CCR5 wt/wt, *n* (%)	194 (91.08)	151 (96.79)		
	CCR5 wt/Δ32, *n* (%)	19 (8.92)	5 (3.21)		
	CCR5 Δ32/Δ32, *n* (%)	–	–		
	CCR5Δ32 allele frequency	0.045	0.016		
	CCR5Δ32 non-carriers, *n* (%)	194 (91.08)	151 (96.79)	0.35 (0.12–0.93)	**0.047**
	CCR5Δ32 carriers, *n* (%)	19 (8.92)	5 (3.21)		
NKG2C gene deletion	Total *n* genotyped	203	151		
	NKG2C wt/wt, *n* (%)	133 (65.52)	90 (59.60)		
	NKG2C wt/del, *n* (%)	58 (28.57)	50 (33.11)		
	NKG2C del/del, *n* (%)	12 (5.91)	11 (7.29)		
	NKG2C del allele frequency	0.202	0.238		
	NKG2C del non-carriers, *n* (%)	133 (65.52)	90 (59.60)	1.29 (0.83–1.99)	0.304
	NKG2C del carriers, *n* (%)	70 (34.48)	61 (40.40)		

Note. C.I: 95%: Wald 95% confidence interval; *n*: sample number; O.R.: odds ratio; wt: wild-type.
CCR5Δ32 allele frequency = (2 × *n* individuals Δ32/Δ32) + (*n* individuals wt/Δ32) ÷ (2 × *n* total individuals).
NKG2C del allele frequency = (2 × *n* individuals del/del) + (*n* individuals wt/del) ÷ (2 × *n* total individuals).
*Chi-square test with Yates's correction. Analysis considering the number of carriers and non-carriers of the variant allele (CCR5Δ32 or NKG2C del) in each group. Statistically significant value is shown in bold.

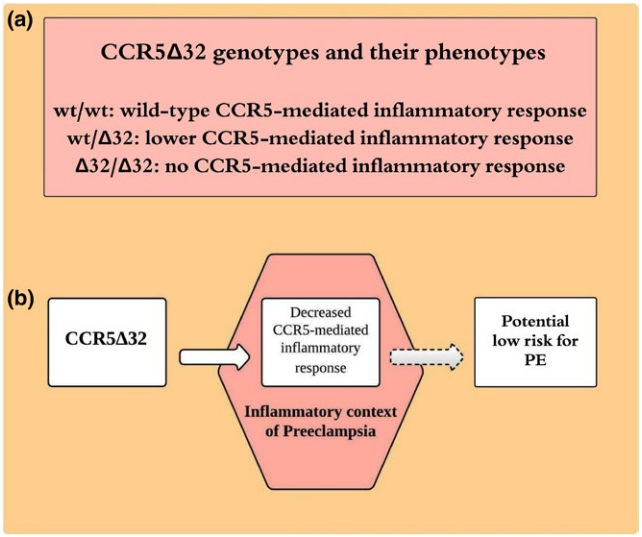

FIGURE 1 Potential contribution of the CCR5Δ32 variant on the inflammatory context of PE development. (a) Different CCR5Δ32 genotypes promote differentiated CCR5 expression on cell surface (Venkatesan et al., 2002; Wu et al., 1997); (b) Chronic/systemic inflammation is an important factor for PE development (Borzychowski et al., 2006; Harmon et al., 2016). Low expression of the CCR5 molecule in maternal cells due to the presence of the CCR5Δ32 allele could minimize the exacerbated inflammation during pregnancy, thus protecting against PE development. This scenario is suggested by our results and is supported by previous studies (Gurdol et al., 2012; Telini et al., 2014)

of CCR5 expression on the cell surface (Venkatesan et al., 2002; Wu et al., 1997) (Figure 1a). In a scenario dominated by the CCR5Δ32 variant, a lower CCR5-mediated inflammatory response could potentially contribute for low PE risk (Figure 1b).

A number of studies evaluating the NKG2C receptor are focused on the context of human cytomegalovirus (HCMV) infection, once HCMV exhibits a pronounced impact on host NK cells, as reviewed by Della Chiesa, Sivorim, Carlomagnom, Moretta, and Moretta (2015). However, the impact of *NKG2C* gene variants on the susceptibility of gestational disorders and other diseases was until now scarcely explored. The *NKG2C* gene deletion has been observed in Asian and Caucasoid populations. In a study performed by Miyashita et al. (2004), the *NKG2C* homozygous deletion presented a frequency of 4.1% in individuals from Japan and a frequency of 3.8% amongst Dutch populations. These values are slightly lower than those here observed, in which the frequency of *NKG2C* homozygous deletion was 5.9% in the control group and 7.29% in the PE group (Table 2).

In conclusion, the present study reinforces the potential protective role of the CCR5Δ32 allele against PE development in the Brazilian population. It is possible that a reduced CCR5 expression on the surface of maternal immune cells due to the presence of the CCR5Δ32 allele contributes to restrain the potential CCR5-mediated inflammation in pregnant women, therefore protecting the CCR5Δ32 allele carriers against PE development. Taking into consideration the influence of the genetic background on PE development,

further investigations focused on different genetic variants may help us to understand the factors that impact PE susceptibility and pathogenesis in different populations.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Parte II

Capítulos VI, VII e VIII

Capítulo VI

Immunogenetic Factors in Autism Spectrum Disorder–Keeping Gene Variants on Stage

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Immunogenetic Factors in Autism Spectrum Disorder–Keeping Gene Variants on Stage

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Abstract

A growing incidence of Autism Spectrum Disorder (ASD) is a typical feature in several populations around the world. ASD encompasses a group of complex early-onset neurodevelopmental pathologies mainly characterized by unpaired communicative and cognitive skills and repetitive/stereotypic behaviors. Currently, it is widely accepted that ASD has a strong genetic component, which is evidenced by a high heritability. Among possible biological routes, increasing evidence has addressed the contribution of the immune system (and its interactions with the central nervous system) as a major component of ASD susceptibility. Imbalances in immune responses during the gestation period or latter in life have been associated with both the incidence and different clinical manifestations of this disorder. Considering this, several studies focus on the characterization of the puzzling developmental and physiological pathways and steps in which disruptions could lead to autistic manifestations, performing evaluations that vary from serum proteins and metabolic pathways measurements to the identification of genetic and epigenetic markers. Here, we reviewed immunogenetic studies in ASD, organized in three different main targets, as follows: (I) genetic variants with a pro-inflammatory impact; (II) MHC genetic variants; and (III) immunometabolism-related genetic variants. Our goal is to highlight the importance of studying variation in genes related to the immune processes in the task force dedicated to clarifying the autism spectrum disorder' etiology. Also, this effort aims to gather in one comprehensive review the bulk of such interesting genetic data, since they are currently quite dispersed in the scientific literature. In this review work, among the three approaches investigated, we found a larger number of studies involving genes related to immunometabolism.

Keywords: genetics; autism; polymorphism; immune system; ASD

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental pathology mainly characterized by repetitive, stereotypical behaviors, and impaired communication skills affecting four times more males than females. In last years, there is a growing incidence of ASD in several populations (for instance, from 20-60/10.000 individuals in 2000's to 1 out of 68 children in 2010's; see Fombone, 2003; Rutter, 2005 and CDC, 2014). This could be partially due to better awareness on the part of the patient's family, changes in diagnostic trends, and also more sensitive diagnostic systems (Meltzer and Van de Water, 2017; Baio et al., 2018); although, this growing incidence also raises questions about the triggering factors of such condition. As for all multi-factorial disorders, the study of ASD is still a challenge that requires the interaction of knowledge from different research areas, beyond the cognitive sciences, and including fields such as genetics and immunology (Betancur, 2011; Jiang et al., 2013; Meltzer and Water, 2017; Liberman et al., 2018). Regarding its genetic aspects, autism can be further divided into simplex and multiplex terms, the first is related to families in which only one child was diagnosed, while the last represents situations where more than one child from the same family is affected. Interestingly, an evaluation of quantitative autistic traits inheritance revealed that subclinical autistic traits can be observed in ''unaffected'' relatives of children with autism, suggesting that the characterization of such quantitative traits and other endophenotypes among close relatives of an affected child may be useful for reducing sample heterogeneity in future genetic and neurobiological studies of ASD (Virkud et al., 2009).

There is strong support for a genetic component in ASD. In the meta-analysis performed by Tick et al (2016) the concordance rate in monozygotic twins was almost complete (98%), being lower for dizygotic twins (53-67%). According to these data, a substantial meta-analytic heritability of 64-91% was estimated. On the other hand, the discordance rate in the incidence of autism in cases of monozygotic twins brings to light the role of environmental triggering for ASD development and manifestation. Considering the polygenic (Weiner et al., 2017) and epistatic (Coutinho et al., 2007) genetic counterpart in autism etiology, it is proposed that environmental factors actually interact with the patient's genetic component, ultimately increasing the disease risk and/or the symptoms' severity observed throughout the spectrum (Gardener et al., 2009).

Given the well accepted strong genetic background of ASD (Muhle et al., 2004; Vorstman et al., 2017), much effort has been made through studies trying to identify genetic contributors to disease manifestation. Genetic association studies were among the first methodologies used in this area, and suggested genes codifying components of different biological functions as involved in ASD (e.g. neurotransmitter metabolism; neurodevelopemental control; neuronal cell adhesion; and genes related to language disorders and social interaction). Nevertheless, the overall findings are inconclusive and there are no result showing a strong association, or being able to be consistently replicated (Baio et al., 2018). Genome-Wide Association Studies (GWAS), which are important strategies to find out the major genetic variants contributing to disease manifestation, has also been applied in ASD molecular studies. The success of these approaches in finding individual susceptibility genes for the disease was limited, but they were

valuable suggesting novel biological pathways for investigation. In addition to GWAS, exome and whole genome sequencing analyses focusing on rare variants have also suggested the contribution of novel genes in ASD etiology, through both inherited and de novo mutations (Jiang et al., 2013). These results come along with whole genome-wide copy number variation (CNV) studies unrevealing de novo as well as inherited regions of rare large effects possible related to ASD (Davis et al., 2018; Iossifov et al., 2014; Levy et al., 2011; Sebat et al., 2007). Regarding epigenetic approaches, the identification of methylation quantitative trait loci (meQTLs) is also a target of investigation in the context of ASD. Interestingly, the investigation of meQTL targets has revealed clues into functional roles, including immune response pathways, that would not be evident via genotype-based analysis in isolation (Andrews et al., 2017). However, both due to the complex ASD phenotypes and to the high genetic variability among populations worldwide, much about the genetic complexity of these neurological pathologies still need to be elucidated. Important points to be clarified encompass the evaluation of gene interactions and the identification of haplotypes, which justifies the use of classical and population-specific genetic studies. Of note, genetic polymorphisms are mainly population-specific and may respond differently to different environmental variations (Ellwanger et al., 2019). Actually, even studies unrevealing the lack of association between specific gene variants in different populations are needed, since they give robustness to important approaches such as meta-analyses.

Impairments in inflammatory responses are a characteristic feature in ASD patients during life, indicating a strong immune component in the disease manifestation (Mazur-Kolecka et al., 2014; Mostafa and Al-Ayadhi, 2013; Mead and Ashwood, 2015). It was suggested that the immune component of ASD relies mainly on the dis-regulation of inflammatory processes, both during pregnancy as well as later in life (**Figure 1**). This is illustrated by cases such as those reported to the cohort from the rubella syndrome in 1950, in which 5% of the infected pregnant woman had children with ASD (Chess, 1971; Chess 1977; Shi et al., 2003). Subsequently, other studies demonstrated that several infections, including influenza virus and measles, could also be associated to ASD development. After the first associations between autism and infections during gestation, it was believed that this condition was directly linked to infections. Nevertheless, the incidence of ASD in children from complicated pregnancies not related to pathogens, such as preeclampsia and gestational hypertension, led to a broader association between ASD and maternal inflammation in general (Wang et al., 2017).

Autism incidence has a higher concordance rate in monozygotic twins (60–92%) in comparison to dizygotic twins (0–10%). Thus, in one hand, there is strong support for a genetic component of the disorder. On the other hand, the discordance rate in the incidence of autism in cases of monozygotic twins brings to light the role of environmental triggering for ASD development and manifestation. Considering the polygenic (Weiner et al., 2017) and epistatic (Coutinho et al., 2007) genetic counterpart in autism etiology, it is proposed that environmental factors actually interact with the patient's genetic component, ultimately increasing the disease risk and/or the symptoms’ severity observed throughout the spectrum (Gardener et al., 2009).

Inflammation is a key process for pregnancy establishment, maintenance, and also one of the key factors involved in the physiological alterations during delivery (Mor et al., 2011). There are increasing

incidence linking the diagnostic of behavioral and psychiatric disorders in children whose mothers experienced infections during pregnancy. The immune signaling pathways triggered by a pathogen involves cell communication molecules whose dis-regulation can impact in all stages of fetal brain development. In fact, a persistent immune activation could be harmful to the developing fetus and thus led to neuronal and psychiatric issues later in life (Careaga et al., 2017). The fact that ubiquitous air pollutants, such as nitrous oxide (N2O), could impair anti-inflammatory mediators gives another evidence for the role of inflammatory processes in ASD, since the exposure to that environmental factor has already been proposed to contribute to ASD etiology (Fluegge, 2017).

Considering this, it is important to study genetic variants that could be involved in the production and regulation of pro-inflammatory molecules by the mother and the placenta during pregnancy. The maternal genetic variants could represent a component of the environmental factors potentially influencing the uterine milieu, subsequently affecting the fetus (Michita et al., 2018). A precious tool for studies on the influence of polymorphisms in inflammation-related genes that could contribute to maternal/placental imbalances in inflammation during pregnancy is the evaluation of triads comprising a case and two parents. Single genetic variants contribute to 1-2% of ASD cases (Abrahams and Geschwind, 2008), but the lack of a direct cause, the genetic variation among human populations, and the multi-factorial characteristics of the disease make it difficult to identify factors that have just a small impact on ASD development. Thus, the investigation of different genetic variants in samples from distinct populations is still welcome and of singular importance. Through this review, we intend to highlight the importance of studying polymorphisms in genes related to the immune processes in the task force dedicated to clarifying the autism spectrum disorder' etiology. Also, this effort aims to gather in one comprehensive review the bulk of such interesting genetic data, since they are presently quite dispersed in the scientific literature and whose joint analysis may bring new insights for future investigations.

Genetic variants with a pro-inflammatory impact in ASD

Several discoveries have associated ASD and alterations in the immune system (Onore et al., 2012). As summarized in **Figure 1**, such alterations included the presence of brain-reactive antibodies (Cabanlit et al., 2007); abnormal T cell responses and activation (Gregg et al., 2007); altered cytokine levels in brain, cerebrospinal fluid (CSF) and peripheral blood circulation (Vargas et al., 2005; Jyonouchi et al., 2014; Molloy et al., 2006); increased levels of circulating monocytes (Sweeten et al., 2003); and dysregulation in Natural Killer (NK) cells activity (Enstrom et al., 2009). In this context, Ross et al. (2013) examined the circulating cytokine levels in DiGeorge syndrome patients, a condition related to an increased risk for autism and schizophrenia. In this study, a correlation between social impairments and elevated levels of the pro-inflammatory cytokines interleukin (IL)-1β and IL-6, as well as the anti-inflammatory cytokine IL-10 was observed. Furthermore, elevated levels of the Th1 cytokines IL-12p70 and interferon (INF)-γ were associated with social and repetitive behavior scores. Increased levels of pro-inflammatory cytokines and chemokines such as IFN-γ, IL-1β, IL-6, IL-12p40, tumor necrosis factor (TNF)-α and chemokine C-C motif ligand

(CCL)-2 were also observed in the brain tissue and CSF in ASD patients (Vargas et al., 2005; Li et al., 2009), which could affect functions of the immune system at the central nervous system (CNS).

In general, cytokines and their receptors are highly implicated in inflammatory processes, CNS development, neuronal plasticity, and cellular pathways at the transcription level (Smith et al., 2012; Boulanger, 2009). Therefore, variants in genes coding these receptors may be involved in neuron development impairments observed in ASD children.

In this line, several research groups have addressed the study of polymorphisms in genes involved in pro-inflammatory processes. The importance of such investigation field relies on finding out variations in immune-related genes that could be associated with the onset and progression of ASD. Subsequently in this section and in **Table 1**, an overview of the genetic variants in genes involved in pro-inflammatory responses already investigated in ASD is presented. Some examples will be detailed in the following paragraphs.

The gene *MET* encodes a member of the receptor tyrosine kinase family of proteins, the MET receptor tyrosine kinase, which is also involved in immune responses (Fruman et al., 2017). Variants in this gene can alter the Toll-Like Receptor (TLR) signaling pathway leading to increased pro-inflammatory cytokine production and monocyte activation (Ashwood et al., 2008). In this context, a study analyzed the gene encoding the MET receptor tyrosine kinase in a family-based study including ASD probands. The study revealed the presence of an autism-associated variant in the *MET* promoter region and a statistically significant higher transmission of the rs1858830 C-allele to ASD affected individuals evidenced by the Transmission Disequilibrium Test (TDT). This *MET* promoter variant is a common G-to-C single nucleotide polymorphism (SNP) located just 20 base pairs 5' to the *MET* transcriptional start site (Campbell et al., 2006). This same variant was assessed by Heuer et al. (2011) in terms of a potential association with immune imbalances in the mothers of children with ASD. This report included a sample of mothers of typically-developing children as well as mothers of ASD children, from the Children Autism Risk from Genetics and the Environment (CHARGE) study in the state of California. A strong association between the *MET* promoter variant rs1858830 "C" allele and the presence of maternal autoantibodies against fetal brain was observed, as well as decreased MET protein expression. Moreover, reduced production of the regulatory cytokine IL-10 was detected in mothers of CC genotype compared with mothers of GG genotype (Heuer et al., 2011).

Forkhead Box P3 (*FOXP3*) encodes a transcription factor pivotal for T regulatory cells (Tregs) functional regulation (Yagi et al., 2004). Considering the immune aspects in ASD linked to T cell dysfunctions, Safari et al. (2017) addressed two gene variants of *FOXP3* (rs3761548 and rs2232365) aiming to evaluate the frequency of these genetic variants in an Iranian population of ASD patients together with age, gender, and ethnic-matched healthy controls. The results revealed a higher rs2232365-G allele frequency in the ASD group, which may represent a Treg cell deficiency in autistic children, corroborating a study with Egyptian affected children (Mostafa et al., 2010).

Integrins are membrane proteins that act as cell-matrix adhesion receptors, being expressed throughout the body. These molecules are important for adequate functioning of neurons, glial, meningeal, and endothelial cells, where they act locally and contribute to development regulation (Milner and Campbell,

2002). Considering the role of integrins in leukocytes migration during CNS inflammatory processes, Correia et al. (2009) evaluated eight integrin alpha 4 (*ITGA4*) SNPs (rs1449263, rs3770136, rs1449260, rs155100, rs3770116, rs3770112, rs2305581, and rs3770105) which were assessed in families with one ASD patient. An association between the SNP rs155100 and autism was found. Furthermore, an association between the *ITGA4* promoter marker rs1449263 and serum levels of autoantibodies in brain tissues of the autistic children was evidenced in this same study. Taken together, these findings further suggest a combined action of genetic variants and the extensive occurrence of autoantibodies in the brain of ASD patients as a potential trigger to disease development (Silva et al., 2004). Other studies have also evaluated SNPs of *ITGA4* and found different associations with ASD, such as the SNP rs12690517 in an Irish population sample composed by triads (Conroy et al., 2009). Ramoz et al. (2008) evaluated 84 linkage-informative SNPs covering the 2q24–q33 locus in a cohort of families with autism and subsets identified with phrase speech delay. Approaching TDT, a preferential transmission of the rs2305586-T allele at *ITGA4* gene was showed. In addition, significant over-transmissions of rs2056202-G allele within the *SLC25A12* gene, rs1807984-C allele and rs1517342-A and rs971257-A alleles within the *STK39* gene were also observed. (Ramoz et al., 2008). Of note, the gene *STK39* encodes a serine/threonine kinase abundantly expressed in the brain that has implications in immunological contexts (Baltoni et al., 2009).

The integrin-β 3 gene (*ITGB3*) was also assessed in family-based association studies. In the study from Napolioni et al. (2011), eleven *ITGB3* SNPs were evaluated in simplex and multiplex European descent families that included ASD patients, unaffected siblings and their biological parents. The analyses revealed *ITGB3* haplotypes associated with autism, being the extensively transmitted to the affected offspring H3 haplotype (rs2317385 allele “G”, rs2056131 allele “G”, rs4525555 allele “C”, rs2015729 allele “G”, rs5918 allele “T”, rs951351 allele “G”, rs15908 allele “C”, rs12603582 allele “G”, rs3809865 allele “A”, rs11650072 allele “C”), identified as a risk factor for autism (Napolioni et al., 2011). In addition, a family-based association study elaborated in a Brazilian sample of autistic patients tested for *ITGB3* SNPs’ association with different ASD outcomes. In this investigation, a preferential transmission of an especific haplotype of *ITGB3* (composed by the alleles rs2317385-G, rs5918-T, rs15908-A, rs12603582-G, and rs3809865-A) was observed in children with ASD. Moreover, an association between echolalia and both the “GG” homozygous genotypeat rs1260358 and the A/G haplotype – composed of rs15908-rs12603582 was verified (Schuch et al., 2014).

Furthermore, evidence showing the role of TLR signaling in ASD has been provided by Nadeem et al. (2017). This study evaluated the influence of TLR4 signaling in the regulation of NOX-2 derived reactive oxygen species (ROS) production via nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) in T cells of ASD children from Saudi Arabia. Intracellular ROS levels were measured in $CD4^+$ T cells obtained from affected children. The analysis showed increased expression of TLR-4 and NFκBp65 (a transcription factor) on $CD4^+$ T cells in ASD patients which meets an up-regulation of NOX-2 expression. Such up-regulation in ROS production was suggested to cause weakened adhesion of cells or tissues in affected children, thus resulting in increased immune cell infiltration in the CNS, which may contribute to neuroinflammation (Mittal et al., 2014).

Bringing out key molecules in CNS development, Toyoda et al. (2007) addressed SNPs of Epidermal Growth Factor (*EGF*), Transforming Growth Factor β-1 (*TGFβ-1*), and Hepatocyte Growth Factor (*HGF*) genes in triads including ASD patients. The study identified two *EGF* gene SNPs (rs4698803 and rs6533485) with a tendency for association with ASD, suggesting a possible important involvement of cell proliferation and differentiation as well as regulation of the synaptic plasticity in autism development. TGFβ-1 is a regulatory cytokine important for an adequate development of inflammatory processes. This molecule also acts in cell growth and differentiation, matrix formation, apoptosis, as well as in cellular homeostasis (Letterio and Roberts, 1998; Aoki et al., 2005). Considering this, a study assessing TGFβ-1 plasma levels in ASD patients and healthy age-matched controls observed lower levels in patients when compared to controls, which may reflect an altered immune response associated with abnormal behaviors and symptoms found in ASD children (Ashwood et al., 2008).

In agreement with the above-mentioned findings, an investigation of candidate genes revealed that *NFkB, Jnk, MapK, TNF, TGF-B,* and *Myc* are part of a network of highly expressed genes in ASD, forming central hubs in the most interconnected of all ASD-derived networks analyzed. Moreover, this interconnected gene network includes fundamental cytokine signaling molecules at their core that were not previously implicated as ASD susceptibility loci and might be important contributors of ASDs' heterogeneity (Ziats et al., 2011).

Taking together, all the available data related to inflammation and ASD points to a strong association among pro-inflammatory genes and pathways and the development of both the disease and its related clinical features. However, although such studies reveal important associations between genes related to inflammatory responses and ASD, the exact mechanisms that led to symptoms manifestation still need to be elucidated.

MHC Genetic Variants in ASD – antigen presentation and further

The major histocompatibility complex (MHC), also known in humans as the human leukocyte antigen (HLA) complex, is located in the short arm of chromosome 6 and encodes molecules that participate in antigen presentation and regulation of inflammatory responses, being directly or indirectly involved in several aspects of autoimmunity, immune-mediated, and infectious diseases (Matzaraki et al., 2017). This complex is a crucial component of the immune system, comprising 20 *HLA* typical and 112 non-typical genes, which are inherited together as extended haplotypes (Torres et al., 2012).

The HLA cluster has the highest polymorphism rate of the human genome. It is divided into three subclasses. The class I region encompasses the classical and highly polymorphic HLA-A, HLA-B and HLA-C genes, along with the nonclassical HLA-E, HLA-F and HLA-G genes, which present limited polymorphism. Together with some less variable genes involved in antigen processing and presentation, the genes *HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQA2, HLA-DQB1, HLA-DQB2, HLA-DRA, HLA-DRB1, HLA-DRB2, HLA-DRB3, HLA-DRB4*, and *HLA-DRB5* make up the class II region. Genes implicated in

inflammatory responses, leukocyte maturation and the complement cascade belong to the Class III MHC/HLA region (reviewed in Dendrou et al., 2018).

The importance of the HLA gene cluster goes beyond the establishment of adequate immune responses, and even the CNS development is subjected to the interference of the MHC. Approaching biochemistry and immunohistochemistry techniques in an animal model, Needleman and colleagues evaluated the expression of MHC I proteins in developing rat visual cortex, and showed that MHC I proteins regulate the activity-dependent refinement of developing visual projections, control synaptic plasticity in hippocampal and cerebellar slices, and synaptic transmission in dissociated hippocampal cultures. Thus, it was suggested that improper function or formation of those synapses could lead to neurodevelopmental disorders, such as schizophrenia and ASD (Needleman et al., 2010). Given the role of the immune system in neurodevelopmental diseases, polymorphisms in the HLA system have already been investigated as possible contributors for ASD etiology, which will be discussed in this section and in **Table 2**.

A close relation with the extended MHC B44-SC30-DR4 haplotype and ASD was suggested in a study performed in the USA, where this haplotype frequency was significantly increased in affected children as compared to controls. Moreover, the presence of this haplotype was also increased in the mothers but not in the fathers of the probands (Warren et al., 1992). Aiming to evaluate the participation of *HLA-A, -B, -Cw, -DQB1, -DRB1*, and 5-*HTTLPR* (human serotonin transporter) genes in ASD, Guerini et al. (2006) enrolled 37 simplex ASD families in a study with a Sardinian sample, although no association was observed. Addressing these same families, a 6-Mb region ranging from the *HLA-DR* to Hemochromatosis (*HFE*) genes was investigated using both microsatellites (Msat) and SNPs analyses, focusing on α and β blocks. Associations with ASD for D6S265*220 and MOGc*131 alleles were observed. In addition, these alleles were more likely to be transmitted together as a haplotype to probands, suggesting that these genes could represent markers with other genetic factors that impact the pathogenesis of ASD (Guerini et al., 2009).

Subsequently, as an attempt to corroborate the previous findings addressing the Sardinian population, this same research group performed association analysis of SNPs, Msats and *HLA* markers surrounding the α and β blocks of the MHC genetic region in a cohort of ASD children and their relatives from peninsular Italy. The results corroborate the previous data in peninsular Italians, a population genetically distinct to those from Sardinia (Guerini et al., 2010). Considering the genetic differences between these two populations, the results from Guerini's group confirm the presence of complex associations between ASD and HLA. The α block analysis included genotypes for Msat D6S265, MOGc, and the SNP rs2857766, while the β block analysis was made with Msat *D6S2810* (*MIB*), and two SNPs (*TNF α*-238 and *TNF α*-308). The *HLA-A*, *HLA-B*, *HLA-Cw,* and *HLA-DR* genes were typed in ASD children and their relatives. Case-control and TDT analyses for intrafamilial transmissions of Msats, SNPs and HLA markers surrounding α and β blocks were performed and a positive correlation between *MOGc**131 and D6S2239*105 alleles with ASD was found. A difference in the distribution of transmitted allele between ASD and healthy sibs was revealed regarding the *MIB**332 allele, which was more frequently transmitted to affected children. The -238 (G) and -308 (G) alleles from *TNF α* , the MIB allele*332, and HLA-B*38-HLA-Cw*12(H1c) and D6S265*218-HLA-A*23-MOGc*131-rs2857766 (G) (H2C) haplotypes were also more frequently transmitted to ASD children. These

results lead to the hypothesis that no specific *HLA* gene dictates the development of ASD, although some variants in different genes could be in linkage disequilibrium with other genetic markers which contribute to disease outcome.

Performing an endophenotypic analysis, Ferrante et al. (2003) found reduced levels of $CD4^+$ naive T cells and an increased number of $CD4^+$ memory T cells in ASD children harboring both *HLA-A2* and *DR11* alleles. Together, these results support the hypothesis of an immune cells endophenotype that characterizes both ASD children and their healthy siblings (Saresella et al., 2009) and reinforce the multifactorial and multigenic modulation on ASD development (Guerini et al., 2011).

The frequency of some HLA-DRB1 alleles in ASD patients was examined in a case-control study also in Cairo, Egypt. The cohort included ASD patients and age and sex-matched children, who were not related to the probands. ASD children had a higher frequency of the HLA-DRB1*11 allele as compared to controls. Also, the HLA-DRB1*03 allele frequency was lower in ASD individuals than in the control group. Moreover, the presence of HLA-DRB1*11 and the absence of HLA-DRB1*03 were associated with a higher risk for autism. Interestingly, significant higher rates of autoimmune diseases were found among the families of ASD children in comparison to the control families, and the expression of the HLA-DRB1*11allele was higher in ASD children that had a family history of autoimmune diseases, comparing to those who didn't. These results indicated a possible immune dysfunction on the offspring probably induced by environmental triggers (Mostafa et al., 2013).

A case-control study with the subjects from the "PARIS study cohort" investigated a possible link between MHC class II-DRB1 and DQB1 alleles, genotypes, and haplotypes and ASD. The findings indicated no statistically significant differences in allelic and genotypic frequencies between cases and controls. Regarding the haplotype analysis, a higher frequency of HLA-DRB1*11-DQB1*07 haplotype was observed in ASD patients as compared to healthy controls. Additionally, the prevalence of HLA-DRB1*17-DQB1*02 was significantly higher in healthy controls as compared to probands, indicating a possible protective role of this haplotype. Considering symptomatology, a higher prevalence of HLA-DRB1*11-DQB1*07 haplotype was found among ASD individuals with higher scores on the Autism Diagnostic Interview-Revised (ADI-R) social domain and ADI-R non-verbal domain. Besides, HLA-DRB1*11-DQB1*07 was observed in higher frequency in patients presenting an IQ below 70 or diagnosed with low-functioning ASD (Bennabi et al., 2018).

The evaluation of six HLA-G isoforms (HLA-G*0101, *0102, *0103, *0104, *0105N, *0106) was performed in ASD children born in peninsular Italy and of Italian ancestry. Mothers and some patients' siblings were also enrolled in this study. Allelic distribution was compared with controls from Brazil (Nardi et al., 2012) and Denmark (Hviid et al., 2002). A higher HLA-G*0105N allelic frequency was observed in ASD children as compared to controls. Conversely, the HLA-G*0101 isoform was significantly less frequent in patients. The same pattern was found when analyzing the alleles on ASD mothers versus the control group (Guerinni et al., 2017).

The 14 base pairs deletion in the 3'UTR of *HLA-G* (HLA-G*14bp polymorphism) has already been associated with reduced expression of HLA-G mRNA (O'Brien et al., 2001; Hviid et al., 2002) and with

reduced levels of soluble HLA-G (Hylenius, 2004), which could affect the efficacy of the immune tolerance mediated by this molecule. Thus, considering that prenatal immune activation is suggested to play an important role in the onset of ASD, Guerini et al. (2015) evaluated the frequency of HLA-G*14bp in a cohort of Italian families with ASD children. For comparisons addressing the mothers of the ASD patients, the historical data from a meta-analysis performed on different groups of healthy mothers (Wang et al., 2013) were used as controls for the HLA-G 14bp+/14bp- distribution. Results showed that both 14bp+/14bp+ genotype and *14bp+* allele were in higher frequency in ASD children and their mothers. This study also related obstetric complications with ASD, since the distribution of the HLA-G 14bp+/14bp+ seen in mothers of affected children was similar to that observed in women with recurrent spontaneous abortions or who have suffered gestational disorders such as preeclampsia. Of note, 45% of mothers enrolled in this study reported repeated miscarriage (Guerini et al., 2015). Considering that miscarriage is a condition related to exacerbated immune activation (Kaminski et al., 2018) accounting with the maternal genetic background (Michita et al., 2016), recurrent miscarriage in mothers of ASD children could represent a strong relationship between maternal immune activation and ASD development (Guerini et al., 2015).

A possible association between the HLA-G*14bp allele and KIR-HLA-C complexes and clinical features such as the electroencephalography (EEG) profile, cognitive, and behavioral scores in ASD children was evaluated in two distinct Italian populations. Data analysis revealed higher scores of autistic behavior in individuals with the KIR2DS1-C2+/HLA-G*14bp+ haplotype. This synergistic polygenic association between *KIR* (chromosome 19) and *HLA* (chromosome 6) genes may account for the spectrum of behavioral traits characterizing ASD, considering the effect of these genes together, rather than separately (Guerini et al., 2018).

In a Saudi Arabian population, Al-Hakbany et al. (2014) enrolled children diagnosed with ASD and healthy adults in a case-control study. All individuals were screened for HLA class I and II through PCR-SSP and Luminex based analysis. An association between genotypes and ASD was found with the following alleles: HLA*A01, HLA*A02, HLA*B07, HLA DRB1*011. The haplotypes A*01-B*07-DRB1*0701-DQB1*0602 and A*31-B*51-DRB1*0103-DQB1*0302 were also observed in a significantly higher frequency in ASD patients as compared to the control group. In addition, this study was the first to reveal an association between HLA B*07 allele and autism, suggesting this allele and the closely linked A*01-B*07-DRB1*0701-DQA1*0602 haplotype, that includes the A*01 and B*07 risk alleles, as a genetic marker for autism in the Saudi population (Al-Hakbany et al., 2014). Discrepant data were found in different populations (Mostafa et al., 2013; Torres et al., 2002), although such disparities could be explained by ethnic differences and interaction of the alleles studied to infectious agents and environmental allergens that vary across geographical regions. Of note, these studies demonstrate the importance of take into consideration ethnic variation and ancestry in ASD development.

In a Thai population, a study investigated the relationship between ASD and *HLA-B* genotypes in individuals diagnosed with ASD and control subjects. An association between different HLA alleles, specifically HLA-B*13:02, HLA-B*38:02, HLA-B*44:03 and HLA-B*56:01, and ASD was observed. Also, HLA-B*18:02 and HLA-B*46:12 alleles showed association with controls, suggesting a protective role

against the disease or showed a higher frequency in controls, suggesting a protective….(Puangpetch et al., 2015). No association with HLA-B haplotypes was observed by Torres et al. (2006); however, they found significant association between HLA-A2-B44 and -A2-B51 haplotypes and ASD subjects – among which those haplotypes were two times more frequent than in controls from the National Marrow Donors Program from U.S. Previously, addressing the same control group, it was demonstrated a higher frequency of HLA-DR4 allele in ASD individuals as compared to controls. In addition, TDT showed that fewer DR13 alleles were inherited from the mothers than expected; regarding the fathers, *DR4* alleles were more frequently inherited than expected (Torres et al., 2002). This data goes in agreement with sex differences in ASD prevalence and symptomatology and may represent additional pieces to the heterogeneity of the disease.

. A study addressed North-American families of ASD affected individuals and typical-developing European descent controls. In this case, a higher frequency of HLA-DR4 was observed in families with an affected individual from a specific geographical region of USA – eastern Tennessee, although no significant differences in the distribution of HLA alleles were seen among samples widely distributed throughout the country (Lee et al., 2006). Interestingly, Johnson et al. (2009) suggested HLA-DR4 as a risk allele for autism, since it has shown high transmission rates from grandmothers to the mothers of ASD children, in a North-American population from New Jersey.

In view of the diversity of findings involving *HLA* polymorphisms and ASD, the need for further and deeply investigations is evident. More than extensive genomic scans on *HLA* clusters in individuals affected by ASD, we need more data from different populations. Such population diversity in genetic studies is always welcome given the already known genetic variability of different human populations. However, considering that human groups from different geographic regions face distinct environmental challenges, several outcomes are also observed in the context of multifactorial diseases such as ASD. Therefore, both the genetic background and the genetic responses to environmental stimuli should be equally considered, especially in the context of *HLA*, which besides being part of the immune system, is the most variable gene cluster of human populations.

Immunometabolism-related Genetic Variants in ASD

High occurrence of metabolic dysfunctions such as mitochondria-associated disorders, obesity, and altered metabolism of polyunsaturated fatty acids were already described in ASD children (Frye and Rossignol, 2011; Hill et al., 2015; Das, 2013). Thus, as summarized in **Table 3**, several studies have approached the role of variants in immunometabolic-related genes in the context of ASD.

Hormone-related genetic variants

Vitamin D is involved in the regulation of calcium and phosphate homeostasis, playing important roles in various immune-related processes, such as the transcription of antimicrobial peptides (Gombart et. al., 2005; Wang et al., 2004), induction/inhibition of T and B cells proliferation and differentiation (Chun et

al., 2014), and tumor suppression (Bikle et al., 2015). Statistically significant lower levels of vitamin D were already observed in ASD children and their respective mothers (Fernell et al., 2010; Wang et al., 2015; Altun et al., 2018). Additionally, polymorphisms in the *VDR* gene have shown associations to bipolar mood disorder (Ahmadi et al., 2012), Alzheimer's disease, and Parkinson's disease (Łaczmański et al., 2015; Lee et al., 2014). Considering the previous investigations in other psychiatric disorders and the reported altered levels of vitamin D in ASD children, genetic variants in *VDR* had also been targeted for investigation in studies addressing ASD.

Among the distinct *VDR* polymorphisms already described, the most extensively investigated are FokI (rs2228570-C/T), BsmI (rs1544410-G/A), ApaI (rs79752320-T/G) and TaqI (rs731236-T/C). The FokI SNP can interrupt a start codon, originating two different forms of the VDR protein, with a difference of three amino acids between them, affecting the protein activity and consequently all vitamin D pathways (Arai et al., 1997). Otherwise, BsmI and ApaI — located in intron 8 — and TaqI — located in exon 9 — affect, respectively, the mRNA stability and protein affinity, although the actual consequences of these variants in both the gene and molecule functions remains inconclusive (Uitterlinden et al., 2004).

A study evaluating *VDR* SNPs conducted in a Polish sample showed higher frequencies of both TaqI "T" allele and ApaI "G" allele in ASD children compared to controls (Cieślińska et al., 2017). In a Chinese sample, TaqI "CT" heterozygous genotype and presence of the "C" allele were associated with ASD (Zhang et al., 2018). Increased risk of ASD was related to the "CC" (TaqI) and "AA" (BsmI) homozygous genotypes in a North-American sample (Schmidt et al., 2015). This last study also found a correlation between ASD risk and two SNPs in two other vitamin D metabolism-related genes, namely the "GG" genotype of rs10741657 in *CYP2R1* gene — that codes for the 25-hydroxylase protein, involved in the synthesis of calcitriol, the active form of vitamin D — and the rs4588 "AA" genotype in the *GC* gene — that codes for the vitamin D-binding protein, involved in vitamin D transport.

Different case-control studies were also performed in the Turkish population. Coşkun et al. (2016) conducted the first of them evaluating a possible association between serum 25(OH)D levels and *VDR* polymorphisms. A correlation between the FokI polymorphism and higher levels was observed in children with ASD. Besides, the G-T-T-T (BsmI-TaqI-FokI-ApaI) haplotype and TaqI "CC" genotype were associated with an increased risk of ASD, while the A-T-C-G and G-T-C-T (BsmI-TaqI-FokI-ApaI) haplotypes were associated with protection against ASD development, in reason of their higher frequency in controls. Balta et al., (2018) evaluated *VDR* SNPs rs11568820 and rs4516035, but no association with ASD was observed. Addressing North-American individuals with ASD, other psychiatric disorders, and controls, Yan et al. (2005) was unable to detect any association between ASD e *VDR* SNPs. In an Iranian population, the frequency of the "CC" genotype from TaqI was significantly higher in controls as compared to in ASD cases, indicating possible protection. Curiously, this study further showed that the "f-T" haplotype (formed by the "T" allele from FokI and the "T" allele from TaqI, respectively) was more frequent among female ASD patients (Mobasheri et al., 2019).

Sex hormones also play an important role in metabolism control, mainly in pathways related to

sexual differentiation. These steroid hormones also act as enhancers of the humoral immunity (estrogens) and immunosuppressors (androgens, progesterone, and glucocorticoids) (Cutolo et al., 2004). Addressing both the Danish Historic Birth Cohort and Danish Psychiatric Central Register biobanks, the concentration of sex steroids (progesterone, 17α-hydroxy-progesterone, androstenedione and testosterone) along with cortisol were assessed and measured in amniotic fluid of woman whose children were later diagnosed with ASD. The results provided the first direct evidence of elevated fetal steroidogenic activity in autism (Baron-Cohen et al., 2015). On the other hand, low levels of progesterone during pregnancy were suggested as responsible for both obstetrical complications and brain changes associated with ASD in the offspring, in a case-control study conducted in a North-American sample (Whitaker-Azmitia et al., 2014). However, there are data that do not corroborate these observations as, for example, an Australian study that showed no significant difference between hormonal levels in controls and men with autistic traits (Tan et al., 2018).

Considering immunogenetic aspects, Schmidtova et al. (2010) approached polymorphisms in the genes of the androgen receptor (*AR*) — number of (CAG)n repeats in the first exon —, 5-alpha reductase (*SRD5A2*) — rs9282858-T/A, and estrogen receptor alpha (*ESR1*) — rs2234693-T/C in ASD boys. In this study, a significantly higher frequency of "AT" genotype from rs9282858 SNP was observed in probands as compared to controls. Besides, a study approached 29 polymorphisms in eight sex hormone-related genes in the Swedish population. Higher scores of ASD traits were found in boys with the "C" allele of rs2747648-C/T from *ESR1*, showing an association with specific modules of restricted and repetitive behaviors, and language/social interaction impairments. Moreover, the "GG" genotype of rs523349-C/G in *SRD5A2* was associated with language/social interaction impairments in girls (Zettergren et al., 2013). Later, approaching a larger sample size, the same research group was not able to corroborate their previous results although they observed an association between the "AA" genotype of rs6259-G/A in sex hormone-binding globulin (*SHBG*) and language impairments in boys (Zettergren et al., 2016).

Cytochromes P-450scc and P-45011beta are fundamental in steroid biosynthesis and are encoded by *CYP11A1* and *CYP11B1* genes, respectively (Hu et al., 2004; Levine et al., 1980). Polymorphisms in *CYP11A1* (rs2279357-C/T) and *CYP11B1* (rs4534-A/G, and rs4541-C/T) were assessed in Chinese families of ASD children. A preferential transmission was observed concerning *CYP11A1* rs2279357 "T" allele, suggesting an association of this SNP and ASD (Deng et al., 2016). An English study evaluated SNPs in 68 genes divided into three categories: synthesis and transport of sex steroids, neural connectivity, and social-emotional responsiveness. Statistically significant results with autistic traits was observed concerning rs4541-T/C and rs5288-G/T from *CYP11B1*, rs1271572-A/C and rs1152582-G/C from estrogen receptor beta (*ESR2*), and rs1902585-C/G from *CYP17A1*. Besides, autistic traits were associated with the SNP rs2873027-C/T from the gamma-aminobutyric acid receptor subunit gamma-3 gene (*GABRG3*), that codes for a subunit of the $GABA_A$ receptors (Chakrabarti et al., 2009).

Mitochondrial pathways-related genetic variants

Mitochondrial signaling pathways play essential roles in ATP production and in the biosynthesis of several macromolecules. These pathways actively participate in the control of both adaptive and innate immune systems by regulating the activation and differentiation of T cells and the function of macrophages (Weinberg et al., 2015). Thus, imbalances in such regulation could contribute to immune dysfunctions observed in ASD.

The NADH-ubiquinone oxidoreductase 1 alpha subcomplex 5 gene (*NDUFA5*) codes for one of the subunits of the first enzyme complex acting in the electron transport chain, the process responsible for producing the majority of the ATP required in most organisms (Wu et al., 2016). In the context of ASD, three *NDUFA5* polymorphisms (rs12539809-A/T, rs12666974-A/T, and rs3779262-A/T) were evaluated in a Japanese sample. Lower frequencies of both the "A" allele (in both rs12666974 and rs3779262 SNPs) and the A-A haplotype (rs12666974-rs3779262) were observed in affected children as compared to controls, possible indicating a role for these alleles in protecting against ASD development (Marui et al., 2011). ASD studies also approached genetic variants in neuroimmune modulator receptors, such as the oxytocin receptor gene (*OXTR*). OXTR responds to oxytocin, a neurotransmitter, which is also an important neuroendocrine modulator of the immune system (Li et al., 2017). A study performed in Chinese Han family triads evaluated four *OXTR* SNPs (rs2254298-A/G, rs53576-G/A, rs2228485-C/T, and rs237911-A/G). A preferential transmission of rs2254298 "A" and rs53576 "A" alleles to ASD children besides an association of the A-A-T-A haplotype (rs2254298-rs53576-rs2228485-rs237911) were evidenced (Wu et al., 2005). With analyses that presented opposite data in European descent family triads from USA, another study observed a preferential transmission of "G" over the "A" allele of rs2254298 for ASD children (Jacob et al., 2007). Montag et al. (2017) also studied *OXTR* polymorphisms (rs53576, rs2254298, and rs2268498-T/C) in Chinese and Germany samples. Their results showed an association between the "TT" genotype of rs2268498 and less autistic traits in both samples. More recently, a Brazilian study investigated two SNPs in *OXTR* (rs1042778-T/G and rs53576) and no associations with ASD was detected after correction for multiple tests (de Oliveira Pereira Ribeiro et al., 2018). Approaching 16 SNPs of *OXTR*, LoParo and Waldam (2014) performed a meta-analysis of the associations of these variants and ASD. Data from 3941 affected individuals from 11 independent samples were addressed. Results showed associations between increased risk for ASD and rs7632287 'A', rs237887 'A', rs2268491 'T', and rs2254298 'A' alleles.

The *SLC25A12* gene codes for the calcium-binding mitochondrial carrier protein (AGC1) — an aspartate-glutamate carrier. Three *SLC25A12* polymorphisms (rs2056202-G/A, rs908670-A/G, and rs2292813-G/A) were evaluated in two independent North-American samples of individuals diagnosed with ASD. In this study, the A-A haplotype (composed by rs2056202-rs2292813) was associated with autistic traits – according to the Repetitive Behavior Scale-Revised (RBS-R) score in both samples (Kim et al., 2011). The rs2056206 SNP of *SLC25A12* was also evaluated in North-American ASD cases through sibship analysis. Herein, the "A" allele had a higher frequency in ASD patients with lower levels of routines and ritual behaviors (Silverman et al., 2008). Previously, a study conducted in heterozygous families showed a preferential transmission of both the rs2056202 "G" allele and the G-G haplotype (from rs2056202 and rs2292813) to ASD children (Ramoz et al., 2004). More recently, a study with a Chinese Han sample of ASD

children found that the “A” allele of rs2292813 from *SLC25A12* was associated with an increased risk for ASD. The same association was observed concerning the “TT” genotype of rs1051266-C/T from *SLC19A1* — a gene that codes for the reduced folate carrier 1 molecule, involved in folate pathways (Liu et al., 2017). Moreover, a Japanese study associated the “AA” genotype of rs1023159-A/G from *SLC19A1* gene with risk for the development of ASD (Mahmuda et al., 2016). With contrasting results, two other studies evaluated the mentioned *SLC25A12* SNPs although no association with ASD was observed, neither in a North-American nor in a Han Chinese sample (Rabionet et al., 2006; Chien et al., 2010), respectively.

Nutrition-related genetic variants

Genes involved in the folate metabolism were also targeted in genetic variant studies in ASD. Folate pathways are related to ATP production, participating in DNA methylation and synthesis (Depeint et al., 2006; Crider et al., 2012). Considering the immunometabolism, the folate cycle is responsible for the production of immunomodulatory molecules, such as inosine and adenosine, which are important in both immune signaling and cytotoxic responses (Bayer and Fraker, 2017). Regarding ASD, significantly higher levels of folic acid were found in mothers of typical-development children in comparison to mothers of affected children in the first month of pregnancy, while a lower risk for ASD was found in children from mothers that received folic acid intake in this same period (Schmidt et al., 2012).

In a case-control study, Mohammad et al. (2009) evaluated five polymorphisms in genes important for the folate pathway: methylenetetrahydrofolate reductase (*MTHFR*) 677C/T (rs1801133-C/T), *MTHFR* 1298A/C (rs1801131-A/C), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) 66A/G (rs1801394-A/G), serine hydroxymethyltransferase (*SHMT*) 1420C/T (rs1979277-C/T), and methionine synthase (*MS*) 2756A/G (rs1805087-A/G). A higher frequency of the “T” allele from *MTHFR* 677C/T was observed in Indian ASD children, suggesting an association with an increased risk of the disease. Besides, the “T” allele from *SHMT* 1420C/T and the “A” allele of *MTRR* A66G were less frequent in ASD children, indicating a possible protective effect against the disease (Mohammad et al., 2009). In agreement, James et al. (2006) found a higher frequency of the “T” allele of *MTHFR* 677C/T in North-American ASD patients as compared to controls from a European descent sample. Higher frequencies of both “CT” and “TT” genotypes in the *MTHFR* 677C/T SNP, as well as an association with the “AC” genotype in *MTHFR* 1298A/C, were observed in ASD children from USA as compared to controls (Boris et al., 2004). In a Chinese Han study, the “TT” genotype in *MTHFR* 677C/T also showed a higher frequency in ASD children than in controls (Guo et al., 2012). Importantly, the presence of the “T” allele reduces the activity of the MTHFR protein (Frosst et al., 1995), being this reduced activity a possible contributor to the putative abnormal metabolic profile of many ASD children. Conversely, no correlation was found between the “T” allele of *MTHFR* 677C/T and ASD when evaluated in a case-control study in South Brazilian and Turkish populations (dos Santos et al., 2010; Sener et al., 2014). An Egyptian study showed an association of the “C” allele of 1298A/C and the “T” allele of 677C/T from *MTHFR* and ASD (El-Baz et al., 2017), while another study from the same region corroborated the 677C/T “T” allele association. Taking together, “CT” and “TT” genotypes of 677C/T were both associated with increased risk for ASD, but not those described for

1298A/C (Ismail et al., 2019).

Considering other nutrition metabolism-related genes, insulin receptors (*INSR*) play an important role by transmitting signals to intracellular pathways necessary to T cell proliferation and cytokine production (Tsai et al., 2018). In the ASD context, insulin-like growth factors (IGF), which are polypeptides that act by binding to INSRs, were already found in significant higher concentrations in children with the disease (Mills et al., 2007). The insulin receptor substrate 1 (*IRS1*) and insulin receptor substrate 2 (*IRS2*) genes were also evaluated in the autism context. A South Korean case-control study approached the SNPs rs4773092-A/G of the *IRS2* gene and the rs1801123-A/G of *IRS1*, where the "G" allele of rs1801123 was significant less present in ASD children, indicating a possible protective factor against the disorder (Park et al., 2016).

Neurotransmission-related genetic variants

Serotonin is an important neurotransmitter and a hormone that relates to the immune system as it mediates the release of IL-1β, neutrophil recruitment, and T-cell activation (Herr et al., 2017). Several studies already showed significantly higher levels of serotonin (hyperserotonemia) in more than 25% of the ASD children (Muller et al., 2016; Gabriele et al., 2014). Considering that integrin β-3 (coded by *ITGB3* gene) is also required for serotonin pathways, Ma et al. (2010) evaluated four SNPs in the human serotonin transporter gene (*SLC6A4* – also named *5-HTT* or *SERT*), namely rs1042173-A/C, rs140700-C/X, rs2066713-G/A, and 5-HTTLPR-Short/Long, and four SNPs in *ITGB3* (rs11657517-C/T, rs5918-T/C, rs5919-T/C, and rs3809865-T/A/G) in families with and without an ASD history. Significantly higher frequency of the *SLC6A4* rs2066713 "G" allele was observed in patients with a negative ASD family history. Conversely, the data revealed that the "A" allele of this same SNP was more frequent in patients from families with a positive history of ASD, but no association survived multiple testing corrections (Ma et al., 2010). Addressing samples from southern Brazil, Schuch et al. (2016) evaluated the polymorphisms 5HTTLPR, rs2066713, STin2, and rs1042173 at *SLC6A4* in ASD children and their biological parents, with no associations found after multiple tests correction.

The above-mentioned polymorphism 5-HTTLPR, located in the *SLC6A4* promoter region, has been widely studied in ASD samples, due to its impact on serotonin transporter expression (Heils et al., 1996). Family-based analyses revealed significantly overtransmission of the short allele "S" for North-American (Devlin et al., 2005; Kistner-Griffin et al., 2011) and Irish (Conroy et al., 2004) ASD children. In South African samples, ASD subjects presented a higher frequency of 5-HTTLPR "SS" genotype as compared to controls (Arieff et al., 2010), while in a Swiss population sample, ASD subjects presented higher frequencies of "S" allele as compared to respective controls (Nyffeler et al., 2014). On the other hand, the long "L" allele was associated with ASD in an Israeli sample (Yirmiya et al., 2001). In the same context, Coutinho et al. (2004) observed hyperserotonemia in a Portuguese ASD sample, which was associated with a haplotype composed of 5-HTTLPR "LL" and the intron 2 variable number of tandem repeats (VNTR) "Stin2.10/Stin2.10" genotypes. An Indian study also found an association between hyperserotonemia in ASD

and the polymorphisms 5-HTTLPR, VNTR Stin2, and rs3813034-G/T: Stin2.10 allele, “Stin2.12-T” and “Stin2.10-T” haplotypes (VNTR STin2-rs3813034), and the “S-T” haplotype (5-HTTLPR-rs3813034) (Jaiswal et al., 2015). Interestingly, an Italian study is not corroborated by this later report, since 5-HTTLPR genotypes were not associated with hyperserotonemia in ASD patients (Persico et al., 2002), further suggesting that genetic heterogeneity of ASD among different populations worldwide should always be taken into consideration. Besides, the impact of the interaction between genetic and environmental factors in ASD should not be ruled out. A study with an Australian sample revealed that boys with the “LL” genotype of 5-HTTLPR and high levels of disaster-related prenatal maternal stress have higher levels of ASD traits, even in comparison with girls harboring the “LS” or “SS” genotypes and maternal peritraumatic dissociation (Laplante et al., 2018).

Glutamate is a neurotransmitter and immunomodulatory molecule that prevents the production of IL-6 and IL-23 (Hansen and Caspi, 2010). Moreno-Fuenmayor et al. (1996) described increased glutamate concentrations in cerebrospinal fluid samples of some ASD patients, while other studies found a statistically significant increase of glutamate in blood serum (Shinohe et al., 2006) and in the auditory cortex (Brown et al., 2013) of ASD patients in different populations. Such findings reinforce the importance of genes involved in the glutamate metabolism as potentially associated to ASD. The glutamate receptor 6 (*GluR6* or *GRIK2*) gene was studied in Chinese families of children with autism through the evaluation of different SNPs (rs995640-A/G, rs2227281-C/T, rs2227283-G/A, and rs2235076-G/A). In this study, a preferential transmission of the “T” allele from rs2227281, the “A” allele from rs2227283, and of the haplotype G-C-G (rs995640-rs2227281-rs2227283) to ASD children was observed (Shuang et al., 2004). Addressing Indian families of ASD patients, Dutta et al. (2007) evaluated three SNPs of *GluR6* (rs2227281, rs2227283, and rs2235076), but no statistically significant results were found.

Gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter, acting in anti-inflammatory pathways by inhibiting the expression of pro-inflammatory cytokines and T cell proliferation through binding to GABA receptors (Bhat et al., 2010; Jin et al., 2013; Wu et al., 2017). GABA activity in CNS has been linked to social behavior (File and Set, 2003), thus it has also been targeted in ASD studies. Importantly, decreased GABA levels were observed in some brain regions of ASD children (Puts et al., 2017; Gaetz et al., 2014). GABA type A ($GABA_A$) are important receptors of inhibitory neurotransmitters and are pivotal in GABAergic pathways (Sigel and Steinmann, 2012). Buxbaum et al. (2002) performed a study with families of children with ASD, where four loci in the gamma-3 subunit (*GABRG3)* gene were investigated (9CA, 155CA-1, 85CA, 155CA-1 and 155CA-2). A preferential non-transmission to affected children of the 87-bp allele from the 155CA-2 region was evidenced in this study. Another study, performed with cases and controls, in a Chinese Han sample approached polymorphisms in $GABA_A$ receptor subunits: two SNPs in *GABRB3* (rs2081648-T/C and rs1426217-G/A), one SNP in the alpha-5 subunit (*GABRA5*) — “C/T” rs35586628, and one SNP in *GABRG3* — rs208129-T/A. Interestingly, the “TT” genotype from rs2081648, the genotypes “CC” from rs2081648, “CC” and “TT” from rs35586628, and both “TA” and “TT” from rs208129 were significantly associated with more severe ASD phenotypes (Yang et al., 2017).

In Korean family triads, four *GABRB3* polymorphisms (rs1426217-G/A, rs2081648-T/C, rs890317-A/C, and rs981778-A/G) were evaluated. The rs2081648 “C” allele was preferentially transmitted to ASD children (Kim et al., 2006). Besides, a case-control study found no association between ASD and six SNPs in *GABRB3* gene (rs4906902-T/C, rs8179184-G/A, rs20317-C/G, rs20318-C/T, rs8179186-C/T, and rs3751582-G/A). Interestingly, the presence of other 22 rare variants (12 at 5’ regulatory, four at intronic, and six at exonic regions – as shown in **Table 3.1**) was significantly higher in ASD patients than in controls (Chen et al., 2014).

Glutathione is a protein whose reduced form is involved in antioxidant processes through ROS neutralization (Dröge et al., 2000). Lower levels of this protein have already been observed in the plasma of ASD patients in comparison to typical developing controls (Geier et al., 2009; James et al., 2004; Hodgson et al., 2014). Considering this, a Nigerian case-control study with ASD children investigated three polymorphisms involved with glutathione pathways: two null polymorphisms in Glutathione S-Transferase Theta 1 (*GSTT1*) and Glutathione S-Transferase Mu 1 (*GSTM1*) genes, and an SNP (rs947894) in Glutathione S-Transferase Pi 1 (*GSTP1*) gene. No association between GSTs genotypes and ASD was found (Oshodi et al., 2017). The results, however, corroborated a previous study showing lower levels of glutathione in the plasma of ASD individuals in comparison to controls (Oshodi et al., 2017).

Inborn errors of metabolism in ASD

ASD may have also a connection with inborn errors of metabolism (IEM), which are genetic disorders related to defects in enzymes of metabolic pathways. The exact rates of IEM in ASD patients are not known, although it is proposed that IEM is present in 2 to 5% of ASD patients. In this context, an association was found between ASD and some metabolic conditions, such as: phenylketonuria, glucose-6-phosphatase deficiency, propionic acidemia, adenosine deaminase deficiency, Smith-Lemli-Opitz syndrome, mitochondrial disorders, and deficiency in branched-chain ketoacid dehydrogenase kinase (Ghaziuddin and Al-Owain, 2013).

When considered together, the results from the studies presented in this section illustrate the multi-factorial and heterogeneous features of ASD. There is a huge variety of polymorphisms evaluated in different genes involved in distinct physiological and pathological processes among different countries and human populations. Such amount of discoveries highlights that immunometabolic pathways are important targets for future research of genetic factors influencing ASD development.

Conclusion

In this review work, among the three approaches investigated, we observed more studies involving genes related to immunometabolism than those in inflammatory or MHC-related genes. We believe that such number of studies investigating immunometabolism-related genes and the greater number of detected associations between SNPs in these genes and ASD is due to the relationship of these variants with

other physiological systems also affected in ASD. For this reason, such genes and SNPs would ultimately add a greater effect on ASD than those with an inflammatory impact or MHC- related ones. All data discussed here strongly indicate that imbalances in immune-related factors are involved in ASD susceptibility. **Figure 2** presents an overview of the immune-related genes and the genetic variants already associated with either protection or risk to ASD development. Polymorphisms in genes involved in triggering and controlling immune responses must be considered when studying complex and multi-factorial diseases, since frequently these genetic variations are population-specific and may differently respond to different environmental features. In addition, ASD is a condition of high heritability, which reinforces the need for further investigations targeting immunogenetic variants in the context of ASD and other complex diseases. Understanding any and all possible influences of a single gene variant can lead to invaluable discoveries and, hopefully, to improvements in life quality for those affected by diseases that require lifelong care and dedication. Fortunately, with the emergence of new techniques applicable in genetic studies, the generation of new scientific data in this field advances ferociously. Given this advance, much more than the direct application of the results observed in scientific publications, one should appreciate scientific production by itself. Considering the theme of this review, the discussion of complex diseases is of great value, since the generation of hypotheses aiming any advance, even punctual, could result in major or minor improvements in daily life, both of which are of paramount importance to those living under special needs – and also to their respective caregivers. Also, if the result of a given research impacts only one person's life, the research in question is justified just as it would if it impacted an entire population. Thus, either through GWAS or simple techniques such as conventional PCR genotyping in gene-candidate approaches, the investigation of genetic polymorphisms is always a starting point for studying diseases of which we still know very little, such as Autism Spectrum Disorder.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Author contributions

VLK defined the topics of the article and wrote the initial manuscript. GLTN, AGR, and MZ wrote the topics. JBS, TR and JABC contributed with opinions on the content of the article and revised the text. VLK created the figures. VLK and JABC revised and edited the final version of the manuscript.

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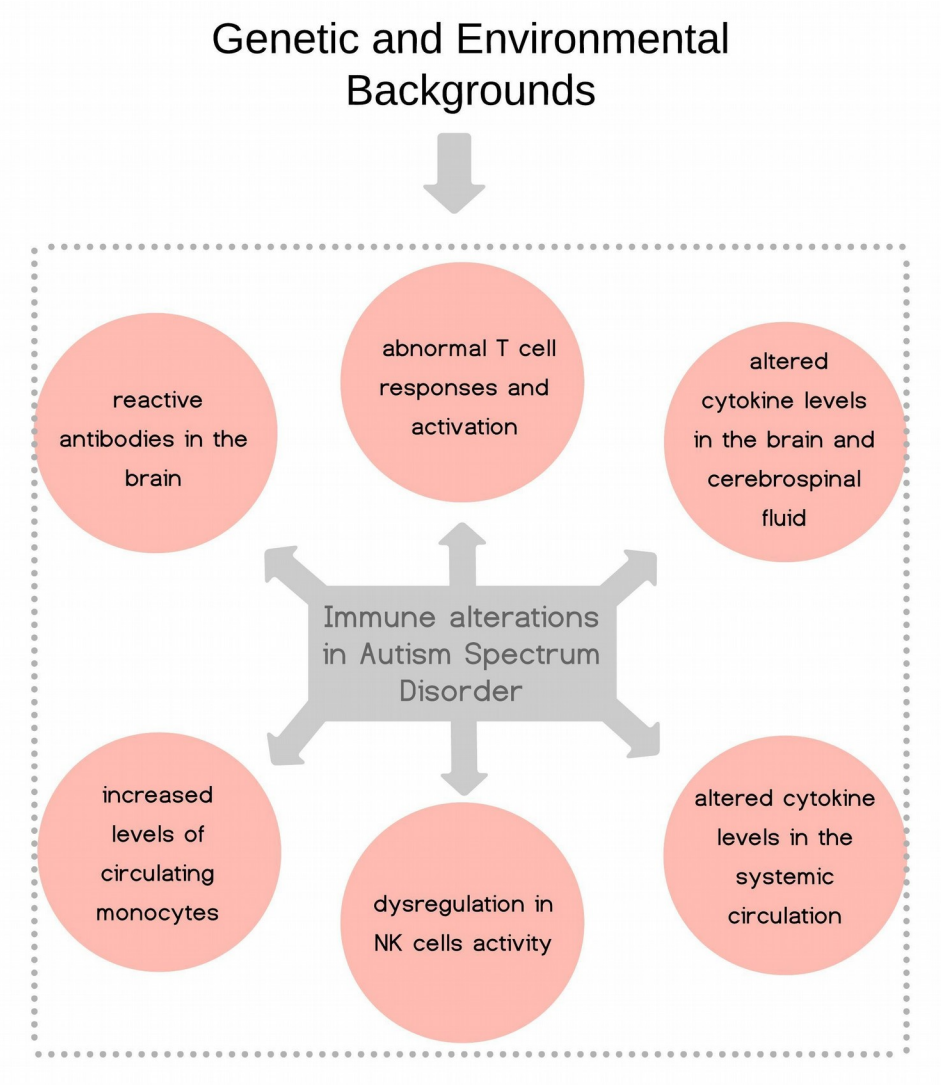
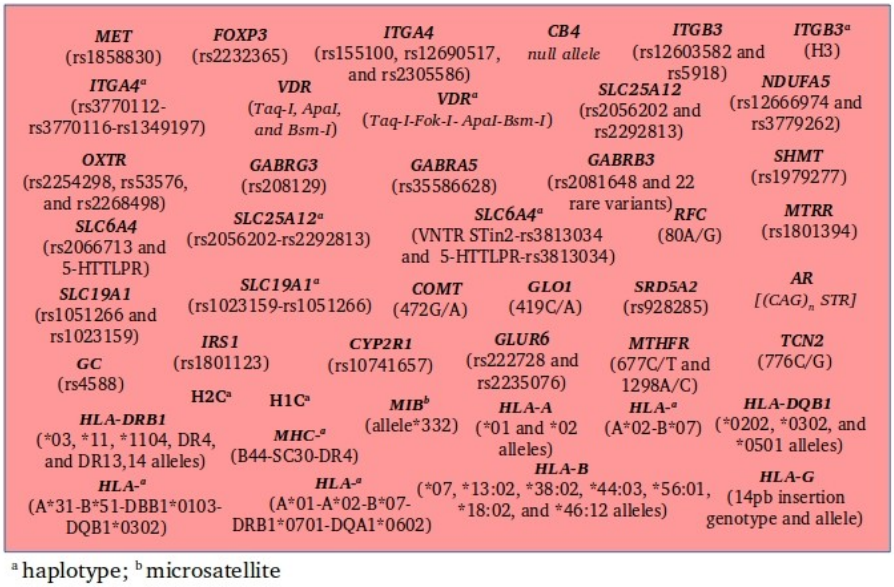


Figure 1. Summary of the most commonly observed immune alterations underlying Autism Spectrum Disorder.



[a] haplotype; [b] microsatellite

Figure 2. Risk and protective genetic variants that presented statistically significant associations with Autism Spectrum Disorder.

Table 1. Pro-inflammatory genetic variants investigated in ASD.

Factors	Sample size	Key findings	Country	ASD diagnostic criteria	Reference
MET (rs11762213, rs13223756, rs41736, rs2023748, rs41737, rs184953, rs1858830 and rs41739)	743 families (265 simplex and 478 multiplex) / 189 controls	Association with ASD: MET rs1858830-C allele in multiplex families and the CC genotype in case-control analyses.	Italy and USA	ADI-R	Campbell et al., 2006
MET (rs1858830)	365 mothers of which 202 had children with ASD	*MET* rs1858830-C allele was more frequent in mothers with antibodies against the fetal brain. MET protein was decreased in mothers with the C/C genotype.	USA	ADI-R and ADOS modules 1 and 2	Heuer et al., 2011
FOXP3 (rs3761548 and rs2232365)	523 individuals with ASD / 472 controls	Association with ASD: rs2232365-G allele.	Iran	[a] Controls were used from previous publications (Olerup et al., 1992 and Lampis et al., 2000).DSM-5 and ADI-R	Safari et al., 2017
C4 and *C4B*	45 individuals with ASD / 79 controls	Association with ASD: *C4B* null allele.	USA	DSM-III-R	Warren et al., 1992
ITGA4 (rs1449263, rs3770136, rs1449260, rs155100, rs3770116, rs3770112, rs2305581, and rs3770105)	164 nuclear families with one individual with ASD	Association with ASD: rs155100-A allele.	Ireland and Portugal	DSM-IV, ADI-R and CARS	Correia et al., 2009
ITGA4	179 family triads and 369 families with two or more individuals with ASD	Association with ASD: rs12690517-C allele and the haplotype rs3770112-rs3770116-rs1349197.	Ireland	ADI-R and ADOS-G	Conroy et al., 2009
84 SNPs covering the 2q24–q33 l*ocus*	252 multiplex and simplex families, including 610 individuals with ASD	Association with ASD: over-transmissions of rs2305586-T allele from *ITGA4*; rs2056202-G allele within *SLC25A12*; rs1807984-C, rs1517342-A, and rs971257-A alleles within *STK39*.	USA	ADI-R	Ramoz et al., 2008
ITGB3 (rs2317385,		Association with ASD: H3	Italy	DSM-IV,	Napolioni

rs2056131, rs4525555, rs2015729, rs5918, rs951351, rs15908, rs12603582, rs3809865, rs11650072)	281 simplex and 12 multiplex families, including 306 individuals with ASD	haplotype.		ADI-R and the italian version of ADOS	et al., 2011
EGF (rs4444903, rs3822288, rs1860129, rs11568994, rs2237051, rs2298999, rs4698803, rs9991904, rs6533485, rs9999824) *TGFβ-1* (rs1800469, rs2241715, rs4803455, rs8179181) *HGF* (rs3735520, rs5745616, rs6467869, rs12707453, rs2286194, rs2074724, rs5745752)	252 family triads with one ASD individual	No statistically significant association was found.	Japan	Not specified at the article	Toyoda et al., 2007

ADI-R - ADI - Autism Diagnostic Interview-Revised; CASRS – Childhood Autism Spectrum Rating Scales; ADOS - Autism Diagnostic Observation Schedule; DSM - Diagnostic and Statistical Manual of Mental Disorders.

Table 2. MHC/HLA genetic variants investigated in ASD.

Factors	Sample size	Key findings	Country	ASD diagnostic criteria	Reference
MHC extended haplotype *B44-SC30-DR4*	21 families of individuals with ASD	The frequency of the *B44-SC30-DR4* haplotype was significantly increased in ASD children as compared to controls as well as in ASD children's mothers, but not fathers.	subjects were of northern European ancestry, but living in Utah/USA	DSM-III-R	Warren et al., 1992
HLA (*-A, -B, -Cw, -DQB1*, and *-DRB1* alleles) and 5-HTTLPR	37 families of individuals with ASD and controls[a]	No statistically significant results were found.	Sardinia, Italy	CARS and other scales	Guerini et al., 2006
Microsatellites (MIB, D6S265, MOGc, D6S2239) and SNPs (two in the promoter of the TNF-alfa, and one in *MOG* rs2857766) in a 6-Mb region spanning from *HLA-DR* to the *HFE*.	37 families of individuals with ASD	Association with ASD: D6S265*220, MOGc*131 and D6S265*224 –MOGc*117– rs2857766(G), MOGc*117 and MIB*346 alleles.	Sardinia, Italy	DSM-IV	Guerini et al., 2009
HLA region (*HLA-A, -B, -Cw, -DQB1* and *-DRB1* alleles) and a 6Mb region that goes from *HLA-DR* to *HFE* gene.	61 families of individuals with ASD	The MIB*332 allelic distribution were more present in autistic individuals. The haplotypes H1C [*TNF-238* (G), *TNF-308* (G), MIB allele**332* and *HLA-B*38-HLA-Cw*12*] and H2C [*D6S265*218-HLA-A*23-MOGc*131-rs2857766* (G)] were more frequently transmitted to ASD children.	Peninsular Italy	DSM-IV-TR	Guerini et al., 2009
HLA-G 14bp insertion and *KIR2DS1-HLAC2* Complex	119 individuals with ASD	No difference was found between these two populations either in demographic or in clinical parameters. HLA-G 14bp+ and *KIR2DS1-C2+/HLA-G*14bp+* was statistically associated with higher ACBS (autistic core-behavior scores). *KIR2DS1-C2+/HLA-G*14bp+* was also associated with hyperactivity.	Sardinia and Peninsular Italy	DSM-5 and CARS	Guerini et al., 2018
HLA-DRB1 alleles	100 individuals	Association of *HLA-DRB1*11* and *HLA-DRB1*03* alleles with ASD.	Egypt	CARS	Mostafa et al., 2013

	with ASD/100 healthy matched children				
HLA class II *DRB1* and *DQB1* alleles	474 individuals with ASD / 350 control	*HLA-DRB1 *11-DQB1*07* haplotype was more prevalent in ASD patients; *HLA-DRB1 *17-DQB1*02* haplotype was higher in control group.	Paris	DSM-IV-TR	Bennabi et al., 2018
HLA-G 14bp insertion in the 3'-UTR	71 families of individuals with ASD	The frequencies of both the 14bp+/14bp+ genotype and 14bp+ allele were significantly higher in ASD patients and their mothers, when compared to non-ASD group.	Peninsular Italy	DSM-IV-TR	Guerini et al., 2015
Comparison of *HLA* class I and class II alleles and haplotypes	35 individuals with ASD / 100 controls	Association of *HLA-A *01*, *02*, *HLA-B *07*, *DRB1 *1104* , *HLA-DQB1 *0202*, *0302*, and *0501* alleles with ASD. The four-loci genotype study showed that *A*01-B*07-DRB1*0701-DQB1*0602* and the *A*31-B*51-DRB1*0103-DQB1*0302* were also associated with autism. The *HLA-A*02-B*07* haplotype associated significantly with autism. Both HLA- *A*01-B*07-DRB1*0701-DQA1*0602* and *A*31-B*51-DBB1*0103-DQB1*0302* haplotype frequencies were significantly higher among autistic patients than controls.	Saudi Arabia	DSM-IV-TR	Al-Hakbany et al., 2014
Frequency of *HLA-B* alleles	364 individuals with ASD / 952 controls	Association of *HLA-B*13:02*, *HLA-B*38:02*, *HLA-B*44:03* and *HLA-B*56:01, HLA-B*18:02* and *HLA-B*46:12* alleles with ASD.	Thailand	DSM-IV	Puangpetch et al., 2015
Autistic Endophenotype	20 individuals with ASD / 15 siblings / 20 matched controls	Proinflammatory, interleukin-10–producing immune cells, CD8_x0003_ naïve (CD45RA_x0003_/CCR7_x0003_) T lymphocytes and CD4_x0003_ terminally differentiated (CD45RA_x0004_/CCR7_x0003_) were higher;	Italy	DSM-IV	Saresella et al., 2009
HLA-DRB1 alleles	103 families	Association with the *DR4* and	USA	ADI-R	Torres et al.,

	of individuals with ASD	*DR13,14* alleles. TDT showed *DR4* as more frequently inherited and *DR13* less inherited by ASD children.		and ADOS	2002
Class I *HLA-A* and *-B* alleles	129 families / 265 controls	HLA-A2-B44 and -A2-B51 haplotypes were associated with ASD.	USA	ADI-R and ADOS	Torres et al. 2006
HLA-DR4 as a Risk Allele	31 families of individuals with ASD	Significant transmission disequilibrium for *HLA-DR4* was observed from maternal grandparents to mothers of probands.	USA	ADOS-WPS and ADI-R	Johnson et al., 2009
HLA-DR4	16 families of individuals with ASD from Tenessee and 31 families from different regions of the USA / 475 controls	Mothers and their sons that belonged to the geographically defined families had a significantly higher frequency of *DR4* compared to control subjects, whereas no significant difference in the distribution of *HLA* alleles was evident between the group of families in different regions of USA.	USA	DSM-IV, CARS and AGRE	Lee et al., 2006
association of *HLA A2–DR11* with CD4 naïve decrease	9 individuals with ASD / 37 controls	A significant decrease in CD4+ naïve and an increase in CD4+ memory T cells in autistic children was observed and were more pronounced in the ASD children bearing the *HLA A2* and *DR11* alleles.	Italy	DSM-IV	Ferrante et al. 2003

ASRS - Autism Spectrum Rating Scales; CARS - Childhood Autism Rating Scale; ADOS - Autism Diagnostic Observation Schedule; AGRE - Autism Genetic Resource Exchange; DSM - Diagnostic and Statistical Manual of Mental Disorders; ADI-R - ADI - Autism Diagnostic Interview-Revised

[a] Controls were used from previously publications (Olerup et al., 1992 and Lampis et al., 2000).

Table 3. Immunometabolism-related genetic variants investigated in ASD.

Factors	Sample size	Key findings	Country	ASD diagnostic criteria	Reference
VDR (Cdx2, TaqI, BsmI and FokI)	201 individuals with ASD and 200 controls	Association with increased risk for ASD: “C” allele and “CT” genotype of TaqI.	China	CARS	Zhang et al., 2018
VDR (Cdx2, TaqI, BsmI, ApaI and FokI)	237 individuals with ASD and 243 controls	Association with increased risk for ASD: “CC” genotype of TaqI and GTTT haplotype (BsmI/TaqI, FokI/ApaI)	Turkey	DSM-5	Coşkun et al., 2016
VDR (BsmI, ApaI, TaqI and FokI)	108 individuals with ASD and 196 controls	Association with protection for ASD: “T” allele of TaqI and “G” allele of ApaI.	Poland	ICD-10	Cieślińska et al., 2017
VDR (rs11568820 and rs4516035)	30 ASD individuals with ASD and 30 controls	No statistically significant association with ASD.	Turkey	DSM-IV	Balta et al., 2018
VDR (BsmI, TaqI, Cdx2 and FokI) *CYP27B1* (rs4646536) *GC* (rs4588) *CYP2R1* (rs10741657)	Families of 384 individuals with ASD and 234 control families	Association with increased risk for ASD: TaqI “CC” and BsmI “AA” paternal genotypes from *VDR*. Association with increased risk for ASD: “GG” genotype of rs10741657 from *CYP2R1*and the “AA” genotype of rs4588 from *GC*	USA	ADI-R and ADOS	Schmidt et al., 2015

VDR (FokI and TaqI)	81 individuals with ASD and 108 controls	Association with increased risk for ASD in female patients: “T-T” haplotype (FokI-TaqI) Association with protection for ASD: “CC” genotype of TaqI.	Iran	DSM-5	Mobasheri et al., 2019
SLC25A12 (rs2056202, rs908670 and rs2292813)	Families of 720 individuals with ASD	Association with restricted repetitive behavior in ASD: “A” alleles of rs2056202 and rs2292813.	USA	DSM-IV-TR	Kim et al., 2011
SLC25A12 (rs2056202 and rs2292813)	355 individuals with ASD	Association with lower levels of routines and rituals behaviors in ASD: “A” allele of rs2056206.	USA	ICD-10 and DSM-IV	Silverman et al., 2008
SLC25A12 (rs2056202 and rs2292813)	411 individuals with ASD	Association with ASD: “G” allele in both rs2056202 and rs2292813.	USA	ADI-R	Ramoz et al., 2004
SLC25A12 (rs2056202 and rs2292813)	Families of 327 individuals with ASD	No statistically significant association with ASD was found.	USA	DSM-IV	Rabionet et al., 2006
SLC25A12 (rs2056202 and rs2292813)	465 individuals with ASD and 450 controls	No statistically significant association with ASD was found.	Taiwan	DSM-IV	Chien et al., 2010
SLC25A12 (rs2056202 and rs2292813) *SLC19A1* (rs1023159 and rs1051266)	201 individuals with ASD and 200 controls	Association with increased risk for ASD: “TT” genotype of rs1051266 from *SLC19A1* and “T” allele of rs2292813 from *SLC25A12*. Association with protection for ASD:	China	CARS	Liu et al., 2017

		G-C haplotype (rs1023159-rs1051266) of *SCL19A1* and the C-C haplotype (rs2056202-rs2292813) of *SLC25A12*.			
SLC19A1 (rs914232, rs3788205, rs1023159, rs944423, rs1888533, rs11700708, rs12627639, rs2838965, rs6518253, rs9974061 and rs2838968)	147 individuals with ASD and 150 controls	Association with increased risk for ASD: “AA” genotype of rs1023159.	Japan	ADI-R	Mahmuda et al., 2016
NDUFA5 (rs12539809, rs12666974 and rs3779262)	235 individuals with ASD and 214 controls	Association with protection for ASD: “A” allele of rs12666974 and rs3779262.	Japan	DSM-IV and CBQ-R	Marui et al., 2011
OXTR (rs2254298, rs53576, rs2228485 and rs237911)	Families of 195 individuals with ASD	Association with increased risk for ASD: “A” allele of rs2254298, “A” allele of rs53576, and A-A-T-A haplotype (rs2254298-rs53576-rs2228485-rs237911)	China	DSM-IV	Wu et al., 2005
OXTR (rs2254298 and rs53576)	Families of 57 individuals with ASD	Association with increased risk for ASD: “G” allele of rs2254298.	USA	ADI-R and ADOS	Jacob et al., 2007
OXTR (rs1042778 and rs53576)	209 individuals with ASD (126 trios and 55 duos families)	No statistically significant association with ASD.	Brazil	DSM-IV, ASQ and CARS	Ribeiro et al., 2018
OXTR (rs2254298, rs53576, rs2268494, rs1042778, rs2268493, rs237887, rs2264891, rs2268495, rs2270465,	3941 individuals with ASD	Associations were found between ASD and rs7632287 (‘A’ allele is risk-inducing), rs237887	The study addressed data from 11		LoParo and Waldman, 2014

rs11720238, rs7632287, rs4564970, rs237885, rs11706648, rs237888, rs4686301, rs53576, rs237894, rs237895, rs4684302)		('A' allele is risk-inducing), rs2268491 ('T' allele is risk-inducing) and rs2254298 ('A' allele is risk-inducing)	independent samples		
OXTR (rs2301261, rs53576, rs2254298 and rs2268494) *HTR2A* (rs6311) *SLC6A4* (5-HTTLPR)	76 individuals with ASD (78 siblings) and 99 controls	Association with high functioning autism: "S" allele of 5-HTTLPR	Switzerland	ICD-10	Nyffeler et al., 2014
SLC6A4 (rs1042173, rs140700, 5-HTTLPR, rs2066713) *ITGB3* (rs11657517, rs5918, rs5919, rs3809865)	Families of 290 individuals with ASD	No statistically significant association with ASD was found after multiple tests correction.	USA	DSM-IV and ADI-R	Ma et al., 2010
SLC6A4 (5-HTTLPR) *DRD4* (VNTR exon III) *COMT* (rs4680)	Families of 34 individuals with ASD	Association with increased risk for ASD: "L" allele and "L/L" genotype of 5-HTTLPR.	Israel	DSM-III, DSM-IV and ADI-R	Yirmiya et al., 2001
SLC6A4 (5-HTTLPR, VNTR STin2)	Families of 84 individuals with ASD	Association with increased risk for ASD: "S" allele of 5-HTTLPR.	Ireland	ADI-R and ADOS-G	Conroy et al., 2004
SLC6A4 (5-HTTLPR and VNTR STin2)	Families of 105 individuals with ASD and 52 controls	Association with hyperserotonemia in ASD: "L/L"-Stin2.10/Stin2.10 (5-HTTLPR-VNTR STin2) haplotype.	Portugal	DSM-IV, ADI-R and CARS	Coutinho et al., 2004
SLC6A4 (5-HTTLPR, VNTR STin2, rs2020936 and rs2020937)	390 families of individuals with ASD	Association with increased risk for ASD: "S" allele of 5-HTTLPR.	USA	DSM-IV	Devlin et al., 2005
SLC6A4 (5-HTTLPR)	109 individuals with ASD and 342 controls	Association with increased risk for ASD: "S" allele and "SS" genotype of 5-HTTLPR.	South Africa	JSAIS, SSAIS, Griffith Mental Development Scale, and the Bender Visual	Arieff et al., 2010

				Test	
SLC6A4 (5-HTTLPR)	Families of 476 individuals with ASD	Association with increased risk for ASD: “S” allele of 5-HTTLPR, “L” allele in maternal genotype. Association with increased risk for ASD and more. severe language impairment: maternally derived “S” allele.	USA	ADI-R	Kistner-Griffin et al., 2011
SLC6A4 (5-HTTLPR, VNTR STin2 and rs3813034)	Families of 169 individuals with ASD and 168 controls	Association with hyperserotonemia in ASD: Stin2.10 allele, Stin2.12-T and Stin2.10-T haplotypes (VNTR STin2-rs3813034) and S-T haplotype (5-HTTLPR-rs3813034).	India	DSM-IV-TR and CARS	Jaiswal et al., 2015
SLC6A4 (5-HTTLPR)	108 individuals with ASD	Association with higher level of ASD traits: boys with “LL” genotype of 5-HTTLPR and high levels of disaster-related prenatal maternal stress; and girls with “LS” or “SS” genotypes and maternal peritraumatic dissociation.	Australia	ASRS – Short Form	Laplante et al., 2018
GABRB3 (rs4906902, rs8179184, rs20317, rs20318, rs8179186, rs3751582 and other 22 rare variants[a])	356 individuals with ASD and 386 controls	Association with increased risk for ASD: 22 rare variants (12 at 5’ regulatory, four at intronic, and six at exonic regions).	Taiwan	DSM-IV	Chen et al., 2014

GABRB3 (rs2081648 and rs1426217) *GABRA5* (rs35586628) *GABRG3* (rs208129)	99 individuals with ASD and 231 controls	Association with severe abnormality in visual response in ASD: “TT” genotype of rs2081648 from *GABRB3*. Association with abnormality in verbal communication in ASD: “CC” and “TT” genotypes of rs35586628 from *GABRA5*. Association with abnormality in imitative behavior and activity level in ASD: “TA” and “TT” genotypes of rs208129 from *GABRG3*.	China	CARS, ABC and ECDQ	Yang et al., 2017
GABRB3 (rs1426217, rs2081648, rs890317 and rs981778)	Families of 104 individuals with ASD	Association with increased risk for ASD: “C“ allele of rs2081648	South Korea	ADOS and ADI-R	Kim et al., 2006
MTHFR [677C/T (rs1801133) and 1298A/C (rs1801131)] *MTRR* (rs1801394-A/G) *SHMT* (rs1979277-C/T) *MS* (2756A/G)	138 individuals with ASD and 138 controls	Association with ASD: “T” allele of 677C/T from *MTHFR*. Association with protection for ASD: “T” allele of rs1979277 from *SHMT* and “A” allele of rs1801394 from *MTRR*.	India	DSM-IV, ABC and DQ	Mohammad et al., 2009
MTHFR (677C/T and 1298A/C)	168 individuals with ASD and 5,389 controls[b]	Association with ASD: “CT” and “TT” genotypes in 677C/T and “AA” genotype in 1298A/C	USA	DSM-IV	Boris et al., 2004

MTHFR (677C/T)	98 individuals with ASD and 70 controls	No statistically significant association with ASD.	Turkey	DSM-IV and DSM-V	Sener et al., 2014
MTHFR (677C/T)	186 individuals with ASD and 186 controls	Association with ASD: “TT” genotype.	China	DSM-IV and ADI-R	Guo et al., 2012
MTHFR (677C/T)	151 individuals with ASD and 100 controls	No statistically significant association with ASD.	Brazil	DSM-IV, ADI-R and CARS	dos Santos et al., 2010
MTHFR (677C/T, 1298A/C) *MTRR* (66A/G) *TCN2* (776C/G), *COMT* (472G/A) *GSTM1*, *GSTT1* *RFC* (80A/G) *GCPII* (1561C/T)	360 individuals with ASD and 205 controls	Association with ASD: “G” allele of RFC 80A/G, TCN2 776C/G, and COMT 472G/A.	USA	DSM-IV, ADOS and CARS	James et al., 2006
MTHFR (677C/T, 1298A/C)	31 individuals with ASD and 39 controls	Association with ASD: “C” allele of 1298A/C and “T”allele of 677C/T.	Egypt	DSM-V TR and CARS	El-Baz et al., 2017
MTHFR (677C/T)	78 individuals with ASD and 80 controls	Association with ASD: “T” allele, and “CT” and “TT” genotypes of 677C/T.	Egypt	DSM-V R and CARS	Ismail et al., 2019

GSTP1 (rs947894), *GSTT1*, *GSTM1*	42 individuals with ASD and 23 controls	No statistically significant association with ASD.	Nigeria	DSM-V	Oshodi et al., 2017
IRS1 (rs1801123), *IRS2* (rs4773092)	180 individuals with ASD and 147 controls	Association with protection for ASD: "G" allele of rs1801123 from *IRS1*.	South Korea	DSM-IV and CARS	Park et al., 2016
GluR6 (rs995640, rs2227281, rs2227283, and rs2235076)	Families of 174 individuals with ASD	Association with ASD: "T" allele of rs2227281 and "A" allele of rs2235076.	China	DSM-IV	Shuang et al., 2004
GluR6 (rs2227281, rs2227283, and rs2235076)	101 individuals with ASD and 152 controls	No statistically significant association with ASD.	India	DSM-IV, CARS and ADI-R	Dutta et al., 2007
AR [$(CAG)_n$ STR] *SRD5A2* (rs9282858) *ESR1* (rs2234693)	101 individuals with ASD and 107 controls	Association with autism: "T" allele of rs9282858 from *SRD5A2*. Association with Asperger: lower repeats of $(CAG)_n$	Slovakia	DSM-IV and ICD-10	Schmidtova et al., 2010

ABC - Autism Behavior Checklist; ASRS - Autism Spectrum Rating Scales; ASQ - Autism Screening Questionnaire; CARS - Childhood Autism Rating Scale; CBQ-R - Child Behavior Questionnaire-Revised; JSAIS - Junior South African Intelligence Scale; SSAIS - Senior South African Intelligence Scale; ADOS - Autism Diagnostic Observation Schedule; DSM - Diagnostic and Statistical Manual of Mental Disorders; ADI - Autism Diagnostic Interview; ADI-R - ADI - Autism Diagnostic Interview-Revised; ICD - International Classification of Diseases

[a] Description of the 22 rare variants are showed in **Table 3.1**.

Table 3.1. Rare *GABRB3* gene variants associated with ASD addressed in Chen et al. (2014)

	Gene region	Mutation ID
GABRB3 rare variants associated with ASD in a sample size of 356 patients diagnosed with ASD and 386 controls from South Korea	5’ region	g.-1571T>C g.-1533_-1526 delCCTCATAGinsTCCATTAGACAA AAGTCTG g.-1528T>C g.-1442G>A g.-1437G>T g.-1090A>G g.-541T>C g.-534C>T (rs4363842) g.-232G>T g.-169T>G g.-142G>T g.-140A>T
	Intronic region	IVS1a+17C>T IVS1a+10G>A IVS1-3C>T IVS2-13G>C
	Exonic region	g.-53G>T c.51C>G c.557C>T c.942C>T c.1006C>T c.1204T>C

Capítulo VII

Inflammation and extracellular vesicles in Autism Spectrum Disorder

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Inflammation and extracellular vesicles in Autism Spectrum Disorder

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Abstract

Autism Spectrum Disorder (ASD) is a set of neurodevelopmental disorders that manifests in early life, impacting behavioral and social skills. Its incidence has been dramatically increasing worldwide. Currently, it is estimated that ~1% of the world population has symptoms of ASD. The exact causes of ASD are still unknown. Despite its unquestionable genetic background, environmental factors also influence the etiology of ASD. Recently, the contribution of the immune system in the development of ASD was raised, and maternal immune activation (MIA) has been suggested as an essential component of ASD development due to the inflammatory background that involves it. Considering MIA as a starting point, in this review we address and summarize the main triggers and enhancers of inflammation during pregnancy that have been identified as significant risk factors for ASD. This article starts a debate about the potential roles of extracellular vesicles in the processes that permeate MIA, which represents the main differential of this review in the context of ASD studies. To achieve this goal, a discussion regarding the roles of extracellular vesicles during pregnancy and its potential influences on ASD is presented, along with a review and update regarding the role of preeclampsia, gestational diabetes mellitus, maternal infections, maternal fever, cytokine unbalances during pregnancy, maternal microbiota, and labor in the context of MIA and ASD.

Key words: Autism Spectrum Disorder; Immune system; Inflammatory responses; Gestation; Cytokines; Maternal immune activation; Preeclampsia; Fever; Microbiota; Exosomes.

1. Introduction

Autism Spectrum Disorder (ASD) is a set of heterogeneous neurodevelopmental disorders that impact behavioral and social skills since early life. ASD is about 4 times more common among boys than among girls and it is estimated that 1% of the overall world population is diagnosed with ASD. From 1998, the Centers for Disease Control (CDC) began tracking the prevalence of ASD and the characteristics of children with ASD in the United States. In 2000, CDC established the Autism and Developmental Disabilities Monitoring (ADDM) network as an attempt to track the prevalence of ASD. Despite the limitations in reason of the heterogeneity in symptomatology, lack of biologic diagnostic markers, and varied diagnostic criteria, the ADDM estimates of ASD prevalence among children aged 8 years in different U.S. communities showed a dramatic increase in diagnostic rates, changing from one diagnostic in 150 children during the years 2000–2002 to one in 68 during 2010–2012 (Baio et al., 2018).

ASD has a strong genetic component and a high concordance rate between monozygotic twins. However, its exact causes are still unknown. Along with genetics, environmental factors are the primary triggers of ASD, especially considering that the rate of ASD incidence is not one-hundred percent concordant in monozygotic twins (Meltzer and Van de Water, 2017; Bai et al., 2019; Hoirisch-Clapauch and Nardi, 2018).

Nowadays, it is known that the immune system is likely to play a role in both development and severity of ASD in reason of its interactions with the central nervous system (CNS) (Gottfried et al., 2015). In this regard, maternal immune activation (MIA) is suggested as a critical environmental factor in ASD development, due to the exposition of the fetus to inflammatory mediators during pregnancy (Meltzer and Van der Water, 2017). Inflammation plays a crucial role during embryo implantation because of its involvement in the remodeling of blood vessel and tissues (Mor et al., 2011). Importantly, pro-inflammatory responses are not systemically predominant during a healthy gestational period; instead, there is a constant immunomodulation, mainly at the maternal-fetal interface, which allows fetal development and protection against pathogens (Kaminski et al., 2019a).

The role of infections during pregnancy and the development of ASD has been a target of investigation, as summarized in **Table 1**. The possible link between maternal infection during gestation and autism was first raised in a study addressing rubella syndrome in 1950, where 5% of the infected pregnant woman had children with ASD (Chess, 1971; Chess, 1977; Shi et al., 2003). Subsequently, additional studies suggested that other infectious agents, including influenza and measles viruses, could also be factors accounting for ASD development. After the first associations between autism and viral infections during gestation, it was believed that ASD was directly associated with infections *per se*. Nevertheless, the incidence of ASD in children born from women with complicated pregnancies, but not related to pathogens, suggests that the association between ASD and maternal inflammation (by different triggers) is a more likely association (Wang et al., 2017).

Gestational disorders characterized by inflammatory unbalances, such as preeclampsia, presents severe consequences for the mother and the fetus (Michita et al., 2018a; Kaminski et al., 2019b). As outlined in **Figure 1**, these and other gestational disturbances might be critical environmental triggers of inflammatory responses that ultimately affect the developing brain of the fetus, potentially leading to ASD development (Meltzer and Van der Water, 2017).

In this article, we summarize the main triggers and enhancers of inflammatory responses during pregnancy that have been associated or suggested as significant risk factors for the development of ASD. A review regarding cytokine unbalances during pregnancy and maternal fever are also addressed. Besides, emerging trends in the field of extracellular vesicles and their impacts on mental and psychiatric diseases, including ASD, is reviewed. Finally, since labor is also an extremely important process for the health of mother and fetus, aspects involving the different categories of childbirth and their possible associations with the development of ASD are raised.

2. Maternal infections that reach the fetus

During the gestational period, there is an intimate contact between maternal and fetal blood. This contact is tightly regulated by immunomodulatory factors produced by the placenta, which avoid infections and maternal immune response towards the fetus (Kaminski et al., 2019a). However, pathogens commonly bypass placental defenses and reach the developing fetus, sometimes causing severe and undesirable effects. Considering pregnancy, the most common pathogens are known as TORCH [*Toxoplasma* sp., "other," Rubella virus, cytomegalovirus (CMV), and Herpes simplex virus (HSV)], but other pathogens have also called attention in the last years, such as Zika virus (ZIKV) (Silasi et al., 2015; Shuler-Faccini et al., 2016, Coyne and Lazear, 2016).

In addition to the classic effects caused by certain infectious agents, especially congenital malformations, follow-up studies have associated some infections during pregnancy with the development of ASD in the first years of life (Chess, 1977; Slawinski et al., 2018; Zerbo et al., 2013; Nielsen-Saines et al., 2019). Interestingly, the birth of a child in winter or spring has already been associated with a higher risk of developing schizophrenia, which is attributed to the high incidence of influenza infection at these seasons (Tochigi et al., 2004).

The first association between ASD and infections during pregnancy was described by Stella Chess through a study involving children with congenital rubella after the rubella epidemic in the United States in 1964 (Chess, 1971). Five years later, a longitudinal study was conducted with the same cohort, reinforcing the association (Chess, 1977). As part of the TORCH group of pathogens, in addition to rubella, CMV infection has already been associated with ASD (Slawinski et al., 2018). Besides the complications involving microcephaly, congenital Zika syndrome was hypothesized and later identified as a risk factor for the development of ASD (Vianna et al., 2018; Nielsen-Saines et al., 2019). This was an important association, illustrating that absence of microcephaly does not warrant

the risk of neurological problems in children of mothers with gestational ZIKV infection (França et al., 2016). Also, pregnant women with one or more episodes of infection with different pathogens have a higher risk of having children with ASD.

As previously mentioned, **Table 1** summarizes the different types of infections during pregnancy that have already been investigated as possible triggers of ASD. The studies discussed here refer to research involving infections and ASD during pregnancy, but other studies also investigated possible associations between particular infections in childhood or adulthood and ASD, such as Varicella zoster virus (Gentile et al., 2014) and HSV (Gillberg, 1986).

3. Maternal activation of the immune system

In addition to the more evident scenario in which infectious agents are directly implicated to impaired neurodevelopment, several evidences point out to a probable less direct effect of infection in the risk of ASD and other neurological diseases in the offspring. An illustrative case-control study with a large sample size revealed that pregnant women with one or more episodes of infection presented a higher risk of having children who would later be diagnosed with ASD. Specifically, the association was observed when pregnant women were diagnosed with infection during hospitalization, mainly due to bacterial infection in the second trimester (Zerbo et al., 2015). This finding corroborated a previous study from Atladóttir et al. (2010) who found that hospitalization of pregnant women due to viral infection in the first trimester and due to bacterial infection during the second trimester were both associated with the diagnosis of ASD in the offspring.

One question still to be answered is whether the mother's immune activation alone would be sufficient to trigger autistic behavior in the offspring. In this regard, an elegant experiment using a mouse model of MIA during mid-pregnancy through influenza infection or through the administration of TLR3 agonist poly(I:C) led to abnormal behavioral responses of the offspring as adults, such as deficits in prepulse inhibition (PPI) in the acoustic startle response and deficiencies in social interaction (Shi et al., 2003). A later study from the same group analyzed the cerebellum of these animals and found a localized deficit of Purkinje cells, a common finding in individuals with schizophrenia and ASD (Shi et al., 2009). So, it is possible that inflammatory mediators released from immune cells during those episodes of infection could negatively influence brain development and result in ASD.

Recently, a study using a MIA swine model suggested that fetal microglia are significantly altered by maternal viral infection by respiratory syndrome virus, presenting a potential mechanism through which MIA impacts prenatal brain development and function (Antonson et al., 2019). Therefore, in addition to the hypothesis of placental barrier breaching by infection, the activation of the mother's immune system due to infections potentially leads to the release of pro-inflammatory

mediators that could negatively influence fetal neurodevelopment. In this regard, several studies have been evaluated the effect of maternal immune activation in the risk of ASD.

3.1. Maternal episodes of fever

One of the main observable effects of immune activation is fever. Fever is the result of increased body temperature resulting from the action of molecules called pyrogens in the hypothalamus. These molecules can be prostaglandins and/or cytokines produced by monocytes/macrophages in response to external stimuli such as infections, environmental changes, and trauma. Due to its role in the maintenance of the physiological homeostasis through immune activation, several studies point to fever as part of the host defenses mechanisms (Cannon, 2013). From an evolutionary perspective, this is considered a highly conserved mechanism because it is a physiological phenomenon observed in different vertebrate and invertebrate species. Also known as pyrexia, fever has an adaptive function of unique importance and is a potent activator of the immune system. Immune outcomes related to body temperature elevation include lymphocyte activation and proliferation, accelerated neutrophil migration, and cytokine production, including interferon (Best and Schwartz, 2014).

Despite being a common symptom during infections by different pathogens, the presence of fever during pregnancy, even in the absence of intrauterine infections (Schlotz and Phillips, 2009), has been suggested as an important risk factor for the development of ASD in the offspring. Zerbo et al. (2013) investigated a possible association between maternal influenza virus infection and the incidence of ASD. Though maternal infections were not associated with ASD, the authors found an association between the occurrence of maternal fever during pregnancy and ASD. Interestingly, Maternal use of anti-pyretic medication did not modify the results of the association between fever and developmental delays (Zerbo et al., 2013).The obtained results were in agreement with those described by Wilkerson et al. (2002), whose data suggested an association between the development of ASD in infants exposed to “high temperatures” during gestation. In another study, Atladóttir et al. (2012) reported an association of maternal fever with ASD risk after febrile episodes lasting 7 days or more. This study also observed a two-fold increased risk of infantile autism in mothers that had influenza virus infection. Finally, a prospecting study conducted in Norway evaluated the incidence of fever in pregnant women and the risk for ASD in 114,500 children born between 1999 and 2009. The development of ASD in children was associated with the occurrence of fever in the second trimester of pregnancy (Hornig et al., 2018). Taken together, the set of results mentioned above points out to an association between febrile episodes and an increased risk of ASD.

3.2. Cytokine unbalances during pregnancy

Amid evidence pointing to immune activation during pregnancy as a major contributor to the risk of autism and ASD, investigating the role of cytokines in this context is critical for the understanding of ASD etiology. The term “cytokines” encompasses interleukins, chemokines, interferons (IFNs), tumor necrosis factors (TNFs), and growth factors. These molecules are small proteins ranging 8–25 kDa in size and play an essential role in several physiological processes including cell growth and proliferation of neuronal tissues, as well as modulation of host immune responses to infection, injury, and inflammation (Xu et al., 2015).

In an experiment using a MIA rat model, administration of IL-6 during pregnancy caused ASD-related symptoms in adult offspring, in contrast to that observed in offspring of *IL-6* knock-out rats injected with poly(I:C) to induce MIA. This study, therefore, indicated that pro-inflammatory cytokine production during pregnancy has a potential influence on the etiology of ASD (Smith et al., 2007). Additional findings of studies with animal models demonstrated that high levels of pro-inflammatory cytokines during pregnancy are associated with higher risk of ASD in the offspring (Ponzio et al., 2007; Hsiao et al., 2013; Choi et al., 2016).

In humans, increased levels of the circulating pro-inflammatory cytokines IL-6, IFN-γ, and IL-1 in pregnant women has been associated with impaired intellectual abilities in the offspring diagnosed with ASD (Jones et al., 2017). Through amniotic fluid analysis of pregnant mothers of children with ASD and controls with typical development, Abdallah et al. (2013) observed higher levels of TNF-α and TNF-β in mothers of affected children. Despite the findings pointing to an important role of pro-inflammatory cytokines in mediating the effects of maternal immune activation on the fetal neurodevelopment (Deverman and Patterson, 2009), there is no consensus regarding the mechanistic action of these molecules, and also whether they cross the placental barrier (Ashdown et al., 2006; Parker-Athill and Tan, 2010). Addressing these issues, a study evaluated amniotic fluid samples from Danish individuals diagnosed later in life with ASD and controls. Elevated levels of MCP-1 were observed in mothers of children with ASD compared to controls (Abdallah et al., 2012). Another study evaluated the levels of 17 different serum cytokines in pregnant women whose children were diagnosed with ASD, with other neurological abnormalities, or classified as typically developing. Comparing cytokine levels between these different groups, mothers of children diagnosed with ASD children had significantly higher concentrations of IFN-γ, IL-4, and IL-5 during pregnancy (Goines et al., 2011).

The impairments in inflammatory responses, including cytokine unbalances, are still observed in ASD children, as well as in adults with the disease, as part of the neuroinflammatory status characteristic of autistic patients, further indicating the immune component in the broad spectrum of clinical manifestations of ASD (Mazur-Kolecka et al., 2014; Mostafa et al.,, 2013; Mead and Ashwood, 2015). In other words, besides evidence indicating inflammation as a risk factor for autism,

the maintenance of this condition is also associated to immune dysfunctions. The management of these immune dysfunctions has already been addressed and extensively discussed as promising treatment strategies (Siniscalco et al., 2018). The already proposed immune treatments in ASD management includes intravenous immunoglobulin (IVIG) infusion (Gupta et al., 2010), corticosteroid therapy (Shenoy et al., 2000), and vitamin D supplementation (Stubbs et al., 2016). The efficacy IVIG infusion has recently been demonstrated in ASD children with immune impairments (Melamed et al., 2018) and positive effects concerning improvements in language and behavior in young autistic children have also been observed in the context of corticosteroid therapy (Duffy et al., 2014). In front of these findings, the importance of specific identification of ASD endophenotypes is evident (Siniscalco et al., 2018). Integrative approaches involving the characterization of inflammatory biomarkers, immune subtypes of cells involved, circulating cytokine profiles, and gastrointestinal status in combination with genetic association studies could be useful for personalized and more efficacious approaches. Mitigation of epilepsy and seizures could also be improved with immune-related approaches once these symptoms have already shown immunological backgrounds (Theoharides and Zhang, 2011).

It is still unknown whether unbalances in the level of circulating cytokines observed in autistic individuals after birth are direct consequences of maternal immune changes to which these individuals may have been exposed during fetal development or if they are only part of the clinical manifestation of the disorder. Vargas et al. (2005) evaluated samples of autopsy-derived brain tissue of autistic patients, as well as cerebrospinal fluid of living patients. In this work, different techniques were used to determine the cytokine expression profile along with the magnitude of neuroglial and inflammatory reactions in the samples of patients with ASD. An intense neuroinflammatory process was identified in the cerebral cortex, white matter, and cerebellum of autistic patients. The study revealed neuroglia-derived macrophage chemoattractant protein (MCP)–1 and tumor growth factor as the most abundant cytokines in the tissues analyzed (Vargas et al., 2005).

Li et al. (2009) evaluated components of the immune response in brain tissue samples from patients with ASD. Compared to matched controls of typical development, samples derived from affected people showed increased levels of IL-8, IL-6, GM-CSF, IFN-γ, and TNF-α. Besides, the Th1/Th2 ratio of the patients was significantly polarized towards the inflammatory Th1 profile and, interestingly, the expected compensatory elevation in IL-10 levels in those samples was not observed (Li et al., 2009). Such compensation via IL-10 production is expected because inflammation is usually composed of two phases and characterized by the rapid production of pro-inflammatory factors, followed by a decrease in their release and a subsequent delayed production of immunosuppressive mediators that limit their production and/or effect, thus avoiding harm to the host tissues. Of note, among these anti-inflammatory factors, IL-10 is widely considered to be the quintessential immunosuppressive cytokine produced within the CNS (Burmeister and Marriott, 2018).

Also as a marker of exacerbated maternal inflammation, high levels of C-reactive protein in pregnancy have already been associated with the development of ASD in offspring (Brown et al.,

2014). Regarding hormones, alterations in their levels have been suggested as contributing factors for ASD development. In this sense, it has been suggested that ASD can be viewed through the perspective of an “extreme male brain”. In brief, this theory is based on demonstrations that autistic people show a masculinized shift in scores on two key sexually dimorphic psychological traits: empathy and systemizing behavior. These characteristics may be due to prenatal exposition to elevated testosterone levels in the womb (Greenberg et al., 2018). Autistic women present high levels of androstenedione, the precursor to testosterone (Schwarz et al., 2011). Extending the findings of elevated prenatal steroidogenic activity in autism (Baron-Cohen et al., 2015), it was recently shown that prenatal estrogens contribute to autism likelihood, affecting both brain development and functioning and, ultimately, sexual differentiation (Baron-Cohen et al., 2019). However, these concepts are still being intensely debated (Underwood, 2019), and deserve further investigation.

The influence of the immune system on the development and functioning of the CNS is unquestionable (Filiano et al., 2015). However, the specific interactions between these two systems and the timing in which they occur are still being elucidated. Cytokines and chemokines are known to be key participants in such interactions. These molecules act directly on cells or may influence the action and migration of different cells of both systems, such as microglia (Prins et al., 2018) and $CD8^+$ T regulatory memory cells residing in the brain (Steinbach et al., 2016). Thus, besides their role in inflammatory responses, cytokines have already been stated as “the common language between the nervous and the immune systems” (Goines and Ashwood, 2013). For example, interleukin (IL)-1β and IL-2 receptors activate and modulate different cellular pathways, such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), both involved in CNS development and damage repair mechanisms (for review, see Xu et al., 2015).

4. Inflammation-related gestational disturbances as risk factors for ASD

4.1. Gestational hypertension

The increasing of blood pressure in pregnancy warrants closer monitoring of maternal and fetal health. Hypertensive disorders is a global public health problem that affects up to 10% of pregnant women (ACOG, 2013). The definition of hypertension in pregnancy varies accordingly to international guidelines, and includes different clinical phenotypes that are often assigned as transient [preeclampsia (PE), gestational hypertension] or chronic (pre-existing hypertension) depending on the onset of symptoms (Braunthal and Brateanu, 2019). Noteworthy, irrespective of pre-existing or *de novo* hypertension, the association of hypertensive disorders with ASD is still inconclusive since hypertension is a common symptom underlying many pathological alterations during pregnancy. Nevertheless, children exposed to hypertension during pregnancy are twice as likely to develop ASD by age 7 (Curran et al., 2018).

In this context, PE is the most frequent hypertensive disorder of pregnancy (HDP), affecting 2-8% of pregnant women (Duley et al., 2009). High blood pressure and inflammation are the hallmarks of PE, usually accompanied by several clinical manifestations affecting both maternal and fetal health (reviewed in Michita et al. 2018a). Although much effort has been made to understand the pathophysiology of PE, delivery still remains the only effective treatment. Besides the significant burden to public health, PE is responsible for up to 40% of preterm births (Bilano et al. 2014). Of note, a higher prevalence of ASD has been reported in preterm population than term infants (Kuzniewicz et al., 2014; Agrawal et al., 2018).

The association of PE with ASD risk has been evaluated in previous meta-analyses (Wang et al., 2017; Dachew et al., 2018; Maher et al., 2018a). PE impacts both maternal and fetal health; for example, an increased risk for vascular related-disorders later in life and postpartum depression is observed among women with PE (Blom et al., 2010). Also, PE may compromise fetal neurodevelopment in several ways due to exposure to maternal systemic inflammation, insulin resistance, nutrient deprivation, and chronic hypoxia (Walker et al., 2015). Intrauterine hypoxia may affect the hypothalamic-pituitary-adrenal axis, increasing the risk for intrauterine growth restriction, cardiovascular disorders, and immunological imbalances in the fetus (Kay et al., 2019). Altogether, exposure to HPD may increase the risk for bronchopulmonary dysplasia, cerebral palsy, developmental delay, and ASD (Mann et al., 2010; Walker et al., 2015; Maher et al., 2018b).

Identifying a causal link between PE and ASD is challenging, since the association between ASD and PE is likely influenced by prematurity, birth weight and other pregnancy-related factors (Mann et al., 2010). Nevertheless, a possible influence of both inflammation and prematurity in neurodevelopment due to PE is discussed elsewhere (Walker et al. 2015; Prins et al. 2018). In this context, exposure to maternal systemic inflammation may alter microglial activation (removal of cellular debris and synaptic pruning), thus increasing the risk of ASD in the offspring (Prins et al., 2018). The role of inflammatory mediators during pregnancy in ASD susceptibility is further reviewed in the following sections.

Considering the role of extracellular vesicles in pregnancy, it is known that the concentration of placenta-derived extracellular vesicles increases in maternal peripheral circulation throughout pregnancy (Salomon et al., 2014). Regarding PE cases, such concentration is even higher, with a predominance of inflammatory factors associated with extracellular vesicles (Tannetta et al., 2017).

4.2. Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is a metabolic disorder in which hyperglycemia develops during the second or third trimester of pregnancy. GDM usually resolves following delivery (American Diabetes Association, 2019). Despite of strong contribution of genetic factors in ASD risk (Tick et al. 2016), early pregnancy exposure to environmental factors is also reported (Bölte et al.,

2019; Rylaarsdam and Guemez-Gamboa, 2019). Intrauterine exposure to hyperglycemia has been consistently associated with ASD (Gardener et al., 2009; Xu et al., 2014; Wan et al., 2018; Yamamoto et al., 2019; Hoirisch-Clapauch and Nardi, 2019). Also, the association of ASD with diabetes is highest for type 1, followed by type 2 and GDM when diagnosed by 26 weeks of gestation. However, when GDM is diagnosed after 26 weeks, the risk of having a child with ASD is similar to the general population, thus implying that glycemia and the timing of exposure are important factors associated with ASD risk (Xiang et al., 2018).

Increasingly glycemia at 24-28 weeks of gestation increases the risk of adverse maternal, fetal and neonatal outcomes, even within normal glycemia ranges for pregnancy (Metzger et al., 2008; American Diabetes Association, 2019). It is suggested that intrauterine hyperglycemia impairs fetal neuronal migration and connections or even leads to long lasting epigenetic modifications in neuronal cells; this could be one of the explanations for the presence of rare SNPs in idiopathic ASD and also why the concordance for ASD in monozygotic twins can be less than 50% (Damm et al., 2016; Wozniak et al., 2017; Hoirisch-Clapauch and Nardi, 2019).

GDM not only increases the risk for ASD, but also increases the risk for type 2 diabetes, cardiovascular disorders, and birth complications (Plows et al., 2018). Importantly, it is widely known that obesity or elevated body mass index is a risk factor for diabetes and PE, both associated with ASD risk (O'Brien et al., 2003; American Diabetes Association, 2019). Moreover, chronic inflammation in obese individuals contributes to metabolic dysfunction (Ouchi et al., 2011). It is possible that such physiological conditions during pregnancy *per se* or in association to any gestational disorder could predispose to ASD development in the offspring (Windham et al., 2019).

A retrospective study was used to quantify exosomes present in maternal plasma of normal and GDM pregnancies. Despite the expected increase in exosome concentration across gestation (Salomon et al., 2014), the study revealed that such increase was significantly greater in GDM cases (Salomon et al., 2012).

5. Maternal production of antibodies against the fetal brain

Another interesting aspect of the connection between the immune system and ASD-related symptoms is the production of maternal antibodies against brain proteins of their children. This type of antibodies derived from women who had autistic children were collected and then injected in pregnant mice. As a result, the offspring of mice that received such antibodies showed altered exploration and motor coordination, among other abnormalities (Dalton et al., 2003). In the same mice model, prenatal exposure to sera with antibodies from mothers of autistic children provoked anxiety disorders, startle reflexes, and lower sociability in animals born from pregnant exposed to sera from mothers of ASD children (Singer et al., 2009; Singer et al., 2016). Also, studies using rhesus monkeys submitted to prenatal exposition to human immunoglobulin G (IgG) derived from mothers of ASD children

presented stereotypies, hyperactivity (Martin et al., 2008), or impaired social behavior (Bauman et al., 2013).

Regarding the importance of such antibodies in humans, it is known that this type of auto-antibody is found in approximately 12% of women who had autistic children (Braunschweig et al., 2008). Studies demonstrated the specificity of these antibodies against 37 and 73 kDa brain proteins using IgG from murine models (Braunschweig et al., 2012).

6. Maternal microbiota

The microbiota has already been suggested as a link between the immune system and prenatal environmental factors that contribute to the occurrence of ASD. In the gastrointestinal tract, resident bacteria form extremely complex ecosystems, and microbial metabolites play major roles in regulating the immune system and also neural development. Thus, for a typical development of the central nervous system, it is believed that the correct maintenance of the diversity and prevalence of different species of these microorganisms is necessary, both in the prenatal (maternal microbiota) and post-natal periods (Madore et al., 2016; Liu et al., 2019; Paysour et al., 2019).

The composition of the maternal gut microbiome contributes to obstetric outcomes with long-term health consequences for both the mother and the newborn (Edwards et al., 2017). The maternal diet during pregnancy impacts on the presence or absence of important immunomodulators ultimately required for a successful pregnancy (Madore et al., 2016). Also, obesity-associated inflammation in pregnancies potentially influences the risk for ASD (Paysour et al., 2019).

The term “Brain-Gut Axis” has been used since the discovery of communication between the brain and the gastrointestinal tract. The immune system is known to exchange information with the brain bi-directionally, along with the autonomic nervous system, the enteric nervous system, and the hypothalamic-pituitary axis. In a healthy individual, all of these pathways act in synchronization since the beginning of CNS development. An imbalance in the number and diversity of microorganisms present in a body part is called dysbiosis. Given the two-way nature of these complex connections, the imbalance in dysbiosis-related inflammatory responses has potential impacts on CNS functioning, body weight, immunity, and behavior (Edwards et al., 2017; Cepeda et al., 2017).

The gestation period is marked by profound changes in the maternal immune system (Kaminski et al., 2019a), affecting the pregnant woman’s body locally, at the maternal-fetal interface, as well as other parts of the maternal organism. In this context, a maternal microbiota is extremely affected during pregnancy. The composition of such a microbiota influences the entire gestation period and may also impact the health of the mother and fetus, having impacts after childbirth for both the women and the newborn (Edwards et al., 2017). In the context of imbalances in IL-6 levels in MIA models, Hsiao et al. (2013) raised the important role of the microbiota by treating the MIA model

offspring. The study demonstrated that treatment with *Bacterioides fragilis* was able to reestablish IL-6 levels along with attenuation of symptomatology (stereotyped behavior and anxiety).

In regard to the diagnosed individuals, analyzing both bacterial gut microbiota the gut mycobiota, Strati et al. (2017) showed an increase in the Firmicutes/Bacteroidetes ratio in autistic subjects due to a reduction of the Bacteroidetes relative abundance. Considering fungi, the study found that the relative abundance of the fungal genus Candida was more than double in the autistic than neurotypical subjects. Based on recently published genome wide association studies of gut microbiota (GWASGM), a search for significant genetic associations between host genes and gut microbiota composition was made in the context of different psychiatric disorders. Regarding ASD, association signals were observed for genus the Bacteroides and Desulfovibrio (Cheng et al., 2019). In agreement, Desulfovibrio species were previously found in significantly higher numbers in stools of severely autistic children than in controls (Finegold et al., 2010; Tomova et al., 2015).

7. The labor

Childbirth is a process marked by extreme physiological stimuli for both the mother and the fetus. Along with the physiological events that permeate all labor, the expulsion of the baby out of the mother's body is a major physical stress. Based on these physiological characteristics, birth is a stressful and even traumatic event for both mother and fetus (Lagercrantz, 2016).

In addition to genetic and environmental causes, it is believed that childbirth is also a process that may influence the increase in the number of new diagnoses of ASD. As already described in previous topics of this review, pregnancy is a complex and extremely regulated phenomenon. After a period of intense immune regulation, normal delivery is preceded by intense information exchange between the body systems of the pregnant woman. Considering that labor is a physiological process involving an inflammatory response (Romero et al., 2006), it is interesting to note that a longer period of normal labor has already been observed in mothers of children who develop ASD (Wilkerson et al., 2002). Forceps-aided delivery has been associated to autism incidence (Deykin and MacMahon, 1979).

Considering the labor and delivering process, a study evaluated fetal exposure to the so-called 'labor and delivery drugs' along with other labor and delivery risk factors. The results revealed that children born of mothers who used a drug or combination of drugs that induce labor are at a higher risk of developing ASD (Smallwood et al., 2016). A follow-up study addressing all live births in Sweden between 1992 and 2005 investigated the association between labor induction and ASD. In this sample, 1.6% of invidulas received an ASD diagnostic after the age of 8. Association of labor induction and ASD was lost when comparison was made within siblings whose births were discordant with respect to induction. Thus, it was indicated that concerns about ASD should not influence into the clinical decision about whether to induce labor (Oberg et al., 2016).

When delivery through the vagina, or “normal delivery”, poses a risk to the pregnant woman, the baby, or both, cesarean delivery is the indicated alternative. However, in recent years there has been a large increase in demand for cesarean deliveries only at the option of the pregnant woman. Cesarean delivery without medical indication shows various risks, as it involves an invasive procedure and general anesthesia of the patient. Thus, studies evaluating the association between cesarean delivery and different phenotypic manifestations in their infants have been done, including in the context of ASD (Danforth, 1985; Wilkerson et al., 2002; O’Donovan and O’Donovan et al., 2018).

Different studies have indicated an association between cesarean delivery and ASD development in children from different countries (Curran et al. 2015; Polo-Kantola et al. 2014; Yip et al., 2017). However, Huberman Samuel et al. (2019) suggested that the associations between risk for autism and cesarean delivery occur in severe manifestations of the spectrum of the disorder, and even when surgery involves general anesthesia of the pregnant woman. In agreement, Chien et al. (2015) have previously reported that neonates delivered by cesarean section with general were more likely to be diagnosed with ASD than those exposed to vaginal birth or cesarean section with regional anesthesia.

Several attempts have emerged to explain associations between cesarean delivery and incidence of ASD. It is believed that deregulation of optimal oxytocin levels may be one of the causes, once perinatal imbalances in oxytocin levels may reflect adverse effects on childhood and adulthood (Gialloreti et al., 2014). Oxytocin participates in the regulation of uterine contractions during labor and also influences the efficiency of birth. Regarding childbirth, oxytocin is released in pulses, gradually increasing its concentration in maternal circulation, with its maximum peak recorded in the first hour after the birth of the child. A direct consequence of planned cesarean section surgery is the absence of exposure of the fetus to these hormone levels. In addition, oxytocin participates in biochemical processes related to mood and social behavior. Supporting this explanation, decreased levels of oxytocin are detected in the plasma of people with ASD compared to typically developing individuals (Husarova et al., 2016).

Besides, oxytocin is a hormone with important participation in the regulation of social skills and interactive activities. In this context, Ben-Ari, (2015) promoted a discussion about the birth-childbirth-development of ASD and postulated that childbirth is a critical moment of stress where there is attenuation or aggravation of the deleterious effects that may have occurred during pregnancy (due to genetic and/or environmental factors). In addition to the issues related to oxytocin and general anesthesia, the type of delivery may impact the individual’s microbiota throughout childhood and adulthood and may participate in the pathogenesis of ASD.

8. Extracellular vesicles and ASD: an emerging topic

The term “extracellular vesicle” (EV) encompasses several lipid enveloped structures released by cells of eukaryotic and prokaryotic organisms through shedding mechanisms. EVs receive different names according to size, shedding mechanism, shape, and content. Some of such names include “microparticles”, “microvesicles (MVs)”, “nanovesicles”, “nanoparticles”, “ectosomes”, “exosomes”, “exovesicles”, and “exosome-like vesicles” (Colombo et al., 2014). The diversity in terms of origin and function requires careful characterization of EVs during experiments. Evaluation of size, shape, and biochemical composition are strongly recommended for identifying EV subtypes (reviewed in Théry et al., 2018).

In multicellular organisms, EVs have been isolated from various biological fluids, including blood, urine, synovial fluid, saliva, breast milk, amniotic fluid, broncho-alveolar lavage fluid, ascites, cerebrospinal fluid, bile, vaginal fluid, and semen (Colombo et al., 2014; Ellwanger et al., 2017). Despite the common features used for EV identification and characterization, it is important to emphasize that EV-associated cargoes differ depending on the organism of origin. Regarding mammals, **Figure 2** illustrates a hypothetical EV, showing the various types of molecules that can be found on the membrane or within EVs released by mammalian cells, including membrane receptors (reviwed in Théry et al., 2018), lipids (Llorente et al., 2013), membrane channels (Stobrawa et al., 2011), pathogen-derived toxins (Husmann et al., 2009), nucleic acids (Kouwaki et al., 2016), pathogens (Liu et al., 2014), immunoglobulins (McLellan, 2009), and immunomodulatory molecules (Stenqvist et al., 2013). Different types of EVs can cross physical and physiological barriers and perform essential roles in cell-to-cell communication. Thus, EVs are critical modulators of the immune response in both healthy and pathological backgrounds (Kaminski et al., 2019a; Ellwanger et al., 2016; Ellwanger and Chies, 2019). The use of EVs as carriers of immunoregulatory molecules is proposed as a promising therapeutic tool under different conditions (Kaminki et al., 2017; Lässer et al., 2018).

Regarding the classification of EVs into the above-mentioned types, we agree with the recommendation of MISEV2018 (Minimal information for studies of extracellular vesicles 2018) which states that “terms such as exosome and microvesicle (...) are historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis” (Thèry et al., 2018). While some articles discussed here have not conducted sufficient or adequate experimentation to disclose the origin and exact classification of EV type, we have chosen to use the terminologies presented in the original publications.

Exosomes are a specific group of EVs showing 40-100nm in size and generated by endocytic pathways. These nanovesicles are highly implicated in pregnancy, being intensely secreted by placental cells and playing important roles in the immunomodulation at the maternal-fetal interface. This communication between mother and fetus via EVs has been reported in the direction from the

syncytiotrophoblast towards maternal circulation (Mincheva-Nilson, 2010; Stenqvist et al., 2013) and modify the susceptibility to various infections (Kaminski et al., 2019a). The concentration of exosomes in the circulation of pregnant women increases over the gestational period. Such content of exosomes also correlate with uterine blood flow and placental weight at deliver (Salomon et al., 2014). Regarding gestational disorders such as PE, the number of circulating exosomes is quite higher, and it has been suggested as a potential tool for pregnancy monitoring (Tannetta et al., 2017).

Upon stimulation, neurons release exosome-like EVs, and the release of neurotransmitters by these nanovesicles likely represent a mechanism of protein sorting and quality control through disposing of such receptors or to regulate excitability in a lysosome-independent manner. Also, experiments involving cell depolarization addressing differentiated neuroblast cultures have shown that these cells contain micro RNAs that are released in exosomes as cargo, which could ultimately be involved in silencing regulation during cellular events such assynaptic plasticity. Besides neurons, other cells of the nervous system act in cooperation with exosomes and other EVs, such as olygodendrocytes, Schwan cells, astrocytes, and microglia (reviewed in Budnik et al., 2016). Of note, microglia consists of brain resident macrophages involved in tissue repair and in protecting the host against infections. In the context of this review, neuroinflammation is a process in which microvesicles have shown intense participation; as examples, in multiple sclerosis (MS) and demyelinating disease, microglia and astrocytes can spread inflammatory signals by the release of EVs associated with inflammatory molecules, such as IL-1β, IFN-γ, TNF, caspase 1 and the P2X7 receptor, among other factors (Budnik et al., 2016). Patients with MS have shown increased numbers of microvesicles in blood and cerebrospinal fluid, and microvesicles have been suggested as biomarkers of different CNS diseases (Carandini et al., 2015). In addition, it is clear that EVs from outside the CNS could exert their effects in CNS cells, as they are able to reach the brain by crossing the blood brain barrier (reviewed by Matsumoto et al., 2017). This characteristic has implications for neurophysiologic disease modeling and also for the design of treatment strategies.

Along with strong indications that exosomes deliver biological information to neurons (Kawikova and Askenase, 2014; Budnik et al., 2016; Sharma et al., 2019), there is growing evidence pointing to a role of extracellular vesicles in psychiatric conditions, including ASD (Tsilioni et al., 2014; Tsilioni and Theoharides, 2018; Saeedi et al., 2019). Both the link between autoimmunity and ASD symptoms and a possible treatment using these EVs were already suggested considering the release of ATP and DNA by mitochondria (Theoharides et al., 2013) and the demonstration that EVs from children with ASD contain a higher amount of mtDNA as compared to normotypic controls (Tsilioni and Theoharides, 2018). Mesenchymal stromal cells (MSC) have shown important functions related to perinatal brain injury protection due to MSC ability to ultimately avoid the activation of brain resident immune cells, thus protecting brain tissues against inflammation-related damages. It was demonstrated that intranasal application of MSC-derived exosomes prior to ischemia significantly prevented perinatal brain injury in a rat model. Thus, MSC exosomes were proposed as a promising

strategy to prevent preterm PBI in human newborns (Thomi et al., 2019). The support for these therapeutic strategies was also previously shown in a model of inflammation-induced preterm brain injury (Drommelschmidt et al., 2017). Regarding MSC-derived exosomes, an *in vitro* model of hypoxia-ischemia revealed neuroprotective and neuroregenerative effects on neuronal cells associated to these EVs (Joerger-Messerli et al., 2018).

It is not a novelty that EVs control immune response. Also, EV cargoes secreted in the intercellular space by different cell types can act as triggers of microglia activation (Robbins and Morelli, 2014). Supporting the hypothesis that EVs are possible mechanistic contributors in the immune dysfunctions that permeate the different ASD manifestations, Tsilioni and Theoharides (2018) reported a significantly increased concentration of EVs in serum of children with ASD as compared to healthy normotypic controls. Such EVs presented mitochondrial (mtDNA) as a cargo, which consists of an important trigger of inflammatory responses both *in vivo* and *in vitro* (Collins et al., 2004). Furthermore, EVs containing mtDNA have shown the ability of stimulating human-cultured microglia to secrete the pro-inflammatory cytokine IL-1β and could potentially act in the brains of children with ASD (Tsilioni and Theoharides et al., 2018). Interestingly, significantly higher mtDNA levels in serum of autistic individuals than in normotypic controls were previously reported (Zhang et al., 2010).

The hypothesis that EVs could drive neuroinflammation deserves additional explanations, such as the signals for the synthesis of inflammatory mediators. It was proposed that EV-mediated IL-1β production via inflammasome would contain mtDNA as a first signal and the neuropeptide neurotensin as the second one; such proposal is based on previous findings of neurotensin capacity to stimulate microglia to secrete IL-1β (Patel et al., 2016) along with the elevated levels of both IL-1β in the brain (Ashwood et al., 2011) and neurotensin in the serum (Angelidou et al., 2010) of children diagnosed with ASD.

Intranasal exosomes-based therapies have been suggested for the treatment of neuronal disorders (Perets et al., 2018; Thomi et al., 2019). Aiming to track the migration and homing patterns of intranasally administrated exosomes derived from bone marrow mesenchymal stem cells in a set of brain pathologies, including stroke, autism, Parkinson's disease, and Alzheimer's disease, Perets et al. (2018) presented a mechanism for longitudinal and quantitative *in vivo* neuroimaging of exosomes based on the superior visualization abilities of classical X-ray computed tomography along with gold nanoparticles as labeling agents. In this context, treatment with MSC-derived exosomes via intranasal administration showed significant behavioral improvement of both genetic and idiopathic autism in mice (Offen et al., 2019).

The traffic of EVs from the placenta to the maternal circulation is already a consensus, as previously discussed. In the opposite direction, the traffic of biological components from mother to fetus has not been the subject of current studies, but some information can be obtained from papers published decades ago. Although considered inadequate and unethical today due to the use of radioactive material, some experiments demonstrated the passage of cells and platelets from mother to

fetus during pregnancy, as raised in **Table 2**. Since there is a passage of different cell types from the maternal bloodstream towards the fetus, it is possible that mother-derived EVs can also reach organs and systems of the fetus, including the CNS, impacting the fetal development.

Considering the scenario mentioned above and the potential impacts of exosomes and other EVs on autistic individuals, some questions emerge:

I) What is the origin of these exosome-like EVs?

II) What is the position of these exosome-like EVs?

III) Do these exosome-like EVs interact with CNS cells?

IV) Is it possible to isolate these increased exosomes-like EVs in autistic patients after infections?

Not all of these questions can be completely answered with scientific data at this point and more research about these aspects is needed. The potential answers to some of these questions and hypotheses are shown in **Figure 3**.

9. Combining the topics

Exosomes and other EVs participate in various immune processes, in both pro- and anti-inflammatory conditions of health and disease. These include natural elevation in exosome concentration in the peripheral circulation of healthy pregnant women (Salomon et al., 2014) along with an intense release of these and other EVs by the placenta at the maternal-fetal interface (Mincheva-Nilson, 2010). In both cases, there is predominant exosome-mediated immunosuppression, where they act mainly through controlling apoptosis of activated lymphocytes that could harm the fetal/placental development (Hedlund et al., 2009; Stenqvist et al., 2013). On the other hand, exacerbated levels of exosomes whose features are mainly pro-inflammatory, characterize the plasma of women with PE (Tannetta et al., 2017). Considering that PE is a risk factor for ASD (Wang et al., 2017; Dachew et al., 2018; Maher et al., 2018a), exosomes along with (or transporting) pro-inflammatory mediators could be a neglected mechanism in ASD etiology, mainly during gestation. In addition, the role of EVs in the communication of different cell types of the nervous system (Budinik et al., 2016) and differences in concentrations of serum exosome between individuals with ASD and normotypic controls (Tsilioni and Theoharides, 2018) further suggest exosomes and other EVs as potential candidates but neglected, components of ASD etiology.

Besides the genetic component of the disorders included in the autistic spectrum, factors of the immune system under the control of a pregnant woman’s physiology can be considered as triggers for the development of ASD. Besides the association of ASD with PE and other pregnancy related-variables, the available data from different approaches still indicate that no single factor contributes to ASD development. Therefore, further studies in well-designed cohorts are required to better explain these aspects.

The epidemiology of ASD is likely underestimated due to a lack of studies from developing countries where maternal-fetal mortality and morbidity is highly associated with vascular disorders. The body of evidence reviewed here strongly suggests that the proper development of the fetal CNS is directly dependent on the correct functioning of both the fetal immune and nervous systems, along with the correct homeostasis of other physiological systems in the maternal and in the fetal counterparts.

In addition to this dynamics between systems, the fetal immune system is directly influenced by maternal immune responses to different biological challenges during pregnancy (Mincheva-Nilson, 2010; Kaminski et al., 2019a). Extracellular vesicles actively participate in the immune adjustments necessary for proper fetal development; and the abundance and content of these vesicles are directly affected by physiological changes during gestation, such as infections and other disorders, like PE. Furthermore, the immunological outcomes in the maternal body, like the responses generated by cytokines and chemokines produced in face of different challenges enhance inflammatory responses at the maternal-fetal interface. Importantly, these responses can be both pro-inflammatory and anti-inflammatory, depending on the context in which such a response was induced.

In addition to the potential influence of inflammation/maternal immune activation on the development of ASD during development of fetal CNS, inflammation is a common feature of individuals with ASD (Siniscalco et al., 2018). Among the immune alterations in ASD, there are presence of brain-reactive antibodies (Cabanlit et al., 2007), abnormal T cell counts and function (Yonk et al., 1990; Mostafa et al., 2010), altered cytokine levels in brain, cerebrospinal fluid and bloodstream (Vargas et al., 2005; Jyonouchi et al., 2014; Molloy et al., 2006), elevated levels of circulating monocytes (Sweeten et al., 2003), and dysregulation in the activity of Natural Killer cells (Enstrom et al., 2009; Bennabi et al., 2019). Considering these observations, inflammation may contribute to the maintenance of the phenotype and symptoms of ASD. Also, inflammation may be a consequence of dysregulations in the neuro-immune-endocrine axis associated with ASD. The relationship between ASD and inflammation can therefore form a feedback loop. The interplay between ASD and inflammation are summarized in **Figure 4**. EV-associated and/or free inflammatory molecules, hormones and inflammatory cells mediate the interactions between inflammation and ASD.

Different molecules participate in the regulation of the maternal immune system at the maternal-fetal interface, whose presence, absence, and abundance are highly influenced by maternal genetic factors (Kaminski et al., 2019b; Michita et al., 2016; Kaminski et al., 2017; Michita et al., 2018b; Vianna et al., 2007) and also by the genetic background of the fetus (Palei et al., 2010; Chedraui et al., 2013). However, how gene variants of the immune system-related genes influence the etiology of ASD has yet to be better demonstrated.

Another interesting fact is the increase in the number of women using birth-inducing drugs in the last 30 years. Being a physiological process that also involves an inflammatory response, labor may be an additional trigger for immune responses accounting for the risk of ASD development.

Regarding cesarean, which involves programmed delivery, the absence of fetal exposure to the physiological stimuli underlying labor (mainly oxytocin) is also another suggested risk factor. Alternatively or additionally, the possible link could not be on cesarean delivery *per se*, but it may be on the microbiome-related differences observed between cesarean and vaginal-delivered children.

10. Key-messages and conclusion

Finally, some take-home messages permeated this review and led us think EVs as neglected mechanistic links on inflammation triggering in ASD:

(I) The immunomodulatory role of exosomes in pregnancy;

(II) The inflammatory background of PE;

(III) The increased levels of exosomes in the maternal circulation of both PE and GDM;

(IV) The indication of both PE and GDM as risk factors for ASD development in reason of MIA;

(V) The role of exosomes and other EVs in microglia activation;

(VI) The increase of EV-associated proteins in the serum of children with ASD;

(VII) EVs could reach the brain and act as inflammation triggers in the context of ASD.

Considering those topics, we suggest that EVs are important mechanistic drivers of the molecular triggers and enhancers of inflammation in ASD. Besides the ability of EVs to protect the inflammatory molecules from degradation, they can also facilitate the gestational intercurrences and should be considered as factors accounting for the development of ASD.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Author contributions

VLK wrote the initial version of the article. RTM, JHE, TDV, JBS, TR, RSR and JABC contributed with opinions on the content of the article and writing the text. VLK and JHE created the figures. VLK and JABC revised and edited the final version of the manuscript.

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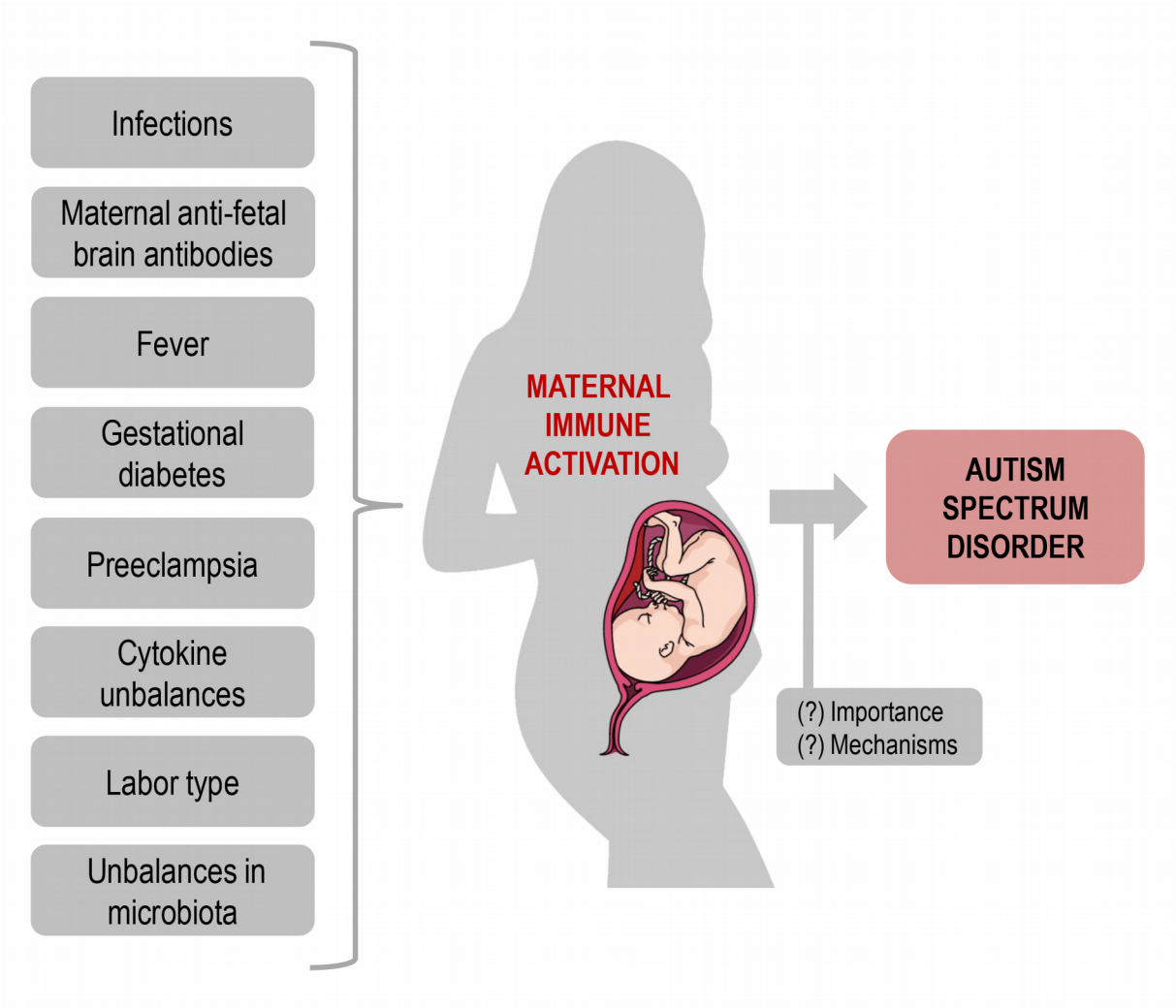

Figure 1. Factors involved in maternal immune activation (MIA) and potentially development of Autism Spectrum Disorder (ASD). Preeclampsia, maternal anti-fetal brain antibodies, gestational diabetes mellitus, fever episodes, unbalances in cytokine systems, infections, unbalances in maternal microbiota and labor type are factors associated with MIA. The development of ASD in the children can be, at least in part, the result of MIA during the gestational period. The importance degree of MIA and the mechanisms by which MIA affects the etiology of ASD must be established in greater detail. This figure was created using a *Mind the Graph* illustration (available at www.mindthegraph.com).

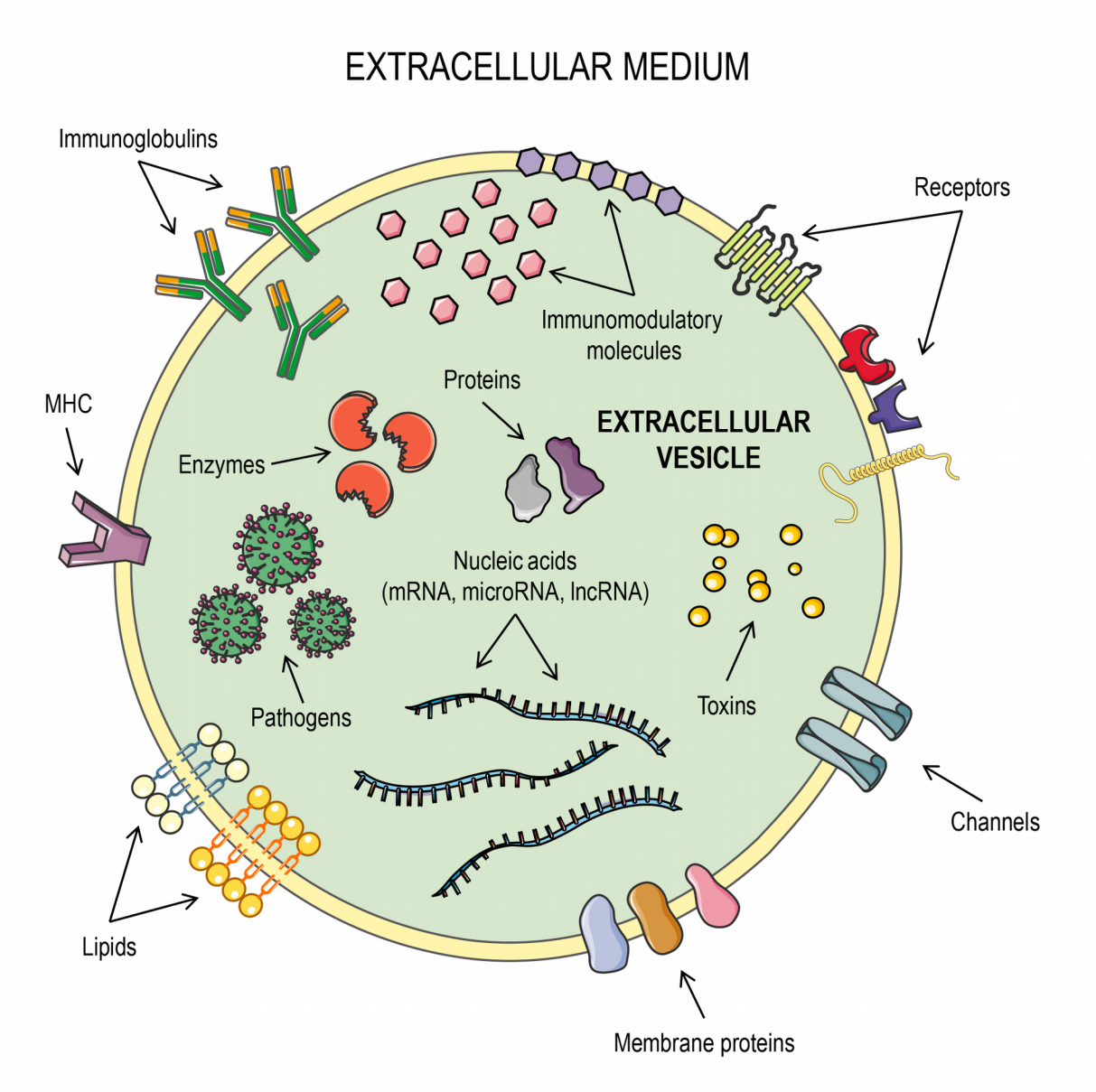

Figure 2. Representation of a hypothetical extracelular vesicle (EV) and its different cargoes. Various types of molecules can be found on the membrane or within EVs released by mammalian cells, including membrane receptors, lipids, intracellular and membrane proteins, membrane channels, toxins, nucleic acids (mRNA, microRNA, lncRNA), enzymes, pathogens, immunoglobulins, MHC, and immunomodulatory molecules. This figure was created using *Servier Medical Art* illustrations (available at https://smart.servier.com, under a Creative Commons Attribution 3.0 Unported License).

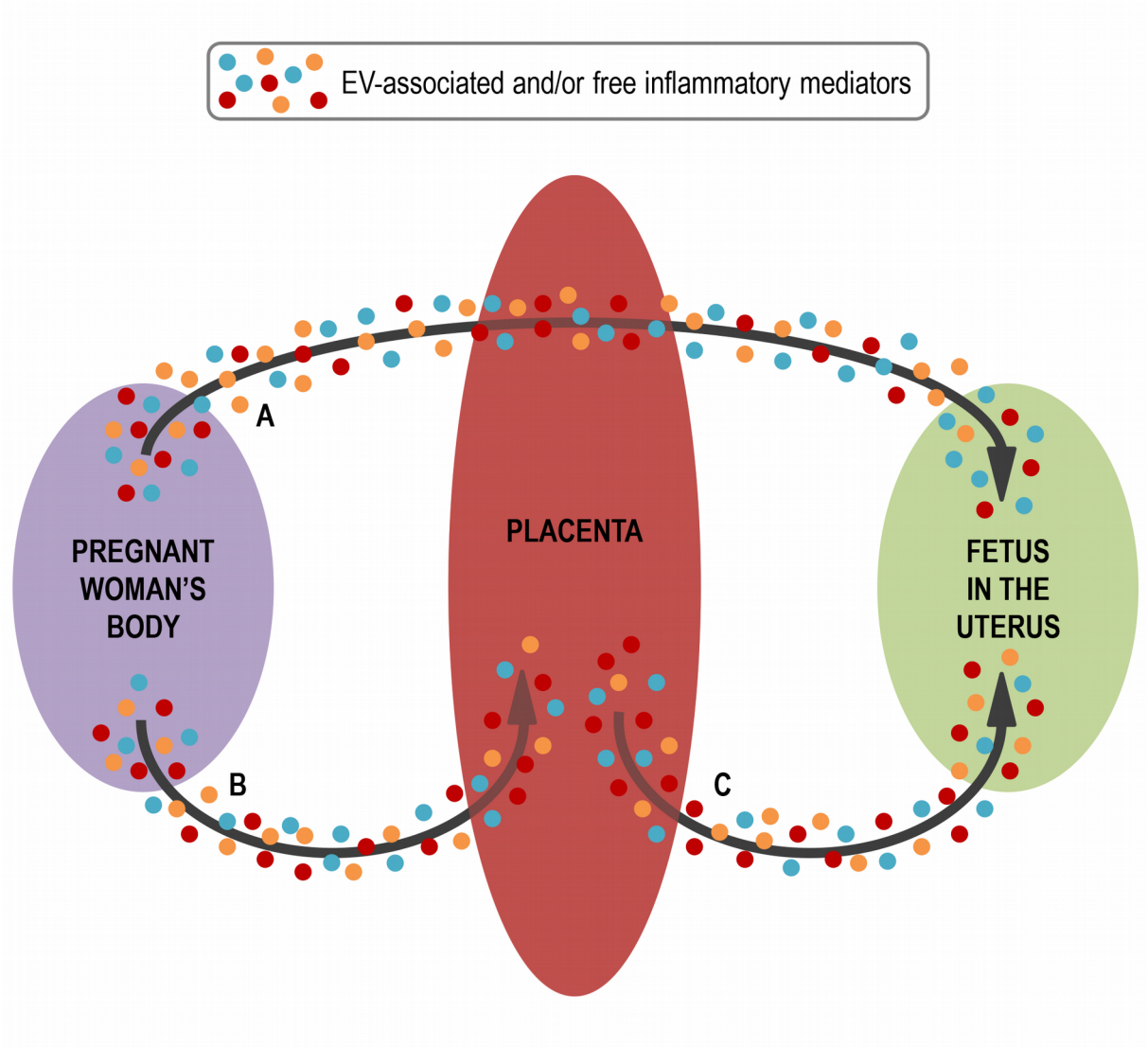

Figure 3. Potential routes of maternal-derived immunomodulatory/inflammatory molecules (Inf-molecules) towards the developing fetus. Inf-molecules may be transported free or coupled to extracellular vesicles (EVs). Inf-molecules/EVs can be directly transported from the maternal circulation to the fetus through the placenta (pathway A). Alternatively, Inf-molecules/EVs can reach the placenta through the maternal circulation (pathway B), stimulating the placenta to release other Inf-molecules/EVs that will exert their effects on the CNS of the developing fetus (pathway C).

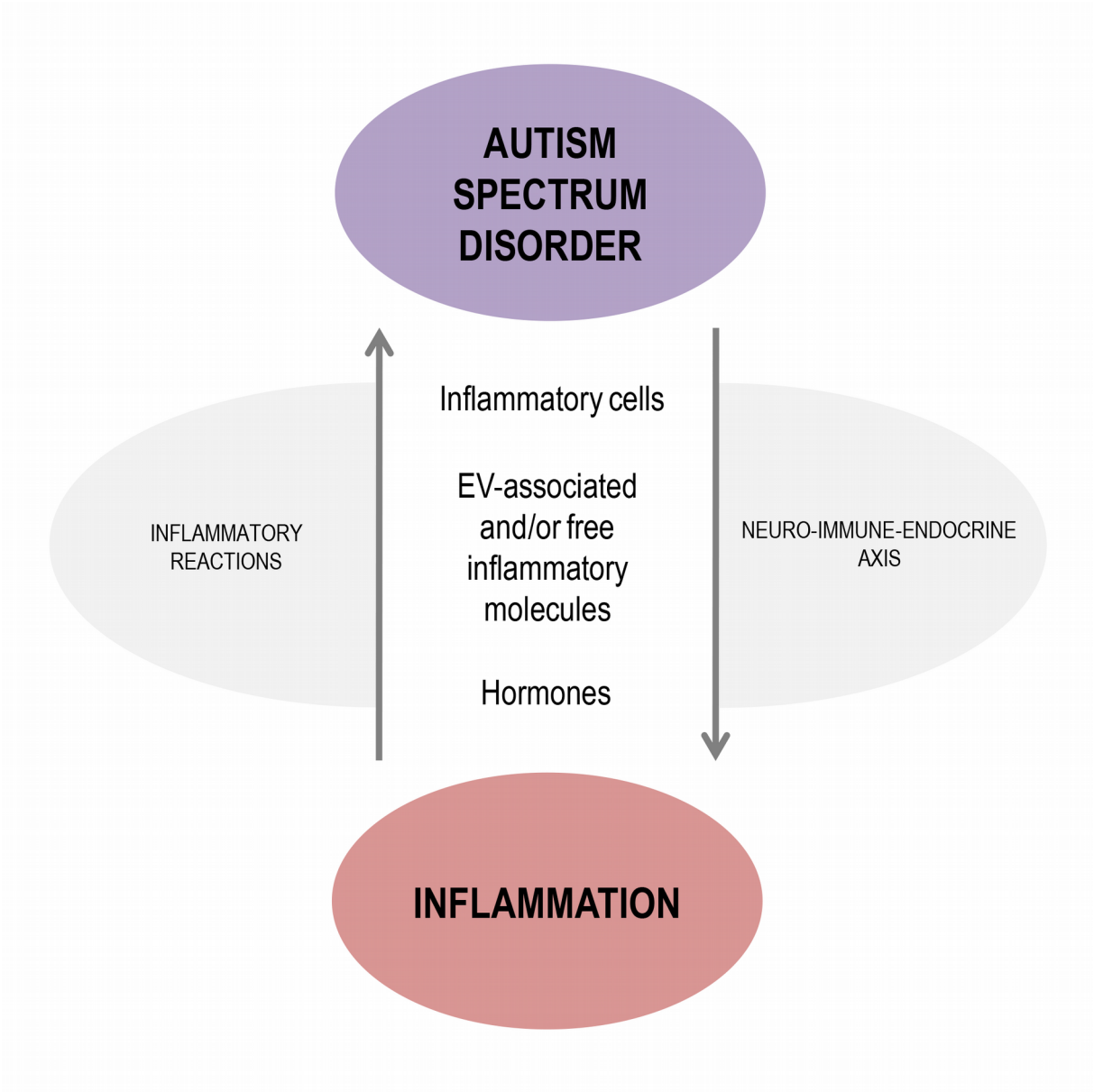

Figure 4. Interplay between autism spectrum disorder (ASD) and inflammation. In addition to the potential influence of inflammation/maternal immune activation on the development of ASD during development of fetal CNS, inflammation is a common feature of individuals with ASD. Inflammation may contribute to the maintenance of the phenotype and symptoms of ASD. Also, inflammation may be a consequence of dysregulations in the neuro-immune-endocrine axis associated with ASD. The relationship between ASD and inflammation can therefore form a feedback loop.

Table 1. Infections during pregnancy or in newborns investigated in the context of ASD.

What was the pathogen and/or the infection investigated?	In which country was the sampling done?	What sampling was used in the study?	How was the information on pathogen exposure obtained?	What were the main findings?	Reference
Rubella virus	USA	Following the 1964 rubella outbreak, 243 children with congenital rubella syndrome were examided.	All samples were part of a follow-up study.	Congenital rubella was associated to autism.	Chess (1971); Chess (1977)
CMV	USA	Mothers of 82 children with ASD symptoms.were tested for CMV IgG and HSV2 IgG in serum.	Samples were tested for CMV IgG and HSV2 IgG in serum.	Maternal CMV infections may influence ASD symptoms.	Slawinski et al. (2018)
Influenza virus	USA	538 children with ASD, 163 with developmental delays, and 421 typically developing controls.	Maternal interviews.	No association was found.	Zerbo et al. (2013)
Influenza virus infection; Vaginal yeast infection; Genital herpes; Labial herpes	Denmark	96,736 children from a population-based cohort where 976 children (1%) were diagnosed with ASD.	Self-reported of the mothers through telephone interviews during pregnancy and early postpartum.	An increased risk of autism in the child after self-reported infection with influenza virus during pregnancy was observed.	Atladóttir et al. (2010)
Influenza, Chickenpox, mumps and rubella infections	USA	163 cases of autism and 355 respective non-diagnosed siblings.	Clinical records and parents' interviews were used for data collection.	Exposure to rubella, mumps and chickenpox during gestation was associated with cases of autism.	Deykin and MacMahon (1979)
Zika virus	Brazil	216 infants followed since the 2015-2016 ZIKV epidemic in Rio de Janeiro.	PCR-confirmed maternal ZIKV infection in pregnancy.	Three children were diagnosed with ASD.	Nielsen-Saines et al. (2019)
Different neurotropic/ polyomaviruses: CMV, EBV, HSV1, HSV2, HHV6, BKV, JCV, and SV40	USA	Postmortem brain tissue from 15 autistic patients and 13 controls.	Nested PCR followed by DNA sequence analysis.	BKV, JCV, and SV40, either singly or in combination, were significantly more frequent in autistic-derived brain tissues.	Lintas et al. (2010)
Two broad categories of infections: organism-specific infections (viral, bacterial, mycosal, parasitic,	USA	The study population was drawn from the Childhood Autism Perinatal Study among the membership of	Data were extracted from Kaiser Perm Northern California clinical databases.	No overall association between diagnoses of any maternal infection during pregnancy and ASD was observed.	Zerbo et al. (2015)

unknown) and organ-specific infections (cardiovascular, ear, eye, gastrointestinal, genitourinary, lower respiratory, upper respiratory, skin, other, unknown).		Kaiser Permanente of Northern California.			
Cytomegalovirus	Japan	Two case reports.	Giant cell analysis in urine and serology for case 1. Serology and PCR test in case 2.	Case 1: Giant cell with intranuclear inclusions characteristic of CMV was found in the urine 2 days after birth. Serum CMV-specific IgM antibodies were positive. Case 2: Serum CMVspecific IgM antibodies were positive, and PCR revealed the presence of CMV-DNA in the urine.	Yamashita et al. (2003)

ASD: Autism Spectrum Disorder; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; BKV: BK virus; HHV6: Human herpesvirus 6; HSV1: Herpes simplex virus 1; HSV2: Herpes simplex virus 2; JCV: JC virus; PCR: polymerase chain reaction; SV40: Simian vacuolating virus 40; USA: United States of America; ZIKV: Zika virus.

Table 2. Experiments showing the transference of factors from maternal blood towards the fetus, based on historical literature.

Method	Main findings	Reference
Red cells tagged with radioactive iron (Fe^{59}) were infused in 7 pregnant woman by autotransfusions.	The infants of 4 of these woman presented radioactive erythrocytes on their bloodstream.	Naeslund et al. (1951)[a]
Transfusion of sickle cell blood was performed in 25 pregnant woman.	Transmission of transfused sickle-trait cells from the mother to the fetus.	Macris et al. (1958)
Erythrocytes from 18 pregnant women were labeled with Cr^{51} and re-injected into these women before delivery.	Of 18 births, radioactive activity was detected in the blood of 13 umbilical cords.	Smith et al. (1961)[a]
Autotransfusions of red blood cells tagged with Cr^{51} were done in 33 pregnant women with signals of preterm delivery.	Transmission of erythrocytes from mother to fetus was observed in 26% of cases.	Zarou et al. (1964)
Elliptocytic blood was infused in 2 pregnant women.	It was observed elliptocytes in the blood of one of the infants along with multiple sites of bleeding and infarction on the placenta.	Hedenstedt and Naeslund (1946)
Red cells tagged with radioactive iron (Fe^{59}) were infused in 29 pregnant women. In addition, 2 other pregnant women received blood from a donor with the sickle cell trait.	From the experiment with Fe^{59}, the infants of 25 women presented radioactive erythrocytes on their bloodstream. Also, sickle cells were observed in the infants of woman who received sickle cells.	Mengert et al. (1955)
Blood cells from 9 pregnant women were collected, exposed to Atabrine dihydrochloride and auto-transfused prior to delivery.	Fluorescent forms were detected in 6 umbilical cords out of the 9 infants born. Besides, fluorescence was detected in granulocytes and platelets in 4 cases and 3 cases present fluorescent lymphocytes.	Desai and Creger (1963)
Erythrocytes tagged with radioactive phosphorus (P^{32}) were injected into 6 pregnant women prior to delivery.	A significant degree of radioactivity was observed in one of the infants.	Naeslund and Nylin (1946)
Placentas from 44 pregnant women with sickle cell were analyzed where sickled erythrocytes served as a marker of maternal blood transferring to the fetus.	Concurrent incidence of sickle cells in maternal and fetal blood was observed in 100% of the cases. The passage of erythrocytes was four times more frequent in umbilical or chorionic veins than in arteries.	Fujikura and Klionsky (1975)

[a] cited by Zarou et al. (1964).

Capítulo VIII

Association between *NKG2* gene variants and epilepsy in Autism Spectrum Disorder

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1. Introduction

Autism Spectrum Disorder (ASD) is a set of neurodevelopmental disorders mainly characterized by repetitive, restrictive and stereotypical behaviors, and impaired communication skills. ASD has been shown to account with a strong genetic component and has a high concordance rate between monozygotic twins, reaching 70-96% (Ronald and Hoekstra, 2011; Bai et al., 2019). Affecting 1 in 42 males and 1 in 189 females, there is a growing incidence of ASD in the modern population (Baio et al., 2018). Many environmental risk factors were also listed as important contributors to ASD, including prenatal injuries such as hypoxia, ischemia, exposure to heavy metals, nutritional deficiency, and maternal immune activation during pregnancy (Modabbernia et al., 2017; Meltzer and Van der Water, 2017).

Several lines of evidence indicate that alterations of the immune system account for ASD development, including the presence of brain-reactive antibodies (Cabanlit et al., 2007), abnormal T cell responses and activation (Yonk et al., 1990; Mostafa et al., 2010), altered cytokine levels in brain, cerebrospinal fluid (CSF) and peripheral blood circulation (Vargas et al., 2005; Jyonouchi et al., 2014; Molloy et al., 2006), increased levels of circulating monocytes (Sweeten et al., 2003), and dysregulation in Natural Killer (NK) cells activity (Enstrom et al., 2009).

Amid the different influences of the immune system in ASD, a significant increase in the number of NK cells in ASD patients in comparison to controls has been described (Enstrom et al., 2009; Ashwood et al., 2011). NK cells are cytotoxic lymphocytes that play a crucial role in the innate immune system. These cells secrete cytokines and modulate the adaptive T cell responses and the function of antigen-presenting cells; they are pivotal for an adequate immune response and can be considered a link between the innate and the adaptive counterparts of the immune system (Long et al., 2013). Regarding ASD, the above-mentioned increase in NK cells number have been suggested as a possible compensatory mechanism as a result of lower cytotoxicity present in NK cells derived from ASD patients (Bjørklund et al., 2016). In this context, a recent study has shown that adults with high-functioning autism have elevated levels of NK cell activation along with other alterations in NK cell functioning, including over-expression of the NKG2C receptor (Benabi et al., 2019).

Considering these alterations described in ASD, the study of genes related to the immune system is an excellent research target both in susceptibility study and in relation to the different clinical manifestations observed in autistic individuals. The natural killer group 2 (NKG2) receptors are a family of C-type lectin-like proteins that can dimerize with CD94 on the cell surface and are expressed mostly in NK cells and also in some subpopulations of T lymphocytes. The different members of this receptor family could induce either suppressive or activating activities (Borrego et al., 2006). In humans, the genes encoding NKG2 receptors are localized at chromosome 12, in the 12p12-13 region and include the following members: *NKG2A*, *NKG2B*, *NKG2C*, *NKG2D*, *NKG2E*, *NKG2F*, and *NKG2H* (Braud et al., 1998; Hikami et al., 2003).

Evidence shows a NKG2C over-expression in NK cells in autistic individuals (Benabi et al., 2019), which led us to investigate the role of complete deletion of *NKG2C* gene in a Brazilian cohort.

NKG2D acts as both an activating as well as a co-stimulatory receptor. It is a type II C-type lectin-like family of transmembrane proteins. Their ligands encompass the UL-16 binding proteins (ULBPs1-4) and the MHC class-I chain-related proteins (MICA and MICB) (Espinoza et al., 2016). Regarding its expression, these receptors are present on NK and γδ-cells, as well as subsets of CD4+ and CD8+ T-cells (González et atl., 2008; Burgess et al., 2008). Despite being usually absent in normal cells, NKG2D ligands undergo rapid up-regulation in cases of physiological insults such as cellular transformation or pathogen infections. NKG2D-ligands interactions triggers cell-mediated cytotoxicity and co-stimulates cytokine production. This receptor-ligand engagement ultimately promotes the elimination of both infected cells and tumors (González et atl., 2008; Burgess et al., 2008). The SNP rs1049174 is located in the 3' untranslated region of the *NKG2D* gene. Using different online algorithms, a study has indicated that the rs1049174 SNP in the NKG2D 3′UTR resides in a conserved region which represents a possible targeting site for miR-1245, a microRNA that was previously reported to downregulate NKG2D expression in NK cells (Espinoza et al., 2012). The SNP rs2255336 is at exon 6 of NKG2D and has been linked to changes in NK cytotoxicity (Hayashi et al., 2006; Iwaszko et al., 2018), and could impact in the ASD immune phenotype.

Taking into account NK cell-related alterations (Enstrom et al., 2009) and differentiated receptor expression of these cells in the context of ASD (Benabi et al., 2019), the present study evaluated the influence of *NKG2C* gene deletion as well as variants on *NKG2D* (rs1049174 and rs2255336) and *NKG2A* (rs2734440) genes in a cohort of southern Brazil composed of 185 children diagnosed with ASD and their respective biological parents.

2. Material and Methods

2.1. Sample

The sample consisted of 185 ASD children and their biological parents. Detailed information on sampling is described in Schuch et al. (2014). Briefly, most family samples were obtained from the Hospital de Clínicas de Porto Alegre (HCPA), the teaching hospital of the Universidade Federal do Rio Grande do Sul (UFRGS) and from the Department of Psychology in the same University. The presence of fragile X syndrome and other genetic syndromes, chromosomal abnormalities and lesional abnormalities of SNC was considered as exclusion criteria. All probands included in the sample were diagnosed as idiopathic ASD cases, according to the DSM-IV and fulfilling the criteria for autistic disorder, Asperger disorder or PDD-NOS, as shown in Table 1. This diagnosis was based on clinical examinations performed by medical professionals in regular appointments (an average of 3–4 appointments) at the Neuropediatric Outpatient Unit from HCPA. This process was always conducted by one neuropediatrician, with a second neuropediatrician participating in the clinical observations and confirming the diagnosis. For all probands, data on the presence or absence of clinical symptoms commonly observed in ASD pa-

tients were also collected. The symptoms evaluated were repetitive behaviors, echolalia, epilepsy (at least 2 unprovoked seizures), mood instability, sleep disorders, aggression (including unprovoked and recurrent aggressive behavior toward self and/or others), psychomotor agitation and sleep disorders. These data were obtained during the regular appointments inquiring the parents and/or caregivers whether the patient exhibited each type of symptom. Of note, the answer was considered positive if the symptom was present before the beginning of the treatment with prescribed drugs.

2.2. Genotyping

The *NKG2C* gene deletion was assessed using conventional PCR according to the methods described by Moraru et al. (2012). Two polymorphisms were investigated in the *NKG2D* gene. The variant rs1049174 (G>C) of *NKG2D* was genotyped using PCR-RFLP. The primers used for amplification were 5'-TTAAGGCTGGAGAATAATGC-3' (Foward) and 5'-TCAGTGAAGGAAGAGAAGG-3' (Reverse). These primers were used to amplify a fragment of 230 base pairs. The amplification parameters were 94°C for 5 min, followed by 35 cycles at 94°C for 30s, 59°C for 30s, and 72°C for 30s, followed by an extension cycle at 72°C for 5 min. PCR products were verified on 2,5% agarose gel. The generated amplicons were digested with the restriction enzyme *Hpy*F3I (*Dde*I) (Thermo Scientific) according to manufacturer's instructions. The rs2255336 (G>C) of *NKG2D* and rs2734440 (A>G) of *NKG2A* were evaluated by real-time PCR using the TaqMan system.

2.3. Statistical analyses

The Chi-square test assesses the deviation from Hardy–Weinberg equilibrium and was also used for association tests between genetic variants and different symptoms presented by the diagnosed individuals (**Table 2**). To analyze whether there was a preference for transmission of allele variants from parents to individuals with ASD, Transmission Imbalance Testing was performed using the Family-Based Association Test (FBAT) software. For correction by multiple tests, Bonferroni Correction were appleid.

3. Results

3.1. Clinical and demographic characteristics

Data from 185 patients were obtained, consisting of 42 informative families for *NKG2C* deletion analyses, 72 for the SNP rs1049174 of *NKG2D*, and 31 families for the SNP rs2255336 of *NKG2D*. The overall information of clinical and demographic data is shown in **Table 1**.

3.2. Allele and genotype frequencies in ASD patients

Both the *NKG2C* gene deletion and the SNPs covered in this study were in Hardy-Weinberg equilibrium. The allele frequencies obtained in the symptomatology analyses are presented in **Tables 2** – **5**.

3.3. FBAT analyses

The family-based analyses of individual SNPs did not detect any association ($p>0.05$; data not shown).

3.4. Associations with clinical symptoms

In this study, association between the *NKG2C* gene deletion and two SNPs in the *NKG2D* gene (rs1049174 and rs2255336) were associated with epilepsy in ASD patients. Regarding *NKG2D* gene deletion, detailed information about the results are shown in **Table 3**. The findings related to the SNPs rs1049174 and rs2255336 are outlined in **Tables 4** and **5**, respectively. No association was found regarding the *NKG2A* SNP rs2734440, as presented in **Table 6**.

4. Discussion

Studies in the ASD context have been showing a dysfunction in NK cells, highlighting the increase in activation (López-Cacho et al., 2016; Bennabi et al., 2019), spontaneous degranulation, and interferon-gamma (IFN-γ) production (Bennabi et al., 2019). Noteworthy, decreased cytotoxicity activity in these cells derived from ASD patients have also been reported (Warren et al., 1987; Vojdani et al., 2008). Enstrom et al., (2009) also found a significantly decrease of NK cell cytotoxicity, in addition to an increase expression of NK receptors and immune molecules like IFN-γ, granzyme B and perforin. A significant increase in NK cells number in ASD patients in comparison to controls have been described (Enstrom et al., 2009; Ashwood et al., 2011), but others studies did not detect this variation (Basheer et al., 2018; López-Cacho et al., 2016).

NKG2C overexpression has been demonstrated in adults with high-functioning ASD (hf-ASD) in comparison with age-matched controls, remaining equal in the 2-year follow-up period (Bennabi et al., 2019). Allele deletion of *NKG2C* (*NKG2C* del) appears to be common in general population, with an allele frequency of 20.2%, 20%, 10.3%, 20.9%, and 33.2% in Japanese, Dutch, Mexican, East- and West-African populations, respectively (Miyashita et al., 2004; Rangel-Ramírez et al., 2014; Goncalves et al., 2016). In Brazil, our group has found a similar frequency of the *NKG2C* allele deletion (20.2%) reported by most of these populations (Kaminski et al., 2019).

The NKG2 member C (NKG2C) protein is codified by *NKG2C* (also called *KLRC2*) gene and acts in both innate and adaptive NK cell pathways. NKG2C has been linked with antiviral responses, es-

pecially infection by human cytomegalovirus (HCMV) (Noyola et al., 2012; Malmberg et al., 2012), playing an adaptive role by specific recognition of HCMV-derived peptides (Hammer et al., 2018), culminating in NK cell activation (Houchins et al., 1997). Despite the suggested association with some viral infection, this increased NKG2C expression was not associated with HCMV and 23 other pathogens seropositivity; no statistically difference in NKG2C expression between HCMV seronegative and seropositive with ASD patients was found. Furthermore, the NK cells derived from hf-ASD patients showed an increased expression of the specific $CD56^{dim}HLA\text{-}DR^{+}NKG2C^{high}KIR2DL1^{+}$ profile, along with higher levels of NK activation (Bennabi et al., 2019). Presence of NKG2C overexpression together and high activation levels of NK cells with the $NKG2C^{high}$ profile suggest an association between ASD manifestation and underlying pathogens.

Congenital infections have been linked with ASD manifestation (Hadjkacem et al., 2016; Ornoy et al., 2015). Besides, the presence of anti-HCMV antibodies was found significantly more frequent in ASD patients (Kawashti et al., 2006; Ornoy et al., 2015); although other studies with bigger sample size did not corroborate such difference (Gentile et al., 2014; Singh 2008; Mora et al. 2009). Otherwise, Gentile et al. found a non-statistically significant trend between HCMV seropositive profile and major values in a severity scale of ASD traits. The impact of viral infections in ASD still is inconclusive, but the above-mentioned findings regarding NKG2C highlight a possible viral-related factor in ASD manifestation. However, this pattern may be a reflection of immune dysfunctions, and deserves further investigations.

The NKG2 member D (NKG2D) is another activating receptor which is encoded by the *NKG2D* gene (or *KLRK1*). In humans, NKG2D is constitutively expressed as a single full-length isoform of a disulfide-bonded homodimer on the cell surface of NK cells and CD8+ T cells. This receptor differs from the others NKG2 because it is expressed along with a hexameric complex with two homodimers of the DAP10 signaling protein. The NKG2D receptor recognizes two classes of ligands: MIC (MICA and MICB), and ULBP (ULBP1–ULBP6), and binds to ligands that are upregulated and presented on the surface of cells undergoing stress, excessive proliferation, or transformation. Such binding allows both NK cells and T cells to recognize and kill these abnormal cells and also to secrete cytokines that ultimately enhance the immune response (Lanier, 2015).

Of note, the inhibitory receptor NKG2 member A (NKG2A), codified by *KLRC1* gene, showed a 10-fold higher affinity by HLA-E in comparison to NKG2C (Borrego et al., 2006), being an important neuromodulatory molecule. It is an inhibitory receptor expressed on $CD56^{hi}$ NK cells, natural killer T (NKT) cells, and in a subset of CD8+ αβ T cells. Neuroinflammation has shown to be an important component in ASD manifestation. Microglia and astroglia activation, and increase in cytokines and chemokines in neuroenvironment (Vargas et al., 2005; Matta et al., 2019) show a probably immune-related brain damage in ASD. Recently, an increase of perivascular lymphocytic cuffs was found in brain tissues of ASD patients, showing signatures of immune cellular responses (DiStasio et al., 2019). Gut dysbiosis also seems to have an impact in immune dysfunctions found in ASD, with an intestinal microbiome dysregulation described (Matta et al., 2019). Despite these findings, no influence of genetic vari-

ants was found in the phenotypes assessed in this study. Further studies should be conducted to identify whether these patterns are associated with ASD, as a cause or a consequence of this disorder.

Our findings indicate an influence of the *NKG2C* gene deletion and two *NKG2D* SNPs in episodes of epilepsy in ASD patients. Supporting our results, reduced NK cell activity in epileptic patients has already been reported (Wang et al., 1989). Also, an increase in NK cell numbers has been reported in measurements performed up to 24 hours after epilepsy episodes (Bauer et al., 2008).
In the context of ASD, studies have already shown NK cell-related changes in diagnosed children, where cell counts in the peripheral circulation of children with autism can reach levels up to 40% higher compared to typically developing children (Warren et al. al., 1987; Ashwood et al., 2011; Vojdani et al., 2008; Enstrom et al., 2009). Despite the high number of ASD patients-derived cells under *in vitro* stimulation, the expected activation of such NKs derived was not observed (López-Cacho et al., 2016). Given all these data, it can be suggested that the high number of NKs cells may reflect their low activity, where a compensatory mechanism is established. Finally, it is noteworthy that the presence of *NKG2C* deletion, which encodes an activating receptor of NK cells, is associated with the presence of epilepsy in the clinical picture of ASD.

5. Conclusion

Immune receptors play an essential role in immune cells, and are the focus of many studies. The evaluation of genetic variants in immune modulators, as well as the investigation of changes in their activity or expression can reveal important findings regarding the impact of these molecules in ASD. The consequences of immune cells and their receptors in the immune alterations observed in ASD still needs to be better understood, along with the potential for new immunotherapeutic treatments to manage ASD symptoms. In summary, and considering the role of the NKG2 receptors in immune regulation, we confirm the importance and the influence of genetic variants in *NKG2C* and *NKG2D* genes in ASD, especially regarding the epilepsy phenotype.

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Table 1. Clinical and demographic data of ASD patients (n=185)

Gender (male)[a]		79.7
Age[b]		9.67 ± 5.11
Ethnicity[a,c]	European	75.7
	Non-European	24.3
ASD diagnosis[a]	Autistic disorder	46.6
	Asperger disorder	7.9
	PDD-NOS	32.0
	Autistic traits	12.4
	Others	1.1
Symptoms[a,d]	Hyperactivity	61.1
	Heteroagression	37.8
	Convulsion	24.9
	Epilepsy	11.4
	Autoagression	45.9
	Panic attacks	30.3
	Mood instability	49.7
	Repetitive moviments	76.2
	Sleep problems	57.3
	Echolalia	61.1
ASQ (n=159)[b]		21.98 ± 5.62
CARS (n=111)[b]		35.7 ± 5.53

ASD: Autism Spectrum Disorders; PDD-NOS: pervasive development disorder-not otherwise specified; ASQ: Autism Screening Questionnaire; CARS: Childhood Autism Rating Scale.

[a] % (n).

[b] Mean ± SD (n=159)

[c] Amerindian and Asian ethnicities were also present in the sample.

[d] The informed percentages and numbers refer to patients presenting each symptom. The different symptoms are not mutually exclusive.

Table 2. Associations between *NKG2C* gene deletion and ASD-related symptoms (n=182)

Symptom	Genotype	Presence, n (%)	Absence, n (%)	OR (CI 95%)	*p*	p_c
Epilepsy	wt/wt type	8 (38.1)	122 (75.8)	OR 5.08 (1.96-13.17)	**0.001**	**0.010**
	wt/del + del/del	13 (61.9)	39 (24.2)			
Sleep disorders	wt/wt type	68 (64.8)	62 (80.5)	OR 2.25 (1.13-4.49)	**0.021**	0.210
	wt/del + del/del	37 (35.2)	15 (19.5)			
Mood instability	wt/wt type	57 (64.8)	73 (77.7)	OR 1.89 (0.98-3.63)	0.071	0.710
	wt/del + del/del	31 (35.2)	21 (22.3)			
Hetero-aggressive behavior	wt/wt type	47 (69.1)	83 (72.8)	OR 1.20 (0.62-2.31)	0.614	1.000
	wt/del + del/del	21 (30.9)	31 (27.2)			
Self-aggressive behavior	wt/wt type	59 (68.6)	71 (74.0)	OR 1.30 (0.68-2.48)	0.511	1.000
	wt/del + del/del	27 (31.4)	25 (26.0)			
Echolalia	wt/wt type	82 (73.2)	48 (68.6)	OR 0.80 (0.41-1.54)	0.505	1.000
	wt/del + del/del	30 (26.8)	22 (31.4)			
Hyperactivity	wt/wt type	75 (67.0)	55 (78.6)	OR 1.81 (0.90-3.62)	0.129	1.000
	wt/del + del/del	37 (33.0)	15 (21.4)			
Repetitive Behavior	wt/wt type	96 (70.1)	34 (75.6)	OR 1.32 (0.61-2.86)	0.570	1.000
	wt/del + del/del	41 (29.9)	11 (24.4)			
Convulsion	wt/wt type	30 (65.2)	100 (73.5)	OR 1.48 (0.72-3.03)	0.345	1.000
	wt/del + del/del	16 (34.8)	36 (26.5)			
Panic	wt/wt type	35 (64.8)	95 (74.2)	OR 1.56 (0.79-3.01)	0.213	1.000
	wt/del + del/del	19 (35.2)	33 (25.8)			

Table 3. Associations between the *NKG2D* rs1049174 SNP and ASD-related symptoms (n=185)

Symptom	Genotype	Presence, n (%)	Absence, n (%)	OR (CI 95%)	*p*	p_c
Epilepsy	GG	4 (19.0)	86 (52.4)	OR 4.69 (1.51-14.53)	**0.005**	**0.050**
	GC + CC	17 (81.0)	78 (47.6)			
Hyperactivity	GG	50 (44.2)	40 (55.6)	OR 1.58 (0.87-2.86)	0.174	1.000
	GC + CC	63 (55.8)	32 (44.4)			
Mood instability	GG	39 (42.4)	51 (54.8)	OR 1.65 (0.92-2.95)	0.106	1.000
	GC + CC	53 (57.6)	42 (45.2)			
Hetero-aggressive behavior	GG	36 (51.4)	54 (47.0)	OR 0.84 (0.46-1.52)	0.649	1.000
	GC + CC	34 (48.6)	61 (53.0)			
Self-aggressive behavior	GG	39 (45.9)	51 (51.0)	OR 1.23 (0.69-2.19)	0.555	1.000
	GC + CC	46 (54.1)	49 (49.0)			
Echolalia	GG	56 (49.6)	34 (47.2)	OR 0.91 (0.50-1.65)	0.765	1.000
	GC + CC	57 (50.4)	38 (52.8)			
Sleep disorders	GG	51 (48.1)	39 (49.4)	OR 1.05 (0.59-1.88)	0.883	1.000
	GC + CC	55 (51.9)	40 (50.6)			
Repetitive Behavior	GG	68 (48.2)	22 (50.0)	OR 1.07 (0.54-2.11)	0.864	1.000
	GC + CC	73 (51.8)	22 (50.0)			
Convulsion	GG	20 (43.5)	70 (50.4)	OR 1.32 (0.67-2.58)	0.497	1.000
	GC + CC	26 (56.5)	69 (49.6)			
Panic	GG	28 (50.0)	62 (48.1)	OR 0.92 (0.49-1.73)	0.873	1.000
	GC + CC	28 (50.0)	67 (51.9)			

Table 4. Associations between the *NKG2D* rs2255336 SNP and ASD-related symptoms (n=91)

Symptom	Genotype	Presence, n (%)	Absence, n (%)	OR (CI 95%)	*p*	p_c
Epilepsy	GG	1 (10.0)	52 (64.2)	OR 16.14 (1.95-133.82)	**0.001**	**0.01**
	GA + AA	9 (90.0)	29 (35.8)			
Convulsion	GG	12 (48.0)	41 (62.1)	OR 1.78 (0.70-4.50)	0.243	1.000
	GA + AA	13 (52.0)	25 (37.9)			
Mood instability	GG	19 (47.5)	34 (66.7)	OR 2.21 (0.94-5.18)	0.087	0.87
	GA + AA	21 (52.5)	17 (33.3)			
Hetero-aggressive behavior	GG	19 (54.3)	34 (60.7)	OR 1.30 (0.55-3.06)	0.663	1.000
	GA + AA	16 (45.7)	22 (39.3)			
Self-aggressive behavior	GG	25 (56.8)	28 (59.6)	OR 1.12 (0.49-2.58)	0.834	1.000
	GA + AA	19 (43.2)	19 (40.4)			
Echolalia	GG	35 (55.6)	18 (64.3)	OR 1.44 (0.57-3.61)	0.495	1.000
	GA + AA	28 (44.4)	10 (35.7)			
Sleep disorders	GG	30 (54.5)	23 (63.9)	OR 1.47 (0.62-3.49)	0.395	1.000
	GA + AA	25 (45.5)	13 (36.1)			
Repetitive Behavior	GG	40 (60.6)	13 (52.0)	OR 0.70 (0.28-1.78)	0.484	1.000
	GA + AA	26 (39.4)	12 (48.0)			
Hyperactivity	GG	25 (53.2)	28 (63.6)	OR 1.54 (0.66-3.57)	0.396	1.000
	GA + AA	22 (46.8)	16 (36.4)			
Panic	GG	12 (48.0)	41 (62.1)	OR 1.78 (0.70-4.50)	0.243	1.000
	GA + AA	13 (52.0)	25 (37.9)			

Table 5. Associations between the *NKG2A* rs2734440 SNP and ASD-related symptoms (n=74)

Symptom	Genotype	Presence, n (%)	Absence, n (%)	OR (CI 95%)	*p*	p_c
Epilepsy	AA	1 (20.0)	34 (49.3)	OR 3.89 (0.41-36.56)	0.361	1.000
	AG + GG	4 (80.0)	35 (50.7)			
Sleep disorders	AA	20 (46.5)	15 (48.4)	OR 1.08 (0.43-2.72)	1.000	1.000
	AG + GG	23 (53.5)	16 (51.6)			
Mood instability	AA	12 (36.4)	23 (56.1)	OR 2.24 (0.87-5.72)	0.106	1.000
	AG + GG	21 (63.6)	18 (43.9)			
Hetero-aggressive behavior	AA	12 (42.9)	23 (50.0)	OR 1.33 (0.52-3.43)	0.634	1.000
	AG + GG	16 (57.1)	23 (50.0)			
Self-aggressive behavior	AA	15 (42.9)	20 (51.3)	OR 1.40 (0.56-3.51)	0.494	1.000
	AG + GG	20 (57.1)	19 (48.7)			
Echolalia	AA	23 (45.1)	12 (52.2)	OR 1.33 (0.50-3.56)	0.621	1.000
	AG + GG	28 (54.9)	11 (47.8)			
Hyperactivity	AA	16 (42.1)	19 (52.8)	OR 1.54 (0.61-3.85)	0.485	1.000
	AG + GG	22 (57.9)	17 (47.2)			
Repetitive Behavior	AA	26 (49.1)	9 (42.9)	OR 0.78 (0.28-2.16)	0.797	1.000
	AG + GG	27 (50.9)	12 (57.1)			
Convulsion	AA	5 (27.8)	30 (53.6)	OR 3.00 (0.94-9.54)	0.065	0.650
	AG + GG	13 (72.2)	26 (46.4)			
Panic	AA	7 (36.8)	28 (50.9)	OR 1.78 (0.61-5.19)	0.425	1.000
	AG + GG	12 (63.2)	27 (49.1)			

Capítulo IX

Discussão geral e Conclusão

DISCUSSÃO GERAL E CONCLUSÃO

Este trabalho apresentou aspectos da imunologia da gestação humana e discutiu sobre a ligação de distúrbios gestacionais com o risco de desenvolvimento de Transtorno do Espectro Autista em crianças nascidas de mães que tiveram gestações com problemas relacionados à inflamação. Diante dos diferentes estudos revisados para comporem os trabalhos aqui apresentados, os dois tópicos a seguir norteiam o fechamento da presente tese.

Sobre os paradigmas imunológicos da gestação

Conforme resumido na Figura 1, a literatura sobre imunotolerância da mãe em relação ao feto é permeada de conceitos que direcionam o pensamento para uma visão simplista que não representa a dinâmica fisiológica de uma gestação. O equilíbrio imune estabelecido na interface materno-fetal vai muito além do "paradigma "Th1/Th2/Th17 e Treg" proposto por Saito et al. (2010). Além disso, nem sempre é possível acessar o *status* imune de uma gestação por medições de fatores presentes no plasma sanguíneo materno.

Porém, é importante ressaltar que, apesar de apresentarem limitações, os estudos que têm avaliado moléculas solúveis presentes na circulação das gestantes são de grande valia. Inclusive, são abordagens fundamentais para que se tenha uma visão mais detalhada da complexidade existente nas relações imunológicas entre a mãe e o feto em desenvolvimento. Como apresentado no Capítulo II, a descoberta do papel desempenhado por vesículas extracelulares na promoção de um ambiente imunossupressor em prol do feto agrega um grau de complexidade sem precedentes ao fenômeno imunológico da gestação. No mesmo capítulo e também na Tabela 1, diferentes moléculas do sistema imune que impactam no desfecho de uma gravidez foram apresentadas, e seus papéis na gestação, brevemente discutidos.

Dentre as novidades evolutivas dos mamíferos, destaca-se a placenta como um órgão transitório com importantes funções imunológicas. Nos humanos, além de constantemente balizar os possíveis ataques por linfócitos maternos ativados e de promover adequadas nutrição e oxigenação para o feto em desenvolvimento, a placenta apresenta características essenciais que evitam infecções no ambiente uterino. A dinâmica das respostas imunes que permeiam a gestação é enriquecida ao passo que analisamos, concomitantemente, as estratégias apresentadas pelos patógenos que, em última instância, podem burlar as defesas presentes na barreira placentária. Ainda, a placenta produz e secreta ativamente diferentes tipos de vesículas extracelulares, a ponto

de já ter sido proposto o estabelecimento de uma “nuvem de exossomos” na interface materno-fetal (Mincheva-Nilson, 2010; Mincheva-Nilsson e Baranov, 2010). No artigo que compõe o Capítulo II, também foram apresentadas as potenciais influências dessas vesículas em ambas infecções transplacentárias e sexualmente transmissíveis (Kaminski et al., 2019a).

Conforme discutido nos Capítulos II e VII, infecções durante a gestação podem apresentar riscos consideráveis para a mãe e para o feto. Além das questões envolvendo infecções, a placenta pode estar relacionada a outras intercorrências gestacionais, que também envolvem fatores imunológicos e oferecem risco para a gestante e para o feto. Nesse contexto, o Capítulo V abordou dois fatores genéticos com potencial de influenciar uma importante doença gestacional, a pré-eclâmpsia. Esse quadro clínico tem caráter multifatorial e acomete de 2 a 8% das mulheres gestantes e tem como principais diagnósticos a presença de hipertensão gestacional *de novo* e proteinúria (Michita et al., 2018). Por ser uma doença complexa, muitas variantes em diferentes genes vêm sendo investigadas no contexto da pré-eclâmpsia e, nesta tese, foram investigadas a deleção completa do gene *NKG2C* e a variante CCR5Δ32, presente no gene *CCR5*. A associação da variante CCR5Δ32 com pré-eclâmpsia foi corroborada, visto que um estudo prévio do Laboratório de Imunobiologia e Imunogenética da UFRGS já havia associado tal variante com menor incidência de pré-eclâmpsia em outro grupo amostral (Telini et al., 2014). Além disso, esse foi o primeiro estudo avaliando a deleção do gene *NKG2C* na população brasileira (Kaminski et al., 2019b).

Diferentes fatores imunológicos são investigados no contexto de problemas relacionados à gestação. Nesta tese, foi avaliado o perfil de citocinas de gestantes e de mulheres que sofreram aborto espontâneo e os níveis dessas moléculas foram comparados nos dois grupos. Conforme os resultados apresentados no Capítulo IV, a única diferença com significância estatística observada foi o aumento de Interleucina 17A (IL-17A) nas gestantes em comparação com os casos de aborto (Kaminski et al., 2018). Os resultados obtidos estão em acordo com dados prévios (Martínez-García et al., 2011) e vão ao encontro do já proposto papel da IL-17 como uma molécula atuante na manutenção de períodos gestacionais prolongados (Chavan et al., 2017). Estudos no contexto de perdas gestacionais são extremamente necessários, visto que cerca de 20% das gestações resultam em aborto espontâneo idiopático (Everett, 1997). Esse estudo foi motivado por evidências que indicam que fatores imunológicos podem influenciar nos casos de aborto espontâneo sem causas definidas (citadas em Kaminski et al., 2019a).

A molécula imunotolerogênica HLA-G possui grande impacto na gestação, e variantes no gene que a codifica têm sido alvo de investigação no contexto de aborto (Michita et al., 2016). Considerando esses aspectos, no Capítulo III é apresentada uma estratégia de contracepção baseada na diminuição da expressão do gene *HLA-G*. A contracepção seria efetivada com a metodologia

apresentada, com base no fato de que HLA-G atua na promoção de tolerância imunológica necessária ao estabelecimento de uma gestação. Além disso, essa molécula é importante tanto para a manutenção da tolerância quanto para a promoção da vascularização que garante a fixação do embrião na decídua.

Sobre sistema imune e Transtorno do Espectro Autista

A segunda parte deste trabalho abordou o papel de fatores imunogenéticos e da inflamação no Transtorno do Espectro Autista (TEA), caracterizado por dificuldades na comunicação e socialização e por comportamento restritivo e repetitivo (O'Hare, 2009). O Capítulo VI é composto por uma revisão de variantes em genes relacionados ao sistema imune que já foram estudados no TEA. Como já discutido, não se sabe a causa exata desse transtorno do desenvolvimento, apesar de já estar estabelecida a contribuição de fatores genéticos e ambientais de forma conjunta. Os genes abordados foram agrupados em três diferentes grupos (relacionados às respostas inflamatórias, relacionados ao MHC e relacionados ao imunometabolismo). Foi observado um maior volume de variantes investigadas e associadas a genes do imunometabolismo, sugerindo que as alterações imunogenéticas no contexto do TEA, embora com pequeno impacto, estão envolvidas em alterações constitutivas na fisiologia dos pacientes.

Nos Capítulos VI, VII e VIII também é extensamente discutido o papel da inflamação no TEA. Respostas inflamatórias alteradas são observadas em indivíduos diagnosticados e são potenciais contribuintes para as diferentes manifestações clínicas da doença (Masi et al., 2017; Bennabi et al., 2019). A ativação imune materna tem sido bastante discutida na literatura como um importante fator de risco para o TEA (Meltzer and Van de Water, 2017). No Capítulo VII, uma abordagem propondo a conexão entre ativação imune materna, TEA e vesículas extracelulares foi apresentada. As bases para tal proposta são a participação de vesículas extracelulares na gestação (tanto com ou sem intercorrências), a observação de perfis alterados dessas vesículas em pacientes com TEA (Tsilioni e Theoharides, 2018) e o potencial imunomodulador dessas vesículas (Kaminski et al., 2019a). É importante destacar que, apesar do provável papel de exposição pré-natal à inflamação como gatilho pro TEA, os fatores inflamatórios podem ser consequência, e não causa, da manifestação do TEA.

Na mesma linha do que foi dito anteriormente, o Capítulo VIII apresenta um estudo inspirado em um trabalho prévio que demonstrou expressão diferenciada do receptor NKG2C em células NKs de indivíduos adultos com TEA (Benabi et al., 2019). Assim, analisamos a deleção completa do gene *NKG2C* e SNPs nos genes *NKG2D* e *NKG2A* em indivíduos com TEA e seus respectivos pais biológicos. Os resultados preliminares desse estudo indicam associação da presença

da deleção do gene *NKG2C* e dois SNPs em *NKG2D* com um mesmo sintoma: epilepsia. Corroborando este estudo, atividade reduzida de células NKs em pacientes epiléticos já foi reportada (Wang et al., 1989). Ainda, aumento no número de células NKs foi reportado em medições realizadas até 24h após episódios de epilepsia (Bauer et al., 2008).

No contexto do TEA, estudos já demonstraram alterações relacionadas a células NKs em crianças diagnosticadas, onde a contagem dessas células na circulação de crianças com autismo de alto e baixo desempenho pode alcançar níveis até 40% maiores em comparação com crianças de desenvolvimento típico (Warren et al., 1987; Ashwood et al., 2011; Vojdani et al., 2008; Enstrom et al., 2009). Apesar do número elevado de células, sob estimulação *in vitro*, não observou-se a ativação esperada das NKs derivadas das crianças com TEA (López-Cacho et al., 2016). Diante de todos esses dados, pode-se sugerir que o número elevado de células NKs pode refletir sua baixa atividade, de forma que se estabelece um mecanismo compensatório. Por fim, destaca-se que o último capítulo desta tese agrega a esta discussão um dado importante: a presença da deleção de um gene que codifica um receptor ativatório de células NK está associada com a presença de epilepsia no quadro clínico do TEA.

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