UNIVERSIDADE FEDERAL DE PERNAMBUCO

LABORATÓRIO DE IMUNOPATOLOGIA KEIZO ASAMI

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA APLICADA À SAÚDE

Associação do polimorfismo da óxido nitrico endotelial (eNOS) T-786C com moléculas do metabolismo lipídico e inflamatório em amostras de mulheres grávidas

Rafaella Adalgisa Silva do Nascimento

Recife,

2013

RAFAELLA ADALGISA SILVA DO NASCIMENTO

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde da Universidade Federal de Pernambuco, como requisito para a obtenção do título de Mestre em Biologia Aplicada à Saúde.

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CRB 1728

Nascimento, Rafaella Adalgisa Silva do
Associação do polimorfismo da óxido nítrico endotelial (eNOS) T-786C com moléculas do metabolismo lipídico e inflamatório em amostra de mulheres grávidas/ Rafaela Adalgisa Silva do Nascimento – Recife: O Autor, 2013.

102 folhas : il., fig., tab.
Orientadora: Danyelly Bruneska Gondim Martins
Dissertação (mestrado) – Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Biologia Aplicada à Saúde, 2013.
Inclui bibliografia e anexos

1. Gravidez 2. Proteína c-reativa 3. Bioinformática I. Martins, Danyellly Bruneska Gondim Martins (orientadora) II. Título

612.63 CDD (22.ed.) UFPE/CCB- 2013- 347

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A comissão examinadora, composta pelos professores abaixo, sob a presidência do ultimo, considera a candidata Rafaella Adalgisa Silva do Nascimento como:

Recife, 30 de Setembro de 2013.

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Prof. Dr. Luiz Bezerra de Carvalho Júnior

Dedico esse trabalho aos meus pais que me apoiaram em todos os sentidos, com muito amor e paciência. E ao meu irmão, pois trabalho todos os dias por ele.

AGRADECIMENTOS

Agradeço primeiramente a Deus, por todas as bênçãos que me concedeu em toda a vida, pelo amor, compreensão e oportunidades de sempre recomeçar.

Agradeço imensamente aos meus pais por todo suporte que me deram, por toda paciência, amor e carinho. Por terem paciência com minhas chatices e sempre me estenderem a mãe pra tudo. Obrigada pelo meu irmão Igor Matheus, a quem destinamos todo nosso sacrifício, amor e cuidados. Amo muito vocês.

Muito obrigada ao professor José Luiz de Lima Filho, por abrir as portas do LIKA e ter depositado confiança em mim e nos meus propósitos de estudo.

Muito obrigada aos professores componentes da banca Dilenia de Oliveira Cipriano e Joao Ricardo de Oliveira pelas contribuições dadas a esse trabalho com intuito de melhorá-lo.

Ao professor Luiz Carvalho, coordenador da pós-graduação, pelos bons exemplos e por seu bom humor inigualável que contagia e acalma a todos.

À Danyelly Bruneska Gondim Martins, minha orientadora, por ser esse exemplo de pesquisadora e professora. Por estar sempre ao lado dos alunos, acompanhado passo a passo nosso desenvolvimento. Você já é uma grande profissional, mas tenho certeza que ainda brilharás muito.

Aos companheiros do grupo Prospecmol, que compartilham todas as experiências no mundo da pesquisa.

A minha amiga Roberta Ventura, por toda amizade, carinho e consideração, por ter ficado ao meu lado dividindo as alegrias e tristezas nesses dois anos que passamos juntas. Muito obrigada Remosa.

A Paula Virgínia, que nem tenho palavras para expressar o quanto sou grata por sua amizade. Admiro demais você amiga, você foi meu amparo e meu consolo nos momentos mais difíceis.

A minha amiga Natalia Oliveira, por sua alegria constante, sua solidariedade e seu carinho que me cativaram tanto. Obrigada pela confiança e por tudo que fizeste por mim.

A Marek Henrique, meu amigo desde o primeiro dia em que cheguei no LIKA. Obrigada por ter sido tão companheiro nesses 2 anos de amizade.

A Kleyton Borges, a patativa, por dividir comigo as tarefas nesse projeto e por me auxiliar e ensinar tanto na formatação desse trabalho.

A Rodrigo Heráclio, principalmente pela formatação das networks e ajuda na parte de bioinformática, mas também por toda paciência e consideração.

A Carla Mola, por todo apoio, solidariedade e paciência que me dedicou nesses poucos meses que nos conhecemos. Obrigada por tudo Carlete.

A Fernando Costa, que me dedicou o amor mais bonito. Obrigada por esse tão especial em minha vida. E a sua família, Graciete e Nadir por se emprestarem como minha também, cuidado tanto de mim. Amo vocês.

A todos os amigos da faculdade Ana Paula, Barbara, Cledna, Ivana, Layanne, Mariana, Henrique, Felipe Torquato e Ricardo Victor que fizeram mais anos de graduação mais felizes e por tantos aprendizados que passamos juntos.

Aos demais amigos de funcionários do LIKA, Eliete, Ilma, Verinha, Edson, Rafael Padilha pela presteza de todos os dias.

Por todo apoio, carinho e lições de vida que cada um me destinou: Muito Obrigada!

“Paciência e Ação

... abracemos o caminho que o Mestre nos aponta, embora muitas vezes, sentindo os ombros agoniados, sob a cruz das responsabilidades crescentes.
Não vacilemos, porém.
Associando paciência e ação, brandura e energia – e às vezes mais energia na brandura,
Sigamos em frente, convencidos de que o senhor não nos desampara. Recordemo-lo sozinho e desfalecente, mas sereno e valoroso e prossigamos de consciência erguida na paz do dever do cumprido. “

Dr. Bezerra de Menezes por Chico Xavier.

Resumo

A gestação é um fenômeno fisiológico que se inicia com a fecundação e progride no sentido de promover um ambiente adequado onde o feto possa crescer e formar um novo indivíduo com todo seu potencial genético expresso. Durante este período, os níveis lipídicos normalmente aumentam para permitir a manutenção da homeostase entre mãe e feto. Níveis lipídicos anormais estão relacionados com disfunção endotelial, reduzindo a produção de óxido nítrico (NO) e causando complicações para mãe e para o desenvolvimento fetal. O NO é principal regulador de eventos feto-placentários sendo produzido a partir da ação de 3 isoformas da óxido nítrico sintase (NOS). Polimorfismos de base única (SNPs) na NOS endotelial (eNOS) tem sido correlacionados à diversas patologias, sendo T-786C um dos SNPs responsáveis por reduzir a expressão da eNOS em 50%. Nosso estudo estabeleceu uma análise sobre estudo sobre o polimorfismo T-786C no gene da eNOS em mulheres no terceiro trimestre de gravidez, observando a possível associação com moléculas lipídicas e com a proteína c-reativa (PCR). Adicionalmente, a partir de ferramentas *in silico*, foram desenhados modelos de interação proteína-proteína (*networks*) para compreensão da importância do metabolismo da eNOS em condições normais e com patologias. Amostras de 92 mulheres grávidas foram submetidas à extração de DNA, identificação do polimorfismo T-786C por PCR-RFLP, e análise dos níveis de colesterol total (CT), HDL, LDL, triglicerídeos (TG) e PCR. Análise genotípica apresentou uma população de 71,73% TT, 26,08% CT e apenas 2,17% CC, estando o alelo C^{-786} presente em 15.21% do grupo estudado. Em relação à análise bioquímica, observou-se significância apenas para PCR quando as pacientes foram agrupadas por níveis sorológicos (normal ou alterado) de acordo com o genótipo da paciente. A CRP atua diretamente no desacoplamento da enzima eNOS prejudicando sua atividade e diminuindo a produção de NO, e servindo como marcador de eventos cardiovasculares. A partir das análises de bioinformática construímos duas *networks*. A primeira, denominada eNOSNet, possui 51 nós e 361 arestas de interação, mostrando proteínas ligantes da eNOS, pequenas moléculas e seus complexos formados. A segunda network, denominada eNOSNetD, relaciona proteínas envolvidas na via normal da eNOS com doenças descritas para o polimorfismo T-786C, tais como: doenças relacionadas ao sistema cardíaco, neurológico, à neoplasias e ao metabolismo lipídico, glicídico, protéico e hormonal. Estes dados podem ajudar na compreensão sobre a progressão de doenças que envolvem o funcionamento da eNOS e seus possíveis tratamentos e diagnósticos.

Palavras-Chave: Gravidez, eNOS, proteína c-reativa, bioinformática.

Abstract

Pregnancy is a physiological phenomenon that begins with fertilization and progresses to promote an appropriate environment in which the fetus can grow and form a new individual with all its genetic potential expressed. During this period, the lipid levels typically increase to maintain homeostasis between mother and fetus. Abnormal lipid levels cause endothelial dysfunction causing complications for mother and developing fetus, reducing the production of nitric oxide (NO) major regulator of fetal-placental events. NO is the main regulator for feto-placentários events, being produced by 3 isoforms of nítric oxide synthase (NOS). Single nucleotide polymorphisms (SNPs) in endothelial NOS (eNOS) have been correlated to many pathologies, being T-786C responsible for reducing eNOS expression in 50%. Our study established a study on analysis of the T-786C eNOS polymorphism in third trimester pregnancy women, analyzing the possible association with lipid molecules and C-reactive protein (CRP). Besides, bioinformatics tools were used in order to obtain models of protein networks that allow the comprehension about the importance of eNOS pathway in physiological and pathological conditions. Samples from 92 pregnant women were subjected to DNA extraction, identification the T-786C polymorphism by PCR-RFLP. The levels of total cholesterol (TC), cHDL, cLDL, triglycerides (TG) and CRP were evaluated. Genotyping analysis showed 71.73% TT, 26.08% CT and only 2.17% CC. The allele C^{-786} was found in low frequency (15.21%) in comparison with the T^{-786} allele (84.78%). Biochemical analysis showed significance for CRP for patients grouped by CRP levels (normal and non-normal) according to the genotyping. CRP acts directly on the uncoupling of the enzyme eNOS impairing its activity and decreasing NO production, besides serving as marker of cardiovascular events. The analysis of bioinformatics allowed the construction of two networks. The first, called eNOSNet, has 51 nodes and 361 edges of interaction and shows eNOS protein ligands, small molecules and their complexes. The second network, called eNOSNetD, is the result of linking proteins from normal eNOS pathway with diseases described for the T-786C polymorphism, like cardiovascular, neurological and hormonal disease, neoplasm and diseases from lipid, protein and carbohydrate metabolisms. These analyses can provide information about the molecular interactions in the states of health and disease related to eNOS pathway and possible treatments and diagnostics.

Keywords: Pregnancy, eNOS, C-reactive protein, bioinformatics.

LISTA DE ILUSTRAÇÔES

Revisão da literatura

LISTA DE ABREVIAÇÕES E SIGLAS

A	Adenina
Asp	Asparagina
AKT	Proteína quinase B
BH4	Tetrabiopterina
Ca+2	Cálcio
Cav-1	Caveolina
C	Citosina
CT	Colesterol Total
cGMP	Monofosfato de guanosina ciclica
Cys	Cisteína
eNOS	Oxido nítrico sintase endotelial
G	Guanina
Glu	Glutamina
HDL	Lipoproteína de alta densidade
HsP90	Proteína de choque térmico
iNOS	Óxido nítrico sintase induzível
LDL	Lipoproteína de baixa densidade
NADPH	Nicotinamida adenina dinucleotídeo fosfatase
nNOS	Óxido nítrico sintase neuronal
NO	Óxido nítrico
NOS	Óxido nítrico sintase
NOSIP	Proteína de interação do óxido nítrico sintase
NOSTRIM	Proteína transportadora do óxido nítrico sintase
Ox-LDL	Lipoproteína de baixa densidade oxidado
PCR	Proteína C reativa
PI3K	Fosfatidilinositol 3 quinase
PKA	Proteína quinase A
PP1	Fosfatase serina-treonina 1
PP2	Fosfatase serina-treonina 2
RCIU	Restrição de crescimento intrauterino

Ser	Serina
SNP	Polimorfismo de nucleotídeo único
T	Timina
TG	Triglicerídeos
Tyr	Tirosina

SUMÁRIO

1. Introdução

O período gestacional é marcado por uma profunda mudança em diversos aspectos na vida da mulher, sejam elas, anatômicas, fisiológicas ou bioquímicas. O organismo materno é responsável pela manutenção e desenvolvimento, um processo complexo que envolve interações sofisticadas entre mãe e feto. Esta relação íntima entre mãe e feto é suportada através da placenta, órgão especializado que assegura nutrição e oxigenação a todas as fases do desenvolvimento fetal. Desta forma, o endotélio vascular íntegro é uma condição necessária para o fornecimento de oxigênio e nutrientes e assim manter o equilíbrio da gestação. Do contrário, danos no endotélio podem levar a condições patológicas durante a gestação.

O óxido nítrico (NO) é um dos principais reguladores da homeostase gestacional devido ao seu importante papel na regulação do tônus vascular, sendo um dos principais mediadores químicos que regulam diversos sistemas biológicos. NO é sintetizado pelas enzimas óxido nítrico sintase endotelial (eNOS), neuronal (nNOS) e induzível (iNOS).

A eNOS é a principal fonte do NO gerado no sistema vascular, sendo regulada por diversas moléculas como caveolina, calmodulina e proteína C reativa. No entanto, variações no gene da eNOS podem prejudicar a atividade desta enzima, como observado para o polimorfismo T-786C, que pode reduzir a atividade da enzima em cerca de 50%. Alterações no mecanismo de ação da eNOS vem sendo associadas com o desenvolvimento de doenças cardiovasculares e metabólicas.

Aliado a este fato, o perfil lipídico anormal durante o período gestacional também contribui para o desenvolvimento de doenças. Os índices de colesterol total,

triglicerídeos, lipoproteínas de baixa e alta densidade apresentam aumento esperado durante a gestação, mas podem ser excessivos tornando-se indicativo de desenvolvimento de patologias como hipertensão gestacional, diabetes gestacional, restrição de crescimento intra-uterino (RCIU), pré-eclâmpsia, aterosclerose e doenças cardiovasculares .

O presente trabalho vem a contribuir, a partir de dados moleculares e de análises de bioinformática, com a discussão sobre os valores de referência lipídicas e de proteína C-reativa em mulheres no terceiro trimestre de gravidez e sobre o efeito do polimorfismo T-786C na região promotora da eNOS como um fator de aumento de risco para intercorrência na gravidez e predisposição à doenças cardiovasculares.

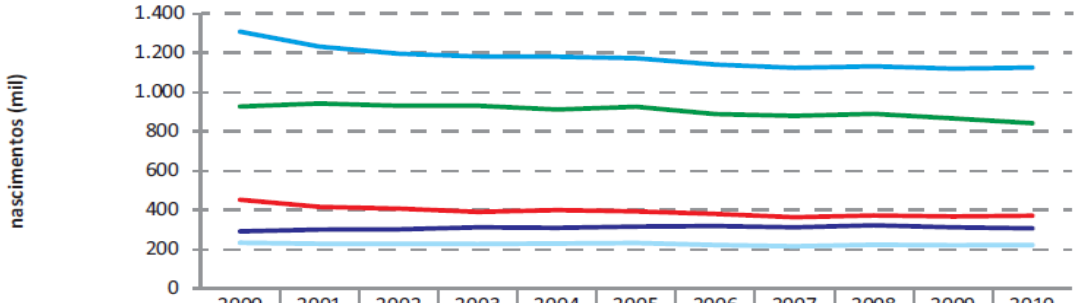
2. Revisão Bibliográfica

2.1 Desenvolvimento gestacional

A gestação é um fenômeno fisiológico que se inicia com a fecundação e progride no sentido de promover um ambiente adequado onde o feto possa crescer e se preparar para vir ao mundo. Esta fase faz parte da vida saudável da mulher envolvendo mudanças significativas no campo físico, social e emocional. A gestação caminha no sentido de gerar um novo indivíduo saudável com todo seu potencial genético expresso. A capacidade de crescer a partir de uma única célula, aumentar além do tamanho também a complexidade do organismo é uma característica fascinante dos seres vivos, que não cessa até a vida adulta (Piccinini et al, 2008; Warner & Ozanne, 2010).

A maioria das gestações avança sem intercorrências, porém uma pequena parcela pode desenvolver problemas que comprometem a saúde materna e a fetal. O Brasil avançou muito nos últimos 30 anos no que diz respeito à atenção ao acompanhamento pré-natal, parto e nascimento através de uma série de programas do governo. Segundo o Ministério da Saúde houve uma redução entre o período de 2000 a 2010 no total de nascidos vivos, 3,2 milhões para 2,9 milhões ao ano. Esse comportamento de queda não foi reproduzido pela região Norte apresentando um pequeno aumento de 5,4% no mesmo período (Figura 1). Esses dados são contraditórios se levado em consideração o atual nível de desenvolvimento econômico e social do nosso país (Ministério da Saúde, 2012).

Gráfico 1 – Número total de nascimentos (em milhares) – Brasil e regiões, 2000 a 2010



	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Norte	290	299	301	311	309	314	317	311	321	310	306
Nordeste	926	942	929	930	910	924	887	878	888	865	841
Sudeste	1.306	1.230	1.195	1.181	1.178	1.171	1.139	1.122	1.130	1.119	1.123
Sul	451	415	406	389	398	392	379	362	371	366	369
Centro-Oeste	232	227	227	225	229	231	221	215	222	220	220

Fonte: SVS/MS//DASIS. Sistema de Informações sobre Nascidos Vivos (Sinasc).

Figura 1. Gráfico demonstrativo da queda da natalidade de acordo com as regiões brasileiras.

Profundas adaptações ocorrem no organismo logo que se inicia a gestação, sejam elas anatômicas, fisiológicas ou bioquímicas, sendo que a maioria ocorre em resposta a estímulos fetais. Mudanças na postura, sistema cardiovascular, respiratório, metabolismo hidroeletrolítico, enzimas, sistema nervoso, níveis hormonais e lipídicos plasmáticos ocorrem tanto para suprir as necessidades energéticas maternas e fetais, como para servirem de precursores estruturais e hormonais. Em condições fisiológicas normais da gestação, triglicerídeos e colesterol materno aumentam progressivamente semana após semana. O organismo materno é responsável pela manutenção desenvolvimento do processo complexo e dinâmico que é a gestação, dependendo estreitamente de sofisticadas interações envolvendo a mãe e o feto (Figura 2) (Herrera, 2002; Warner & Ozanne, 2010).

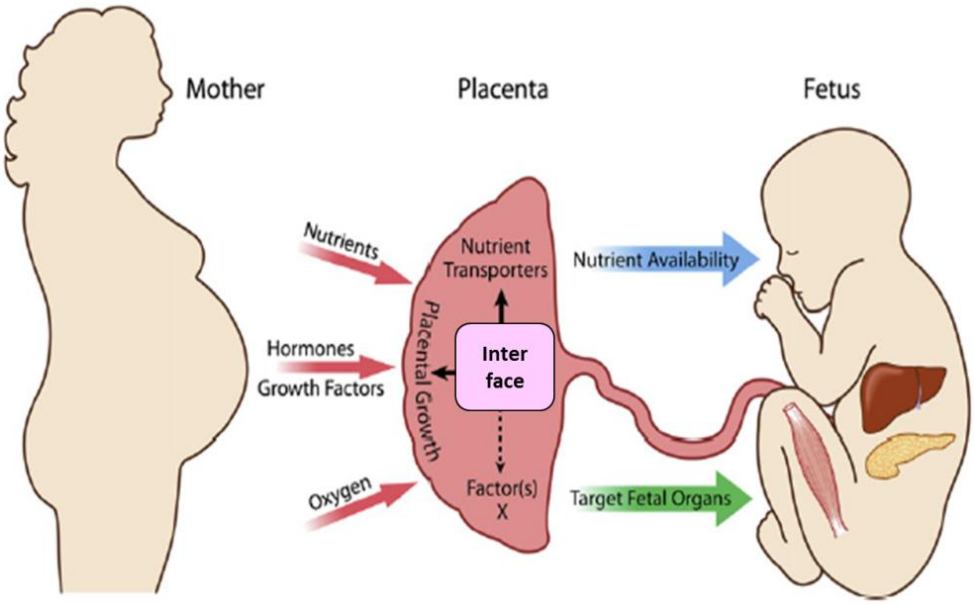


Figura 2: Desenho esquemático da troca de nutrientes, hormônios, fatores de crescimento e oxigênio do ambiente materno para o desenvolvimento fetal. Adaptado Jansson, 2012.

A interação entre mãe e feto é suportada através da placenta, órgão especializado dos mamíferos, composta por uma rede de capilares, veias e artérias que assegura nutrição e oxigenação a todas as fases do desenvolvimento fetal, mantendo a homeostase através de uma gama de funções fisiológicas como, por exemplo, proporcionar uma barreira imunológica entre a mãe e o feto, mediar a transferência de gases respiratórios, água e nutrientes além da capacidade de produzir hormônios, citocinas e moléculas de sinalização necessários a gestação (Jansson et al, 2007).

A integridade vascular mantem o fluxo sanguíneo e o suprimento de gases e nutrientes, atendendo a demanda energética e mantendo o equilíbrio da gestação normal. Danos no endotélio vascular podem ocorrer devido a certos desequilíbrios na liberação e atividade de mediadores vasculares, como o óxido nítrico (NO), ocorridos durante a gestação (Brolio et al, 2010).

O NO é um dos principais vasodilatadores que participa das adaptações uterinas hemodinâmicas na gravidez. A desregulação nos níveis de NO pode acarretar em problemas para saúde da mãe como a hipertensão e pré-eclampsia, mas também para o feto como hiperfusão útero-placentária ou hipóxia placentária, o que pode levar a um estado de restrição de crescimento intrauterino (Tanbe & Khalil, 2010). Desta forma, o recém-nascido pode apresentar alteração de tamanho com relação à idade gestacional (Figura 3) e predispor-se ao desenvolvimento de doenças cardiovasculares ou metabólicas na vida adulta, seguindo a hipótese de Barker e colaboradores (Barker et al., 1989).

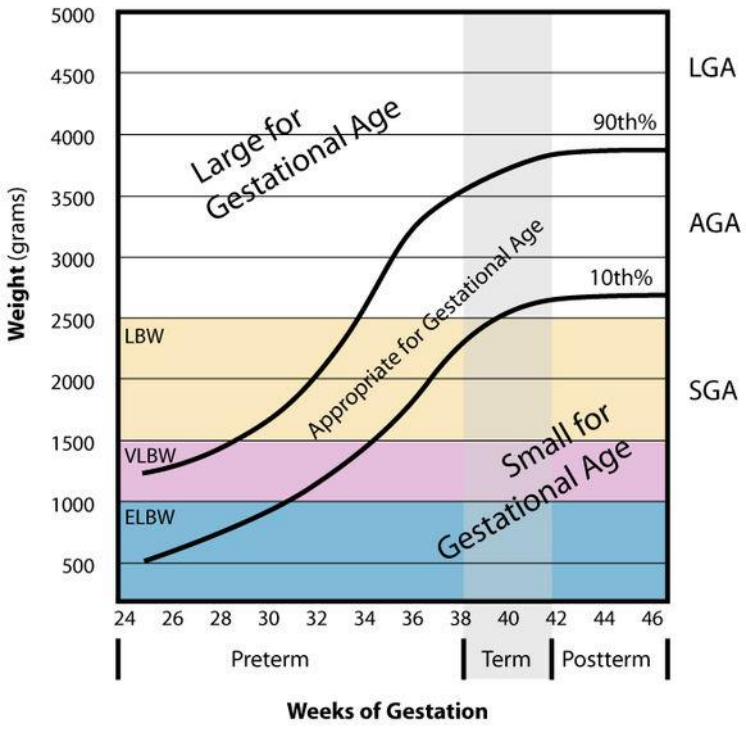


Figura 3. Classificação do recém-nascido de acordo com o peso de nascimento. O RN abaixo de 2500 g (percentil 10%) é classificado em pequeno para idade gestacional (PIG), acima de 4000 g (percentil 90%) em Grande para idade gestacional (GIG). Valores intermediários são determinados para adequados para idade gestacional (AIG). As faixas em rosa representam peso baixo no nascimento (LBW), em lilás peso muito baixo no nascimento (VLBW) e azul peso extremamente baixo no nascimento (ELBW). (Fonte: Yehudamalul, 2010).

2.1.1 Alterações do perfil lipídico na gestação

O desenvolvimento do período gestacional é marcado por um aumento da obtenção de energia adicional, a fim de suprir o crescimento fetal e o desenvolvimento dos tecidos materno e da placenta (Bartels & O'Donoghue 2011). Durante este período, diversos mecanismos adaptativos ocorrem devido à mudança no estado hormonal e energético, como as alterações no metabolismo lipídico e no metabolismo inflamatório.

Os índices de colesterol total (CT), triglicerídeos (TG), lipoproteína de baixa densidade (LDL) e lipoproteína de alta densidade (HDL) vem sendo intensamente estudadas em mulheres grávidas associadas com parâmetros como idade gestacional e o risco de desenvolvimento de doenças como pré-eclâmpsia, aterosclerose, hipertensão gestacional, doenças cardiovasculares além da disfunção endotelial que sinaliza para diversos processos patológicos (Merabishvili et al., 2006; Lippi et al., 2007; Diareme et al., 2009).

Os lipídeos são macromoléculas essenciais à gravidez, sua concentração é naturalmente aumentada nesse período, uma vez que ocorrem mudanças físicas e hormonais, e este servirá como substrato para diversos hormônios placentários (Plosch et al., 2007; Ziaei et al., 2012). O colesterol é um componente estrutural importante da membrana celular, enquanto diminui sua fluidez controla sua permeabilidade. Além disso, é um precursor direto de hormônios esteróides que são produzidos pela placenta (Byanes & Dominiczak 2009). Atua como componente essencial para o desenvolvimento feto-placentário, sendo positivamente relacionado com a taxa de crescimento do feto. O colesterol materno é ativamente transferido para o feto através da membrana, contribuindo para o nível lipídico fetal (Woollett, 2011).

Na fase anabólica da gestação, correspondente ao início e a fase intermediária, ocorre acumulo de depósitos de lipídeos proporcionando sua mobilização quando ocorre a transição para fase catabólica favorecendo o tecido materno a utilizar os lipídios como fonte de energia para proliferação celular e o rápido crescimento fetal (Bute, 2000; Toescu et al., 2004; Ghio et al., 2011). A placenta utiliza o colesterol para síntese de esteróides e os ácidos graxos são usados para formação da membrana e oxidação placentária. Este é ativamente transferido pela placenta através de receptores de lipoproteínas presentes na superfície sinciotrofoblástica materna. O colesterol absorvido é liberado pelas células endoteliais na circulação fetal formando uma partícula de liproproteína de alta densidade (HDL). O HDL possui papel anti-inflamatório e inibe a oxidação do LDL em oxLDL, fator chave em lesões arterioscleróticas (Palinski, 2009; Woollet, 2011; Stefulj et al., 2009; Navab et al., 2011). Os triglicerídeos acumulados não conseguem atravessar a barreira placentária, porém os receptores de lipoproteínas presentes na placenta, lipases, fosfolipoproteínas A2, e lipases intracelulares proporcionam o transporte e liberação de ácidos graxos poli-insaturados maternos transportado como triglicerídeos para o compartimento fetal. Perfis lipídicos anormais têm sido associados com mudanças no transporte placentário durante a gravidez (Plosch et al., 2007; Ziaei et al., 2012).

Durante o primeiro trimestre de gestação, os níveis lipídicos se assemelham com os de mulheres não-grávidas, ocorrendo um desvio significativo nesses valores durante o segundo e o terceiro trimestre de gestação (Lippi et al, 2007). Nestes períodos, os níveis de TG aumentam de 2 a 3 vezes, acompanhado por um aumento em menor grau de CT, HDL e LDL. Porém em alguns casos, os níveis lipídicos se tornam anormais formando um quadro de hiperlipidemia que pode ser encarada como resposta aos eventos fisiológicos maternos, por exemplo, hipercolesterolemia

suprafisiológica materna, ou indicativo do desenvolvimento de alguma patologia como pré-eclampsia e diabetes mellitus gestacional (Brizzi et al., 1999; Sattar et al., 1999). Essas condições patológicas estão amplamente associadas com disfunção endotelial, apesar da falta de informação sobre na literatura sobre os parâmetros lipídicos (níveis de referência) durante a gestação. Existem evidências de que altos níveis de colesterol no sangue modificam a função endotelial, levando à redução da biodisponibilidade do NO e, consequentemente, a redução da reatividade vascular e vasodilatação (Leiva et al., 2011; Koklu et al., 2007).

2.2 **Óxido Nítrico (NO) e sua função biológica**

O NO é um gás de radical livre e lábil, capaz de difundir livremente através da membrana celular, o que facilita a sua atividade biológica sendo considerado um importante mediador parácrino do organismo humano (Nishank, 2013). Este gás biossintetizado em diversos tipos celulares, inclusive no endotelio dos vasos sanguíneos, onde desempenha um papel chave na regulação do tônus vascular (Figura 4). Após a produção e liberação a partir de células endoteliais para o lúmen vascular, NO difunde rapidamente através das membranas de músculo liso interagindo com o guanilato ciclase no grupo heme, para formar 3-5-monofosfato cíclico (cGMP). O cGMP promove uma sinalização por meio de proteínas quinases que conduzem ao aumento da absorção de cálcio pelo retículo endoplasmático quando então, ocorre a dilatação propriamente dita (Allen et. ai., 2012; Shah et al., 2013; AlFadhli, 2013 e Gagala et al, 2013).

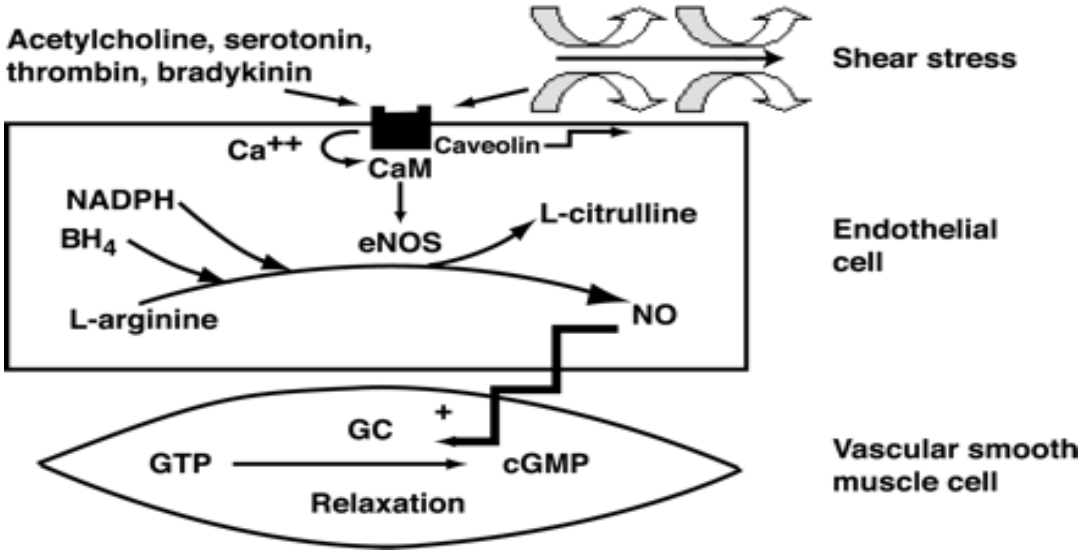


Figura 4. Desenho esquemático da produção do óxido nítrico (NO). Ativadores se ligam à receptores na membrana e promovem a regulação intracelular da enzima oxido nítrico sintase endotelial (eNOS). O NO age nas células de musculo liso vascular promovendo a vasodilatação. (Fonte: Jean Davignon and Peter Ganz, 2004).

O NO atua ainda como mediador da homeostase no sistema cardiovascular, no qual doenças como a aterosclerose são prevenidas devido a sua ação, que impede eventos como a adesão de células brancas do sangue, agregação/adesão plaquetaria e a inibição da proliferação de células musculares lisas. A inibição da adesão plaquetária protege o músculo liso contra o fator de crescimento derivado de plaqueta, impedindo assim eventos subsequentes na aterogênese como a formação de placas de fibras. Assim, o NO é caracterizado como o mediador mais importante na defesa do sistema vascular (Figura 5) (Förstermann et al., 2006).

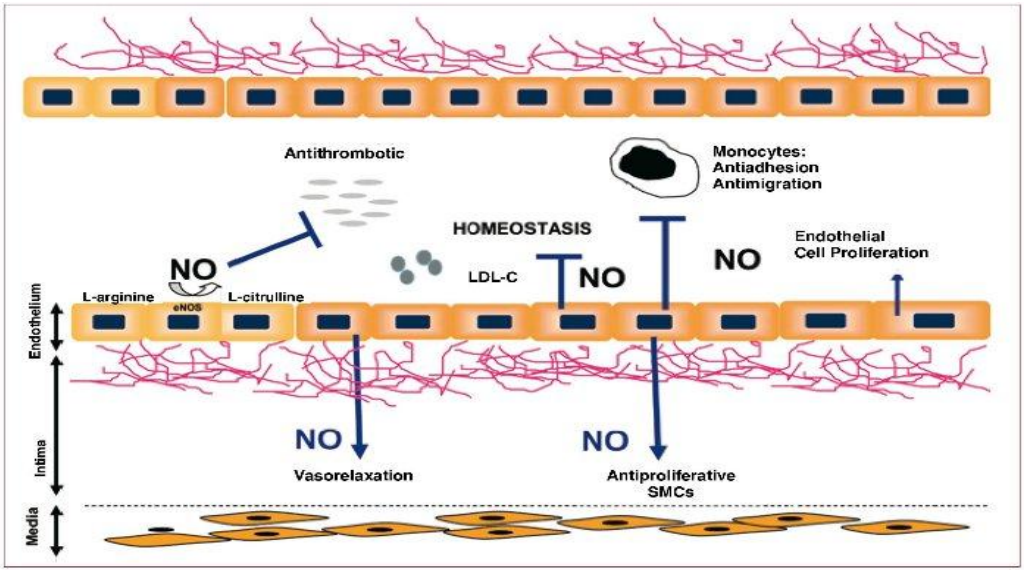


Figura 5: Esquema do papel vaso-protetor do NO. (Fonte: Badimón, 2009).

NO também pode ser produzido por macrófagos, agindo na proteção contra processos infecciosos, inibindo a adesão dos leucócitos à parede do vaso à medida que interfere com a capacidade da molécula de adesão CD11/CD18 dos leucócitos em ligar-se à superfície endotelial ou suprimindo a expressão deles. NO atua ainda no sistema nervoso central (SNC), gastrointestinal, respiratório e geniturinário (Shah et al., 2013).

2.3 Regulação da oxido nítrico *versus* Óxido Nitrico Sintase endotelial

A síntese de NO é catalisada por um grupo de enzimas chamadas óxido nítrico sintase (NOS). Existem três isoformas que compartilham 50% de homologia e são codificados por genes diferentes, conhecidas de NOS: óxido nítrico sintase endotelial (eNOS), óxido nítrico sintase neuronal (nNOS) e a óxido nítrico sintase induzível (iNOS) (Shah et al.,2013; AlFadhli 2013 e Gagala et al, 2013).

A eNOS é a principal fonte de NO endógeno gerado no sistema vascular, seu gene está localizado no braço longo do cromossomo 7 (7q35-36), contém 26 exons e 25 introns. Os grupos de proteínas NOS dos mamíferos são flavoproteínas que têm um grupo heme para onde os elétrons são transferidos a partir de um NADPH, regulados por proteínas calmodulina e auxiliado por co-fatores, tais como tetrahidrobiopterina (BH4). A atividade da eNOS é controlada pelos níveis de Ca^{+2} e outros co-fatores intracelulares. Em células endoteliais, sob condições fisiológicas, o NO é catalisado pela eNOS a partir da oxidação da L-arginina auxiliado por co-substratos tais como NADPH e oxigênio (Allen et. al., 2012).

Estímulos fisiológicos e fisiopatológicos levam a respostas como mecanismos de regulação pós-traducionais que estimulam ou inibem a atividade dinâmica da enzima. A regulação e atividade biológica da eNOS são estabelecidos por mecanismos de controle interdependentes através da interação com diversas moléculas pequenas e proteínas que desencadeiam vias de sinalização para produção de NO (Dudzinski & Michel, 2007).

Durante o processo de ativação, a eNOS é direcionada para uma região da membrana denominada cavéola, rica em colesterol e esfingolipídios, com a presença marcante da proteína caveolina-1 (Cav-1) (Govers & Rabelink, 2001). Esta fase parece ser crítica para as interações com proteínas e outras moléculas necessárias a sua ativação (Figura 6). A miristoilação e a palmitoilação conferem grupamentos acil a eNOS, permitindo sua forte adesão a membrana caveolar. No processo de miristoilação, a eNOS é catalisada por uma N-miristoiltransferase reconhecendo uma sequencia N-terminal específica, enquanto que a palmitoilação ocorre nos resíduos Cys15 e Cys26 estando sujeito a ação de um thiopalmitoil, que induz a

depalmitoilação e a translocação da enzima para o citosol (Dudzinski & Michel, 2007).

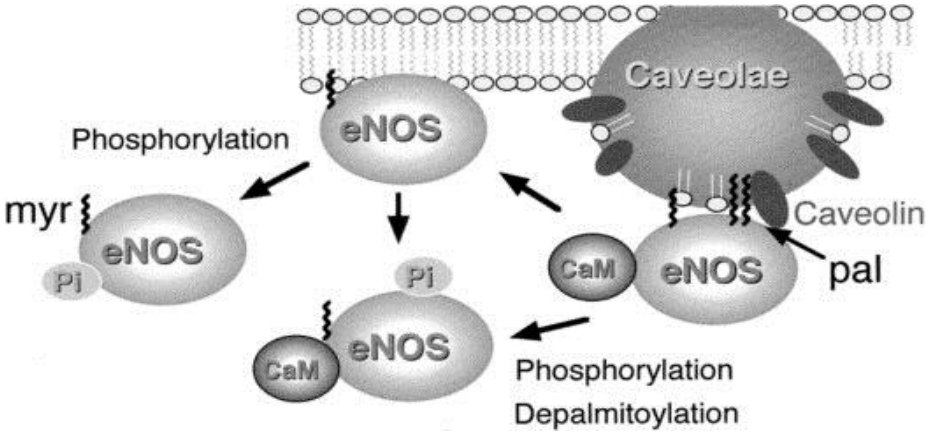


Figura 6: Desenho esquemático da regulação da eNOS na região Caveolar. Interações com a proteína camodulina (CaM), adição de fosfatos (Pi) e dos grupos palmitoil (pal) e miristoil (myr). (Fonte: Hisaaki Taniguch, 1999).

A atividade da eNOS depende intimamente dos níveis intracelulares de cálcio, uma vez que forma um complexo com a proteína calmodulina para facilitar a troca de elétrons entre os domínios redutase e oxidase da eNOS (Shaul, 2002; Dudzinski & Michel, 2007) A Cav-1 é constitutivamente expressa e atua inibindo temporariamente a eNOS, o que impede seu acesso ao complexo cofator cálcio/calmodulina, essencial a ativação. Desta forma, a interação Cav-1/eNOS é necessária para o controle de liberação de NO em condições basais (Razani et al., 2002; Sessa, 2005; Garcia et al., 2012). A calmodulina rompe a interação inibitória Cav-1/eNOS para ativar a enzima.

A proteína de choque térmico 90 (Hsp90), por sua vez, atua no tráfego e dobramento da eNOS, estimulando sua atividade por cooperar com o aumento da afinidade da enzima pela calmodulina, equilibrando a produção de NO. Além disso, a ação da Hsp 90 também é requerida para o processo de fosforilação com Akt quinase (Takahashi & Mendelsohn, 2003). O tráfego da eNOS conta com a contribuição de

proteínas como a NOSIP, que se liga ao terminal carboxil da enzima e auxilia na sua translocação da membrana caveolar para membranas intracelulares. Da mesma forma, a NOSTRIN forma um complexo ternário NOSTRIM/Cav-1/eNOS na membrana plasmática, formando um dos mecanismos regulatório a qual a enzima eNOS está submetida. A super-expressão de NOSTRIN transloca a eNOS para vesículas intracelulares acompanhada de uma redução na atividade da enzima (Zimmermann et al., 2002; Schilling et al., 2006).

Outros processos regulatórios pós-traducionais como fosforilação e desfosforilação influenciam a atividade da enzima. Os resíduos de serina e treonina constituem locais reguladores, podendo ser estimuladores (Ser 1177, Ser 635 e Ser 617) ou inibitórios (Tyr 495 e Ser 116). A desfosforilação em resíduos inibitórios (Tyr 495 e Ser 116) pode ativar a enzima, enquanto que nos resíduos estimuladores (Ser 1177) pode atenuar retornando a atividade basal. Fosfatases, como as proteínas serina-treonina 1 a 2A (PP1 e PP2A), participam da regulação da eNOS principalmente por desfosforilar Ser116 e ativar a enzima (Figura 7) (Harris et al., 2001).

A eNOS também sofre ação de agonistas como a bradimicina que induz de forma potente a formação do NO endotelial por mediação do receptor B2 da bradimicina, através da ativação da via de fosforilação por PKA no resíduo de S1177 (Bae et al., 2003).

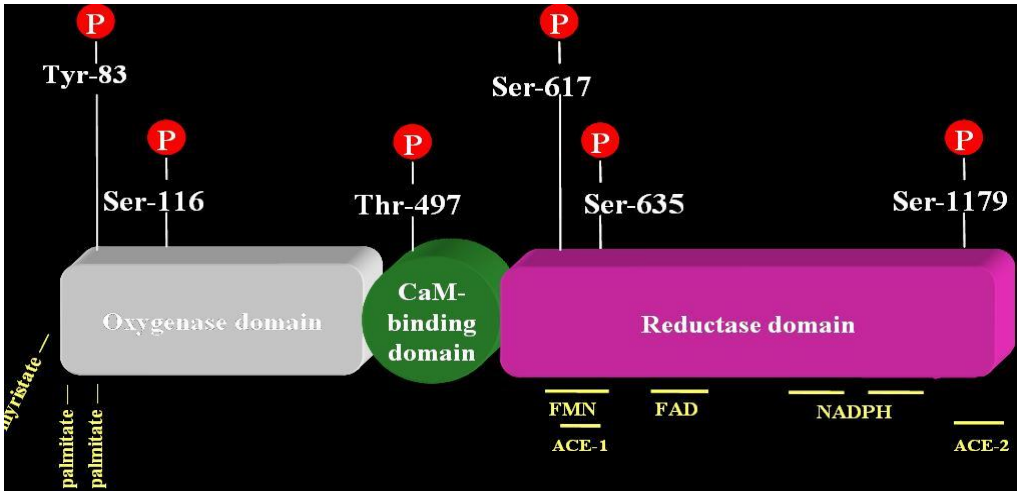


Figure 7: Desenho esquemático representando os sítios de regulação pós-traducionais na eNOS Ser: Serina, Thr: treonina e Tyr: Tirosina, flavinas mono e dinucleotídeo (FMN e FAD) e interações proteína-proteína. (Fonte: *Molecular and cardiovascular physiology laboratory*, 2011).

O receptor acoplado a proteína G, intimamente ligado do lado citosólico com a proteína heterotrimétrica G, permuta sinais possíveis no controle da atividade da eNOS, mobilizando o cálcio intracelular e a cascata desencadeada pela fosfatase PI3K. Moléculas como Bradicinina (receptor B2), acetilcolina, histamina, adenosina e proteína trombina estimulam quantidades de cálcio intracelular. A organização intracelular também é uma característica marcante na ativação da eNOS. A rede que forma o citoesqueleto de actina é utilizada a partir da cavéola para translocar a enzima reversivelmente entre citoplasma e o Golgi. Desta forma, a eNOS se mantém estabilizada nos filamentos do citoesqueleto e em contato direto com o substratos de arginina (Dudzinski et al., 2006).

Desde a ultima década, estudos sobre o envolvimento da proteína C reativa (PCR) na regulação da eNOS estão em ascensão na literatura (Rifai & Ridker, 2001; Paternoster et al., 2006; Bisoendial et al., 2010; Asemi et al., 2013). Acredita-se que a PCR atue na inibição da bioatividade pelo desacoplamento da eNOS mediante

aumento de espécies reativas de oxigênio que ocasionam uma menor taxa de dimerização da enzima e fosforilação do resíduo de serina S1177 na eNOS e diminuição da disponibilidade de BH4 (Singh et al., 2007). A PCR é membro da família pentraxina (pentaxins) que pode promover a disfunção endotelial por induzir o desequilíbrio entre os fatores teciduais e os inibidores desses fatores nas células endoteliais, além de promover a adesão plaquetária (Chen et al., 2009; Schwedler et al., 2007). Esta proteína parece desempenhar um papel importante em todas as fases de doenças cardiovasculares, por exemplo, a partir das lesões iniciais até o estágio agudo (Corrado et al., 2010), sendo considerado um marcador de inflamação com fator preditivo para eventos cardiovasculares (Kavsak et al., 2007; Shimada et al., 2009).

Em células endoteliais, a PCR aumenta a expressão de moléculas de adesão, citocinas inflamatórias e proteínas quimiotáxica de monócitos, diminuindo a liberação de prostaciclina e a ação de ativadores de plasminogênio tecidual (Figura 8) (Verma et al., 2006), além de promover a formação espécies reativas de oxigênio acometendo a enzima a um estado de desacoplamento levando a prejuízos na liberação do NO, diminuindo as taxas de relaxamento dos vasos e consequentemente, disfunção endotelial (Whitsett et al., 2006; Griendling et al., 2000).

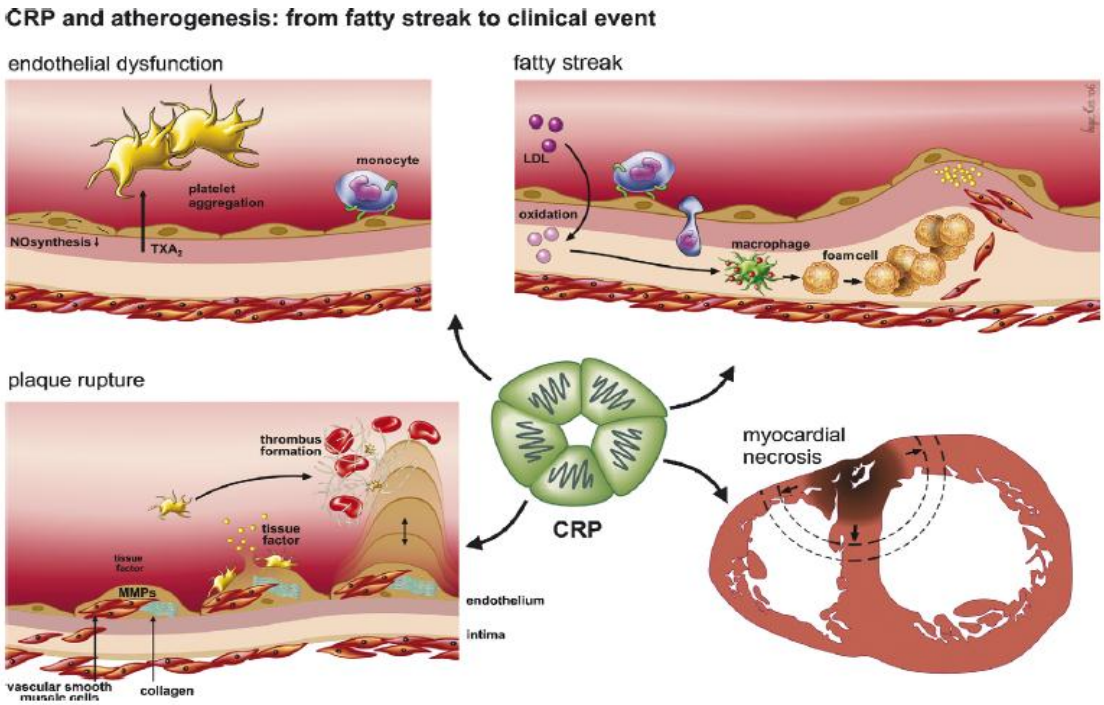


Figura 8: Desenho esquemático do papel da PCR como fator pró- aterogênico. (Fonte: Bisoendial, et al., 2007).

A eNOS se caracteriza como uma enzima de grande importância devido a bioatividade do NO ser responsável pela homeostase de tantos processos fisiológicos e evitando o desenvolvimento de patologias. Mutações no gene da eNOS, tais como os polimorfismos, podem alterar a informação contida no gene responsável pela sua atividade e prejudicar a sua ação protetora vascular (Zago et al., 2009). Apesar de pouco compreendida, a relação entre polimorfismos de DNA e fenótipos humanos apresentados como a susceptibilidade à doenças, o uso de técnicas de biologia molecular tem permitido à realização de estudos sistemáticos.

2.4 Polimorfismo: Variantes genéticas na disfunção vascular

As variantes genéticas da eNOS também têm sido intensamente estudadas como fatores de risco para doenças cardiovasculares e metabólicas (Förstermann et

al., 2006). A regulação do gene da eNOS afeta diretamente a bioatividade do NO endotelial (Jaramillo et al., 2008), pois leva a redução ou excesso de sua produção contribuindo para muitos processos patológicos como a disfunção endotelial nos diversos sistemas onde o NO atua (Santos et al., 2010).

Diversas mutações no gene da eNOS foram descritos na região do promotora, éxons e íntrons. Os polimorfismos mais estudados na literatura atual são SNPs (Polimorfismo Único Nucleótideo) que têm uma diferença em único nucleotídeo na sequência de DNA. Um nucleotídeo (A, C, T ou G), difere em uma determinada sequência para outra ou entre os cromossomas homólogos no mesmo indivíduo. SNPs aparecem em uma frequência alélica de, pelo menos, 1 a 5% da população. A ativação ou inibição da transcrição gênica pode ser alterada pela presença de um SNP na região promotora prejudicando a ligação de fatores de transcrição. O SNP T-786C da região promotora (5'- região flanqueadora) é caracterizada pela alteração de uma timina (T) por citosina (C) na posição -786 capaz de reduzir a atividade promotora em aproximadamente 50%, chamando atenção para o relevante papel fisiológico desse SNP (ROPERS, 2007; Zhang et al., 2008). Outra variação conhecida no gene da eNOS é a substituição de aminoácidos 894G>T, no qual a substituição de uma G por uma T no exon 7 leva a troca de um glutamato por um aspartato na posição 298 da proteína (Glu298Asp) (Piccoli et al., 2008). Esses polimorfismos vêm sendo amplamente associados com doenças cardiovasculares, diabetes, hipertensão gestacional, pré-eclâmpsia, estresse oxidativo e etc.

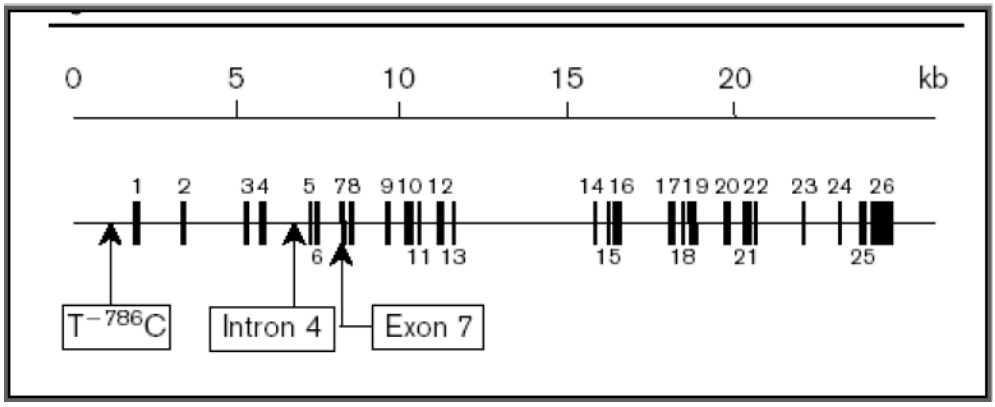


Figura 9: Esquema do gene eNOS humano e polimorfismos citados na literatura. (Fonte: Tanus et al, 2001)

2.5 Disfunção endotelial

O endotélio é a monocamada interna dos vasos, altamente especializado e faz interface entre o sangue os tecidos subjacentes. A disfunção endotelial é um processo fisiopatológica sendo uma variável chave no processo de desenvolvimento de doenças cardiovasculares e outras complicações em decorrência da exposição a fatores de risco já bem conhecidos e a pré-disposição genética (Krause et al., 2013).

Estímulos mecânicos e químicos induzem o endotélio a produzir e liberar substâncias vasoativas e fatores de crescimento que regulam a sua função. No entanto, altos níveis de colesterol, tabagismo, sedentarismo, e diabetes predispõem o indivíduo a uma possível disfunção endotelial, no qual há perda de equilíbrio entre os agentes vasodilatadores, vasoconstrictores, fatores e inibidores de crescimento (Chhabra, 2009). Uma característica marcante da disfunção do endotélio é a alteração de resposta à estimulantes dependente de endotélio, como por exemplo, a acetilcolina que estimula a eNOS a produzir NO e subsequente relaxamento do músculo liso pelo aumento da produção de cGMP (Krause et al., 2013).

A perda da capacidade de produzir e liberar NO são características da disfunção endotelial sendo fortemente relacionado como fator de risco para doença cardiovascular (DCV) e um tipo de marcador para doenças crônicas como diabetes de mellitus (DM), hipertensão, hipercolesterolemia e doença renal (Myatt, 2010; Versari et al., 2009; Libby et al., 2011).

Em gestações com ocorrências de pré-eclâmpsia, diabetes de mellitus gestacional, e restrição de crescimento intra-uterino (RCIU) a síntese ou a biodisponibilidade de NO são alterados, o que resulta em mudança no fluxo sanguíneo através da placenta prejudicando o desenvolvimento e crescimento gestacional (Sobrevia & Casanello, 2011). Como o tônus vascular da placenta é regulado pela presença de moléculas vasoconstritoras e vasodilatadoras, a disfunção endotelial placentária tem sido associada com estudos de disfunção vascular reforçando a hipótese de que a vasculatura fetal na placenta pode ser afetada pela alteração da síntese de NO e estresse de nitratos (Leiva et al., 2011). Sendo o NO o principal vasodilatador feto-placentário, sua síntese e efeitos são diminuídos na placenta com RCIU (Krause et al., 2011; Krause et al., 2013), o que pode levar à diminuída oferta nutricional. Este evento pode levar a alterações permanentes em vários aspectos do desenvolvimento do sistema nervoso central, principalmente se ocorrer durante os períodos mais precoces do desenvolvimento (Camelo e Martinez, 2005).

2.8 Bioinformática

Com o advento do genoma humano no início do século abriu-se intensas discussões sobre e ações sobre a nova era da biologia, a era pós-genômica onde predominou o desenvolvimento e aperfeiçoamento de tecnologias que permitem o entendimento e o aprimoramento das novas ciências ômicas (Figura 10) (Espíndola, et al., 2010).

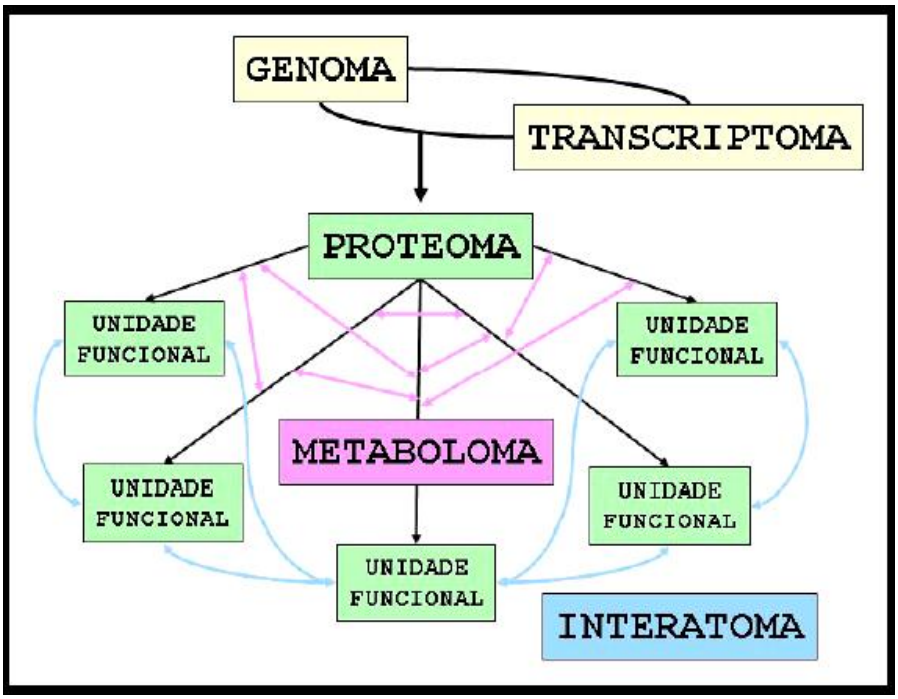


Figura 10: Representação esquemática das principais ciências ômicas. (Fonte: Espíndola 2010)

Desde então, dados biológicos vem sendo produzidos em uma taxa surpreendente, os bancos de dados onde se abrigam essas informações dobram a cada ano. Esses dados fundamentam os projetos relacionados ao estudo de expressão gênica, estrutura das proteínas codificadas pelos genes, como os produtos interagem um com o outro e as diversas informações que vão sendo produzidas, tornando-se

essencial a utilização de sistemas e softwares computacionais para pesquisa biológica (Luscombe et al., 2001).

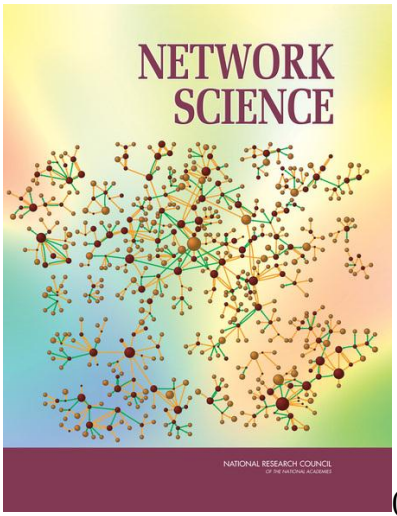
A análise global dos sistemas biológicos é definida pela expressão ômicos, esta reúne uma gama disciplinas interligadas que requerem seu próprio conjunto de instrumentos, técnicas, softwares e banco de dados. A transcriptômica, proteômica e metabolômica contam com o avanço da bioinformática que atua como instrumento de análise de dados de alta capacidade e troca de informações sobre sistemas e códigos biológicos gerenciados por programas computacionais que constroem sistemas *in silico* que simulam as vias e eventos naturais e seus produtos específicos (Wingender et al., 2007; Espíndola et al., 2010).

Apesar de todos os estudos e sequenciamentos já realizados, o conhecimento sobre a complexidade dos sistemas biológicos a nível molecular, em eucariotos ou procariotos, ainda são muito limitados. Sendo assim, a bioinformática explora caminhos no sentido de aumentar substancialmente informações produzidas por seus projetos (Yandell & Majoros, 2002)

A bioinformática permite organizar dados de uma forma que pesquisadores possam acessá-los e submeter novas entradas por eles produzidos, assim como desenvolver ferramentas e recursos que auxiliem na análise desses dados (Berman, 2000). A bioinformática pode utilizar recursos como *text mining* que permite a obtenção de dados não-estruturados a partir de um banco de dados organizado com auxílio de algorítimos. O processo permite a identificação de identidades biológicas e suas interações o que facilita a análise dos dados extraídos. A partir do *Data mining* é possível utilizar diferentes recursos para identificar novas informações ou padrões que sejam úteis no entendimento das identidades biológicas (Espíndola et al., 2010).

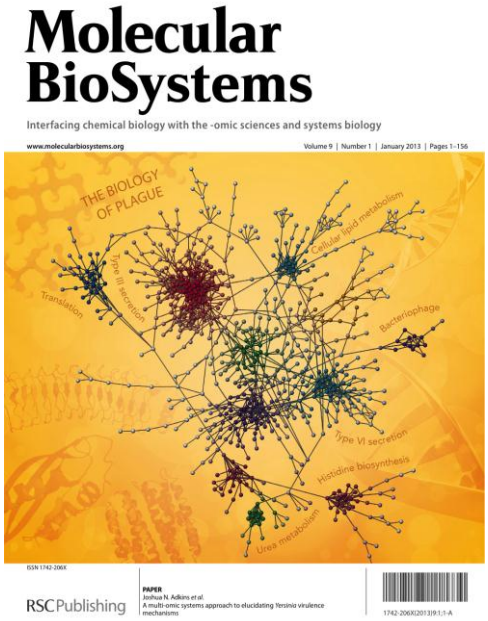
Para determinar uma associação funcional aos dados biológicos experimentais são empregadas na construção de redes que compreendem as interações entre proteína-proteína, grupos de genes mostrando a correlação significativa da expressão e interações genéticas comparando indivíduos mutantes e o aparecimento de patologias. Essas abordagens aumentam a confiança e o entendimento das interações ganhando um contexto lógico nas associações como uma visão complementar aos paradigmas tradicionais (Babu et al., 2009; Janga et al., 2011).

A grande disponibilidade de dados de sequencias genômicas possibilita um estudo comparativo produzindo informações importantes sobre o papel realizado por determinadas moléculas, processos de desenvolvimento e mecanismos de defesa em condição patológica (Eichler & Sankoff, 2003; Rubin et al., 2000). Técnicas laboratoriais convencionais como proteoma por si só, não são suficientes para a compreensão da maquinaria complexa em que os componentes celulares estão inseridos, as características fundamentais entre os organismos e as variações metabólicas que ocorrem em cada etapa do desenvolvimento não são decifradas por simples estudos genéticos e proteicos independentes. Desta forma, analisar todas as relações entre as moléculas de interesse permite ampliar os conhecimentos sobre complexidade funcional, diversidade dos sistemas biológicos e suas interações (Ng et al., 2003). A arquitetura dessas interações e sua organização são bem representadas na forma de redes, como por exemplo, redes de interação de proteínas (Figura 11) onde são apresentadas as vias bioquímicas e regulação genética (Bolser & Park, 2003).



(A)

(B)



(C)

Figura 11: Capa das revistas Science, 2005 (A), Genome Research, 2009 (B) e Molecular Biosystems (C) mostrando associações em rede em estudos genéticos com associação a doenças.

A análise das interações moleculares em rede tem revolucionado as análises genéticas, mostrando não somente a importância das funções protéicas, mas também

suas inter-relações. Desta forma, a visão da biologia vem sendo amplianda quanto ao entendimento da progressão de doenças, diagnóstico e tratamento (Kann, 2007). Isso, devido aos fatos de que os métodos computacionais, incluindo programas, softwares e banco de dados, se apresentam como uma ferramenta mais rápida do que as tecnologias experimentais atuais, para mapear, atribuir e prever as interações moleculares pretendidas por toda sequencia genômica (Pellegrini et al., 1999; Tan, 2004).

A idéia principal dessas redes de interação biomolecular é trabalhar na previsão de novos genes associados a doenças, uma abordagem promissora na tentativa de integrar dados em uma rede física ou funcional (Ideker & Sharan, 2008). Goh et al. (2007) criou uma network de doenças humanas/associação de genes humanos onde cada gene conhecido era documentado no banco de dados Online Mendelian Inheritance in Man, encontrando tendências entre as interações das proteínas envolvidas e seus produtos formados (Goh et al., 2007).

Os métodos em rede no estudo de doenças humanas estão recebendo uma atenção cada vez mais significativa, a fim da interpretar os efeitos da ação dos genes sobre a população humana, se caracterizando como um poderoso meio para mapear esses mecanismos moleculares. Apesar disso, ainda é necessário a complementariedade de informações, uma vez que os dados em networks ainda escassos. Além disso, as estruturas computacionais existentes ainda são inadequadas para lidar com a explosão de dados e informações (Ideker & Sharan, 2008).

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3.0 Objetivos

3.1 Objetivo Geral

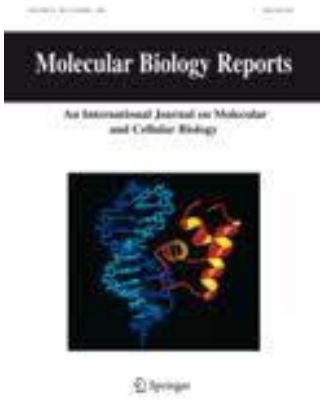
Associar o polimorfismo T-786C da região promotora do gene óxido nítrico sintase (eNOS) com os níveis lipídicos através do colesterol total, HDL, LDL e triglicerídeos, e níveis da proteína C-reativa em amostras de mulheres no terceiro trimestre de gestação.

3.1 Objetivos Específicos

- Extrair o DNA das amostras do grupo das mães e dos recém-nascidos;
- Desenhar primers para identificação do polimorfismo T-786C da região promotora da eNOS;
- Determinar a presença do polimorfismo da eNOS T-786C de região promotora;
- Associar os níveis lipídicos (Colesterol total, HDL, LDL e triglicerídeos) e proteína C-reativa com a presença do alelo C^{-786};
- Desenhar a rede de proteínas envolvidas no funcionamento da eNOS a partir de ferramentas de bioinformática;
- Prever a associação de mutações no gene da eNOS no desenvolvimento de patologias, a partir de ferramentas de bioinformática aliadas à *data minning* e *text minning*.

Manuscrito a ser submetido a revista Molecular Biology Reports

(Fator de impacto: **2,506 em 2012 Qualis B1 na área de Ciências Biológicas I)**



Influence of T-786C polymorphism over lipid and C-reactive protein profile in third semester pregnant women

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Abstract

Changes in lipid metabolism are needed in the course of normal pregnancy for homeostasis between mother and fetus, in addition to maintaining the integrity of the vascular endothelium. Abnormal lipid levels cause endothelial dysfunction causing complications for mother and developing fetus, reducing oxide production oxide (NO) major regulator of fetal-placental events. Our research aimed at the study on T-786C polymorphism in the eNOS gene, the main source of NO, and molecules associated with its regulation, such as lipids and C-reactive protein (CRP). Samples from 92 pregnant women were subjected to DNA extraction; identification of T-786C polymorphism was performed by PCR-RFLP. Total cholesterol, cHDL, cLDL, triglycerides and hs-CRP levels were evaluated according to genotypes, but no statistical significance was observed, except for hs-CRP. This molecule acts directly on eNOS regulation by decreasing its bioactivity. Then, further studies should be performed to improve the knowledge about the role of CRP in pregnancy and the prediction of endothelial dysfunction and cardiovascular disease.

Keywords: Lipids, polymorphisms, Nitric Oxide, C - reactive protein.

Introduction

Pregnancy is associated with significant change in the functions of normal liver, despite the precise mechanisms underlying these various alterations are not clear in every case. Homeostasis between mother and fetus is supported by the integrity and regulation of endothelium [1].

A hyperlipidemic third-trimester is a result of great increase in Triglyceride (TG) level, with cholesterol and phospholipid levels increasing more modestly. LDL levels raises during the course of pregnancy, which seems normal despite some studies demonstrate no change during the course of pregnancy. Non-uniform findings are also related to lipid role in maternal and fetal diseases, like LDL role in pre-eclamptic, showing increased, decreased, or equivalent level compared to healthy pregnant controls [2]. Similarly, high levels of maternal TC are associated with pre-term birth (PTB) [3] [4, 5], as well low levels [6].

Indeed, modifications in lipid profile directly can cause endothelial dysfunction [7] leading to a reduction in disposal vasodilators molecules, mainly nitric oxide (NO·), which is the main regulator of fetal-placental events [8]. NO· is a free radical gas, labile and able to diffuse freely across the cell membrane, which facilitates its biological activity [9]. Functionally, NO· is a potent inhibitor of platelet aggregation and leukocyte adhesion to the vessel wall, protecting against the onset of atherogenesis and prevents fibrous plaque formation [10].

In endothelial progenitor cells (EPCs), NO· synthesis is catalyzed by endothelial nitric oxide synthase (eNOS) through the oxidation of L-arginine to L-citrulline aided by co-substrates such as NADPH and oxygen. eNOS is the predominant NOS isoform in the vasculature, among three isoforms which generates NO [10]. Then, the decreased eNOS

expression is also related to endothelial dysfunction, despite several studies have shown that cardiovascular risk factors are associated with an increase rather than a decrease in eNOS expression [10].

eNOS is regulated by multiple molecules, like caveolin, high-density lipoprotein cholesterol (HDL), estrogen and C-reactive protein (CRP). CRP, a prototypic marker of inflammation, has been shown to predict cardiovascular events in apparently healthy persons [11]. In fact, CRP appears to be a stronger predictor of vascular death than LDL cholesterol and adds prognostic value to conventional Framingham risk assessment [12]. Through its action on eNOS, CRP directly quenches the expression of eNOS and diminishes NO· production, and may serve to impair EPCs function and promote apoptosis through receptor for advanced glycation end-products (RAGE) [13], also regulating endothelial cell growth and migration [14].

eNOS gene is located on chromosome 7 (7q35-36), contains 26 exons constitutively expressed [15]. Gene polymorphisms in eNOS and resultant impacts on eNOS expression levels have been associated with increased risk of hypertension, as well as a variety of conditions affecting the coronary circulation, including coronary artery disease and coronary spasm. Several studies also associate eNOS combined haplotypes of the T-786C, Glu298Asp, and intron 4 polymorphisms with incidence of hypertension and plasma NO metabolites [16]. This study aimed to investigate eNOS T-786C polymorphism in pregnant women in third-trimester associated to CRP and lipid levels.

Materials and Methods

Study population and sample collection

This transverse study evaluated 92 mothers aged between 18 and 40 y/o with gestational age between 34 and 40 weeks attended at Hospital Candida Vargas - João Pessoa, Brazil. This study was received approval from Ethics Committee (CEP-HAM No. 83/05), being conducted after each woman provides written consent.

DNA Extraction

The blood samples were subjected to DNA extraction and stored at -20^{o}C for subsequent analysis of genetic polymorphism. The genomic DNA was extracted using the Wizard® SV Genomic DNA Purification kit (Promega, USA) following the manufacturer's instructions. The quality of extraction was assessed using the reporter gene beta-globin.

Primer design for eNOS T-786C polymorphism and *in silico* PCR

CLCBio software was used for primer design and prediction data as annealing temperature, self-annealing, primer dimmer and hairpin. Primer forward (5'- TGG AGA GTG CTG GTG TAC CCC – 3') and reverse (5'- GGA GGG TTG GGC AGA AGG TGA -3') amplified a fragment of 494 bp. The amplified products were subjected to enzymatic digestion using the restriction enzyme *Msp*I. CC genotype was determined by the presence of 354, 94 and 48 bp

fragments, while TT genotype presented only 354 and 140 bp fragments. CT genotype presented 354, 140, 94 and 48 bp.

T-786C polymorphism analysis

Analysis of eNOS polymorphism was performed by PCR for 25μL final volume containing: 2μL DNA extracted (80 - 100ng/μL), 2μL of each primer (10pmoles), 6,5μL ultrapure water and 12,5μL GoTaq@ Green Master Mix (Promega, USA). PCR conditions were as follows:

95 °C for 2 min; 30 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s; followed by final extension at 72°C for 5 min. The amplified products were subjected to enzymatic digestion using *Msp*I, following manufacture instructions. RFLP analysis was performed by electrophoresis on 1% agarose gels, stained with ethidium bromide.

Biochemical analysis

The levels of total cholesterol (TC), cHDL, cLDL and triglycerides (TG) were measured in serum using commercial kit (mg/dL). C-reactive protein was measured using high-sensitive commercial kit (mg/L). The results for theses molecules in third semester pregnancy were evaluated according to the reference levels previous described [17].

Statistical analysis

Statistical analyzes were performed using Prism 6.00 for Windows from GraphPad Software (San Diego, California, USA). Values are expressed as means ±S.D. χ^2 test was used to test categorical variables, and the Hardy–Weinberg equilibrium was used to test the variant frequencies. OR (*odds ratios*) and 95% CIs (confidence intervals) were calculated and value of $p < 0.05$ was considered significant.

Results

T-786C eNOS in lipid and CRP levels in 3th trimester pregnancy

A total of ninety-two healthy pregnant women were selected for study the genotypic distribution of the polymorphisms T-786C located in the eNOS promoter region. The distribution of the three genotypes is under Hardy-Weinberg equilibrium ($p<0.05$). Genotyping analysis shows 66 patients homozygous (TT=71.73%), 24 heterozygous (CT=26.08%) and 2 homozygotes (CC= 2.17%) (p=0.01). The allele C^{-786} was found in low frequency (15.21%) in comparison with the T^{-786} allele (84.78%).

In maternal serum total cholesterol (TC), triglycerides (TG), cHDL and cLDL were measured and compared between TT group and allele C carrier group (Table 1), but no differences were observed.

Otherwise, CRP levels showed a borderline for significant data according to genotypes (Table 1). Then, the comparison between each group showed to be significant (Fig. 1), except for different genotypes in normal group ($p=0.3412$) and in non-normal group ($p=0.3662$).

Table 1. Biochemical analysis for triglycerides (TG), total cholesterol (TC), cHDL, cLDL and high-sensitive C-reactive protein (hsCRP) according to the patient genotype. *p*-value was considered significant <0.05.

	Reference value for 3th trimester pregnancy	**TT**		**C***		***p*-Value**	***Odds ratio* (CI 95%)**
		N	(Min.; Máx.)	N	(Min.; Máx.)		
TG (mg/dL)	Normal (131-453 mg/dL)	53	131; 406	22	131; 338	0.7703	0.7413 (0.2175-2.527)
	Non-normal	13	72; 551	4	70; 620		
TC (mg/dL)	Normal (219-349 mg/dL)	33	219; 330	15	222; 330	0.6437	0.7333 (0.2935-1.832)
	Non-normal	33	147; 212	11	156; 210		
cHDL (mg/dL)	Normal (48-87 mg/dL)	33	48; 83	14	48; 87	0.8187	0.8571 (0.3451-2.129)
	Non-normal	33	24; 91	12	27; 90		
cLDL (mg/dL)	Normal (101-224 mg/dL)	51	101.2; 223	20	103.2; 188.4	1.0000	1.020 (0.3467-3.001)
	Non-normal	15	27.6; 100.2	6	63; 231.8		
hs-CRP (mg/L)	Normal (0.4-8.1 mg/L)	31	2; 7.3	18	2; 7.7	0.0657	0.3937 (0.1502-1.031)
	Non-normal	35	8.2; 96	8	9.3; 63		

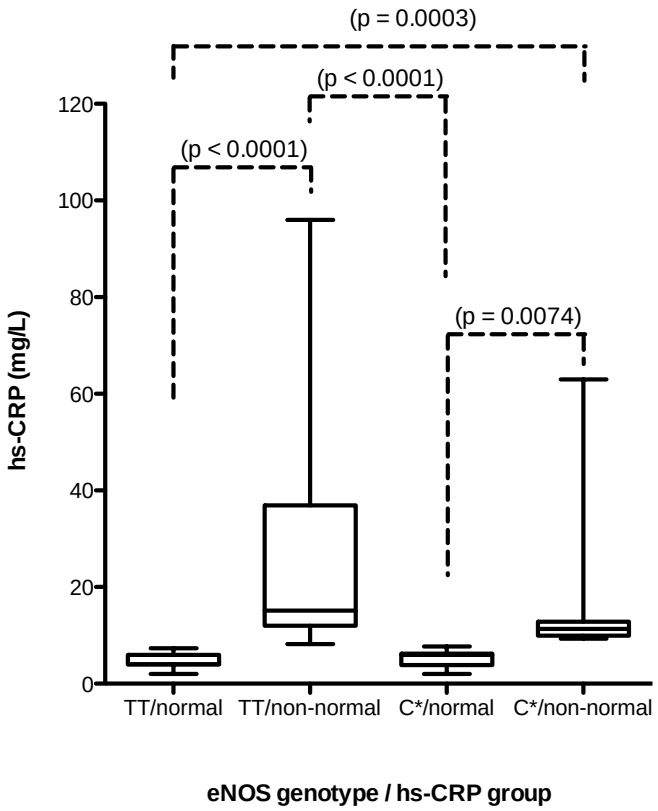

Figure 1. Distribution of eNOS genotypes according to the groups of high sensitive C-reactive protein classified as normal and non-normal levels.

Biochemical values in 3th trimester pregnancy

TG values showed variation from 70 to 620 mg/dL, with median of 194.5 mg/dL and four TG values higher than 500 mg/dL (IQR, 147-255.8) (Figure 2). A total of 65.22% (60/92) patients showed TC median of 222 mg/dL, being 44/92 patients below the reference values assumed in this study (IQR, 184.5-256.3) (Figure 3). cHDL values were in the range of 24-91 mg/dL, being 48.9% patients outside the range, but only 2 patient showed values above the top reference (IQR, 40.25-55) (Figure 4). Regarding to cLDL levels, it was observed a great

variation from 27.6 mg/dL to 231.8 mg/dL, with median of 123.5 mg/dL and 21.73% (20/92) patients showing low cLDL values (IQR, 101.5-161.3) (Figure 5).

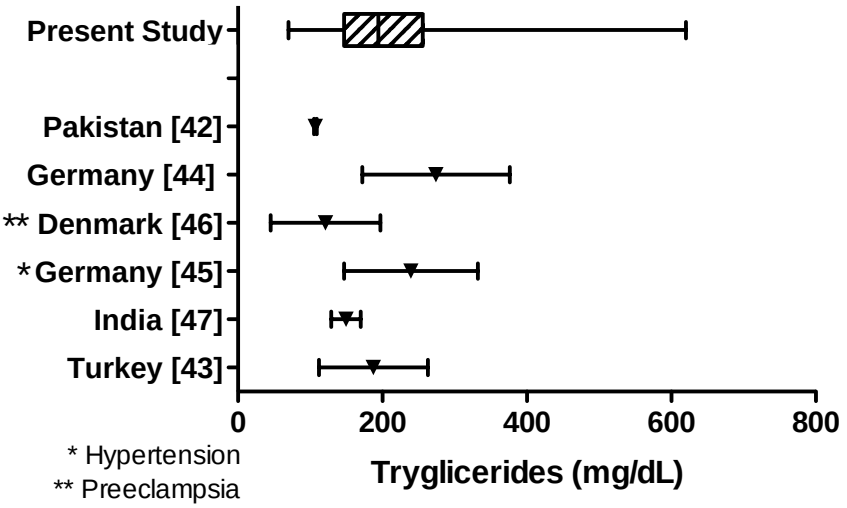

Figure 2. Triglycerides profile from pregnant women in the third trimester in comparison with the values reported for other populations.

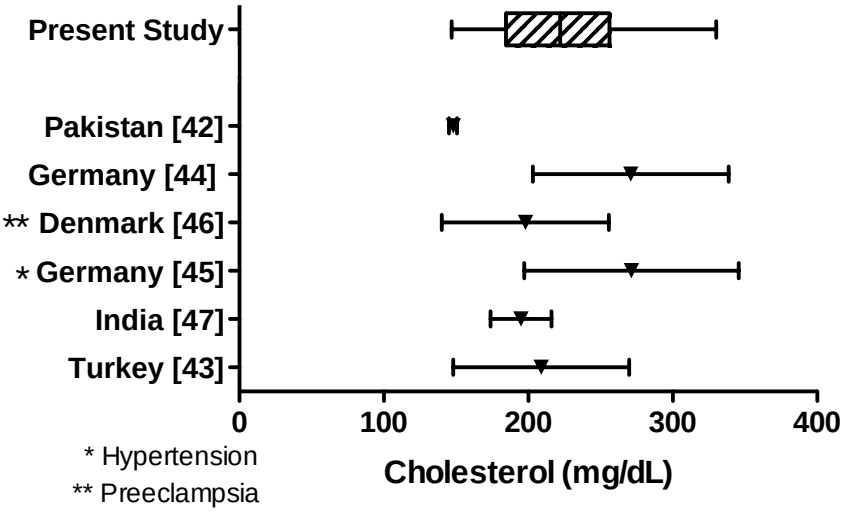

Figure 3. Total cholesterol profile from pregnant women in the third trimester in comparison with the values reported for other populations.

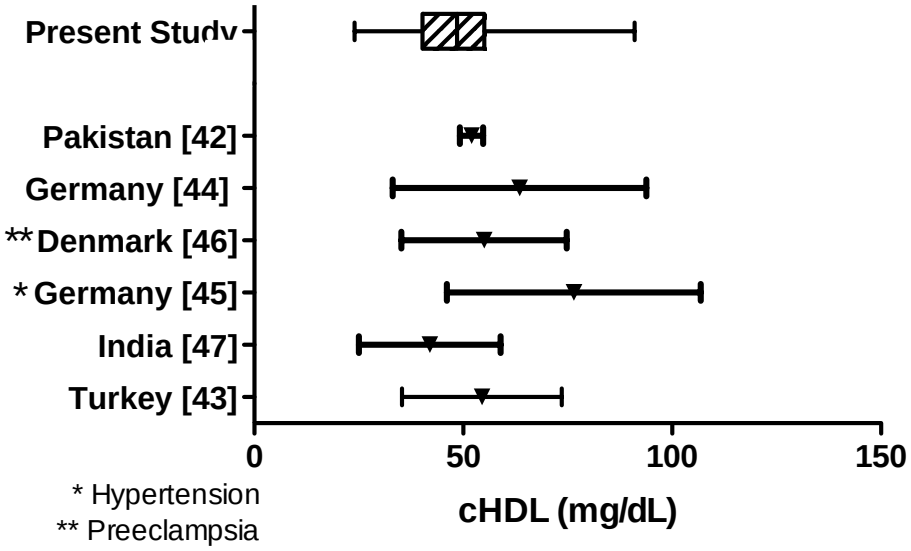

Figure 4. cHDL profile from pregnant women in the third trimester in comparison with the values reported for other populations.

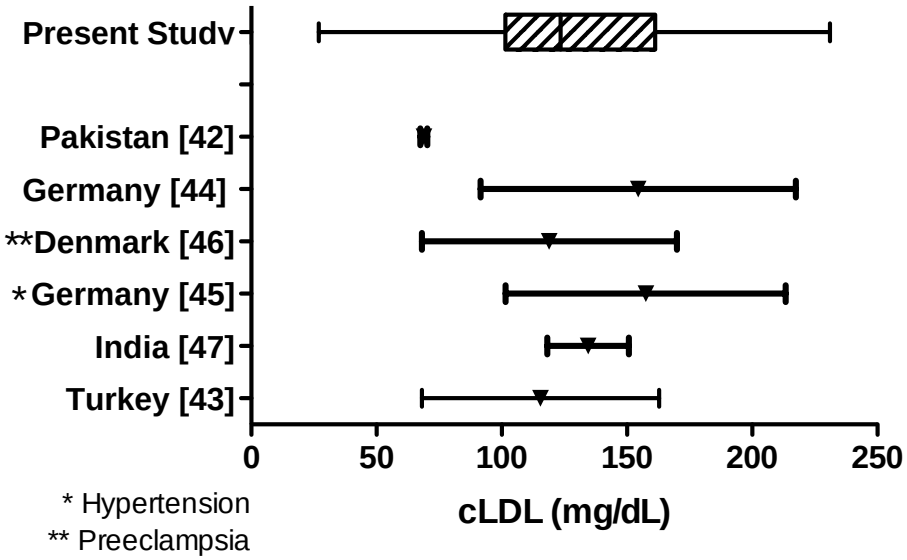

Figure 5. cLDL profile from pregnant women in the third trimester in comparison with the values reported for other populations.

Hs-CRP levels showed large range, varying from 2 to 96 mg/L being 46.74% patients with high level of this molecule (IQR, 4-12) (Figure 6). The comparison between lipid values or hs-CRP and gestational weeks in the pregnant women showed significance for TG and cHDL. hsCRP analysis also revealed significance, showing high values for women in 34-36w and 38-40w (Table 2).

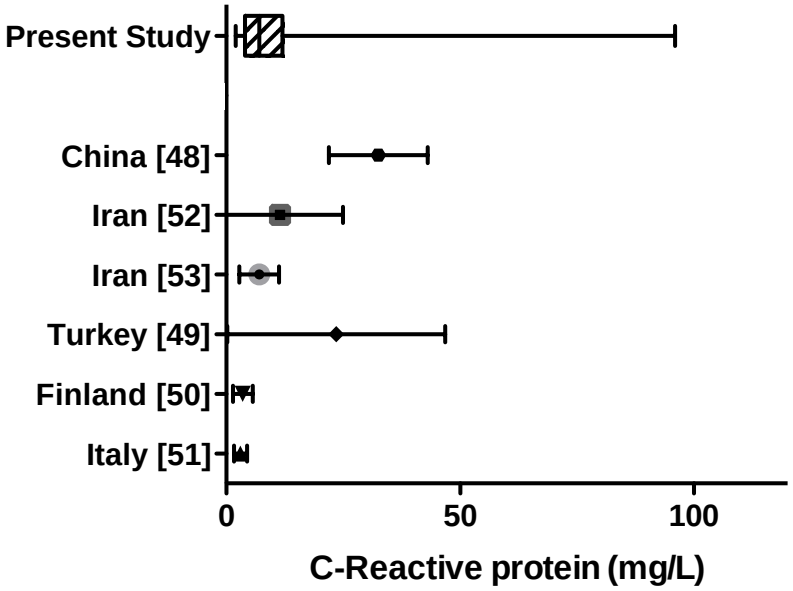

Figure 6. High sensitivity C-reactive protein profile from pregnant women in the third trimester in comparison with the values reported for other populations.

Table 2. Biochemical profile of pregnant analyzed according to gestational age, showing mean and *p* value in each group.

Variable	**34-36 Weeks** (N=8)	**37 Weeks** (N=6)	**38-40 Weeks** (N=78)	***p-value***
TG (mg/dL)	220.5 ± 100.6	238.7 ± 155.0	212.3 ± 99.68	0.0005*
TC (mg/dL)	222.9 ± 53.25	241.8 ± 58.23	222.4 ± 46.32	>0.01
cHDL (mg/dL)	46.0 ± 9.35	42.33 ± 13.72	49.35 ± 13.25	0.0093*
cLDL (mg/dL)	132.8 ± 46.73	151.8 ± 44.21	131.3 ± 40.60	0.0721
hs-CRP (mg/L)	10.28 ± 15.94	6.35 ± 3.22	14.06 ± 15.77	<0.0001*

Discussion

Cardiovascular diseases (CVDs) are a major cause of complications in pregnancy and the number of patients who develop cardiac problems during pregnancy is increasing [18]. Lipid metabolism during pregnancy had been investigated in several studies, reporting regular increase of lipoproteins and cholesterol levels during pregnancy with triglycerides showing the largest increase [2, 19, 20]. These modifications in lipid profile are related to endothelial dysfunction leading to a reduction in disposal vasodilators molecules, including eNOS for NO· production [21]

Table 3. List of pathologies related to T-786C eNOS polymorphism in different populations.

Country	Study design	Pathology	p-value	Reference
Saudi Arabia	287	Coronary artery disease	< 0.0001	[27]
Brazil	417	Gestational hypertension/pre-eclampsia	0.0071	[28]
Brazil	205	Pre-eclampsia	> 0.05	[23]
Canada	n.a.	Cardiac insufficiency	0.2731	[29]
Chile	224	Coronary artery disease	0.777	[30]
China	2179	Hypertension	< 0.05	[31]
India	1170	Type II Diabetes	< 0.05	[15]
India	283	Type II Diabetes	0.004	[32]
Iran	502	Coronary artery disease	0.041	[33]
Italy	860	Atherosclerosis	0.42	[34]
Mexico	582	Pre-eclampsia	> 0.05	[35]
Tunisia	661	Hypertension	0.004	[36]
USA	761	Coronary artery disease	0.47	[37]

n.a. – not available.

Estrogen can also act as transcription factor, gradually modifying the cellular program, including by enhancing eNOS activity [22]. Genomic and nongenomic effects of estrogen on eNOS and antioxidant activities of estrogen are discussed as potential mechanisms of interest in coronary circulation [16]. Then, polymorphisms of the eNOS gene can result in significant associations with incidence of hypertension in health and pregnant women [23]. Indeed, study in adults showed significant association between eNOS polymorphisms and metabolic syndrome in adults, adolescents and children; thus supporting the idea that genetic variations in the eNOS gene are associated with features of metabolic syndrome, and may predispose to insulin

resistance, hypertriglyceridemia, and low HDL-cholesterol concentrations [24, 25].

eNOS T-786C polymorphism was previously related to be associated with coronary disease, hypertension, pre-eclampsia and diabetes mellitus type 2, despite conflicting results (Table 3). The importance of this polymorphism has been demonstrating great importance for clinical support in cardiology area, once pharmacological research showed the restoration of nitrite levels in obese women treated with simvastatin modulated by T-786C polymorphism, with 15% better results in C-allele carrier patients [26].

The eNOS pathway represents a crucial factor for endothelial cell function and may be compromised by C-reactive protein, a biomarker for endothelial dysfunction [38]. It inhibits the activity of eNOS via uncoupling the enzyme [39], blunts eNOS phosphorylation at Ser1179 [40] and decreases its bioactivity [41]. CRP also determines the interaction of eNOS with other proteins, in a negative way, interacting with complex required for its activation leading to endothelial dysfunction [39].

There is no standard profile for lipid levels in the third semester among pregnant women, showing variation according to the population studies. In Pakistan [42] and Turkey [43] the maximal value for TG were below 300 mg/dL, while in Germany [44] the value reached to 400 mg/dL; being still lower than the values found in our analysis. TC values were highest Germany patients shows the highest TC and cLDL values [44, 45], patients from Denmark [46] and Turkey [43] showed the lowest values for both molecules. In India, pregnant women with hypertension showed the lowest value for cHDL [47], as observed in our patients, which could inhibit eNOS expression and lead to endothelial dysfunction.

CRP values shows different profile among the studies, with increased values for patients from China [48] and Turkey [49] compared to Finland [50] and Italy [51]. Two different analysis in Iran also showed different profile [52, 53]. However, no previous study reported so high value for CRP as observed in our population, showing a great risk for the end of the pregnancy and for future cardiovascular disease.

In conclusion, CRP is an important molecule related to eNOS pathway that needs further analysis to allow the comprehension of its role as prediction marker of gestational problems and cardiovascular events in the future. The effect of high levels of CRP should also be investigated in the newborn.

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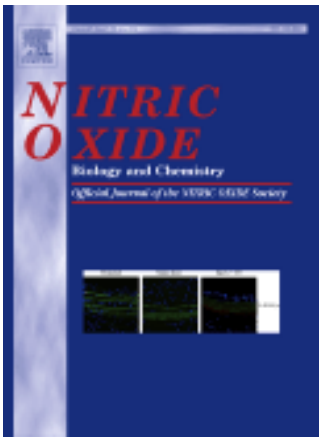

Manuscrito a ser submetido à revista

NITRIC OXIDE: BIOLOGY AND CHEMISTRY

Fator de impacto: 3.548 em 2011,

Qualis A2 na área de Ciências Biológicas I

A protein-protein interaction network for endothelial nitric oxide synthase and cardiovascular disorders

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Abstract

Bioinformatics points as one of the most promising biological sciences applied research of the last decade. The mapping through protein-protein networks enables a greater understanding of the biological context related to the genes of interest. In this context, the relationship between endothelial nitric oxide synthase (eNOS) regulation and development of diseases could be predicted from data obtained from different non-integrated data. In this work, it was performed a systematic search for eNOS polymorphism concerning cardiovascular and metabolic diseases. From this, two networks were obtained in order to understand the participation of different proteins in eNOS pathway, under normal and pathological conditions. eNOS network (eNOSNet) showed 51 nodes and 361 edges of interaction with eNOS protein ligands, small molecules and their complexes. A eNOS network under pathological conditions (eNOSNetD) showed its great importance in cardiovascular disease. However it was also related to neurological, neoplasic and hormonal diseases, being linked to the metabolism of lipids, carbohydrates and protein.

Keywords: Bioinformatics, Polymorphism, Nitric Oxide.

Introduction

Nitric oxide (NO) is an endogenously produced gas, one of the simplest short-lived free radicals and diffusible enough to act as a signaling molecule in the whole organism (Fleming, 2003; Gopi et al., 2010; Choudhari et al., 2013) One of its most important activities is the regulation of vascular tone and blood pressure, identified in 1987 as a factor of endothelium-derived relaxing by Ignarro et al. NO is responsible for a variety of cellular and physiological processes including angiogenesis, cell proliferation, degradation of the extracellular matrix and migration of endothelial cells. Furthermore, it has anti-inflammatory function by inhibiting the adhesion of leukocytes on the vessel wall. The loss of endothelial function seems to be a crucial step in the appearance of distinct pathologies (Kubes et al., 1991; Ignarro et al., 2002; Gopi et al., 2010).

NO is synthesized by enzymes belonging to the group of nitric oxide synthase (NOS) present in mammalian cells and three different genes encode: NOS 1 or neuronal NOS (nNOS), NOS 2 or inducible NOS (iNOS) and NOS 3 endothelial NOS (eNOS) (Alderton et al., 2011). nNOS and eNOS are constitutively expressed isoforms, being regulated by calcium and calmudolin and post translational modifications. Otherwise, iNOS is regulated by cytokines stimulation and produces greater amounts of NO than the other two isoforms (Asano et al., 2003; Napoli et al., 2006).

eNOS is a homodimer with the ability to convert L-arginine and O2 to L-citrulline and NO. Two structural domains were identified in this molecule, NOS oxygenase and reductase. The domain reductase receives electrons released from NADPH and transfers through molecules into the oxygenase domain or N-terminus. The transfer of electrons is allowed for binding calcium/calmudolina complex in a specific binding site. In oxidase domain there is a catalytic site responsible for NO production and binding of BH4, L-arginine and heme iron (Jan et al., 2011). The enzyme requires dimerization and stabilizing the dimeric form for activation, a key step to catalyze the reaction of formation of NO (Zou et al, 2002; Grijalva et al., 2008).

The reductase domain, or C-terminal, has binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH. Through the oxidation of NADPH to NADP+ electrons are released one at time, also mediated by FAD, toward the heme group in the oxygenase domain where occur the reduction of molecular oxygen in O^- and the conversion of arginine linked to L-citrulline and NO (Andrew et al., 1999; Gielis et al., 2011).

The relationship between eNOS regulation and development of diseases is intimal once NO develops regulatory functions, and protective mediators in various tissues. The enzyme eNOS is found in endothelial cells, myocytes and vascular smooth muscle cells, being the primary source of NO generated in the vascular system, thus changes in eNOS expression or even in related proteins can lead to a decrease in the production and consequent bioavailability of NO and endothelial dysfunction. Endothelial dysfunction is a crucial pathophysiological that occurs in the early stages of many diseases, serving as an early marker for cardiac system diseases, diabetes, preeclampsia among others (Koukoura et al., 2012).

In this paper we visualize through a network the interactions between eNOS and the major regulatory proteins through bioinformatics tools in order to understand the mechanism that involves the various pathologies associated.

Methods

Literature and Data minning

It was performed a systematic review of the literature on the subject through the Pubmed and ScienceDirect databases for the period between 2000 and 2013. The terms used for searching in English were: “polymorphism of eNOS gene”, “cardiovascular diseases”, “metabolic diseases” and “diseases related to eNOS polymorphisms”. The titles, abstracts, and full text were carefully read to exclude papers that showed other results than cardiovascular diseases.

In silico analyses

The open source software Cytoscape (http://www.cytoscape.org/) was used for importing, data from databases like UniProt databases, Ractonme, MINT, NCI / Nature Pathway Interaction Database, IntAct and BioGrid, setting up and analyzing the data. It was build a proteins network related to eNOS with nodes (vertex) representing proteins, small molecules (oxygen, nitric oxide, etc.) or complex (stable interaction between two or more proteins) and edges (which are relationships established between two nodes).

eNOS network consists of regular protein ligands, small molecules and their complexes, presented with JGraph Layouts, being called eNOSNet (eNOS Network). The relationships between SNP T-786C eNOS and the proteins related to eNOS were used to build eNOSNetD (eNOS Network and Diseases) through Gaph 0.8.2 software. The edge "protein and change" was used only if the target protein had been described in the literature as having correlation with

the disease. Therefore, proteins that had high-grade edge value are assumed as having multiple relationships with various diseases. Diseases that showed high-degree edge value are assumed to involve a large number of proteins in eNOS network. eNOSNetD follows the behavior of a bipartite graph, where the proteins interact only with diseases, without self-interaction among diseases or proteins.

Results

A total of 780 eNOS polymorphisms, being associated to many pathologies including: cardiovascular diseases, metabolic syndrome, glaucoma, neurodegenerative diseases, migraine and erectile dysfunction, among others. An accurate analysis showed only 27 SNPs recognized as clinical correlated. However, only 14 SNPs could be direct related to cardiovascular and metabolic diseases in 70 articles (Table 1).

dbSNP ID	Disease	N	Population	Statistical correlation	Technologies	Reference
rs1800783 (allele A/T)	Hypoxic-ischemic encephalopathy	110 cases 128 controls	Croatian	Yes	RT-PCR	"Kuzmani_ _amija et. al., 2011"
rs1800779 (allele G/A)	Hypertension	18,436	American	No	Immobilized probe-based assay	"Conen, et. al., 2008"
	T2D	24,309	American	No	Immobilized probe-based assay	"Conen, et. al., 2008"
	Hypertension	18,738	American	No	Immobilized probe-based assay	"Conen, et. al., 2009"
rs2070744 (allele T/C)	Atherosclerosis	316 cases 544 controls	Italian	No	PCR-RFLP, RT-PCR	"Fatini, et. al., 2004"
	CAD	761	American	No	TaqMan	"Zhang, et. al., 2006"
	PE	230 cases 352 controls	Mexican	No	RT-PCR	"Várquez, et. al., 2013"
	CAD	142 cases 145 controls	Saudi arabian	Yes	RT-PCR, PCR-RFLP	"Alkharfy, et. al., 2010"
	CAD	112 cases 112 controls	Chilean	No	PCR-RFLP	"Jaramillo, et. al., 2010"
	Gestational hypertension, PE	122 cases 295 controls	Brazilian	Yes	RT-PCR, chemiluminescence	"Muniz, et. al., 2012"
	Hypertension	288 cases 373 controls	Tunisian	Yes	PCR-RFLP	"Jemaa, et. al., 2011"
	PE	107 cases 98 controls	Brazilian	No	RT-PCR, chemiluminescence	"Sandrim, et. al., 2010"
	CAD	228	Turkish	No	PCR-RFLP	"Alp, et. al., 2009"
	Heart failure	169	Canadian	No	PCR-RFLP	"Zakrzewski-Jakubiak, et. al., 2007"
	T2D	970 cases	Indian	Yes	PCR-RFLP	"Shah, et. al., 2013"

		200 controls				
	T2D	162 cases 121 controls	Indian	Yes	RT-PCR	"Narne, et. al., 2013"
	CAD	241 cases 261 controls	Iranian	No	PCR-RFLP	"Salimi, et. al., 2012"
	Hypertension	1061 cases 1118 controls	Chinese	No	PCR-RFLP	"Xin, et. al., 2009"
rs1799983 (allele G/T)	PE	230 cases 352 controls	Mexican	No	PCR, RT-PCR	"Várquez, et. al., 2013"
	Stenosis	932	Korean	Yes	PCR-RFLP, DNA sequencing	"Yoon, et. al., 2005"
	CAD	761	American	No	TaqMan	"Zhang, et. al., 2006"
	Metabolic syndrome	632	Brazilian	Yes	PCR-RFLP	"Piccoli, et.al., 2008"
	Nephrotic syndrome	86 cases 114 controls	Turkish	No	PCR-RFLP	"Alasehirli, et. al., 2009"
	CAD	142 cases 145 controls	Saudi Arabian	Yes	Allele-specific PCR, PCR-RFLP	"Alkharfy, et. al., 2010"
	CAD	112 cases 112 controls	Chilean	No	PCR, PCR-RFLP	"Jaramillo, et. al., 2010"
	CAD	116 cases 119 controls	Egyptian	Yes	PCR	"Abdel-Aziz, et. al., 2012"
	Gestational hypertension, PE	122 cases 295 controls	Brazilian	Yes	RT-PCR, chemiluminescence	"Muniz, et. al., 2012"
	Oxidative stress	560	Korean	Yes	TaqMan	"Kim, et. al., 2012"
	PE	101.042	Danish	Yes	RT-PCR	"Lykke, et. al., 2012"
	CAD	253 cases 174 controls	Indian	No	PCR-RFLP	"Rai, et. al., 2012"
	Isquemic stroke	558	Chinese	Yes	RT-PCR	"Yan, et. al., 2011"

	PE	107 cases 98 controls	Brazilian	No	RT-PCR, chemiluminescence	"Sandrim, et. al., 2010"
	Oxidative stress	236 cases 237 controls	Germany	No	PCR and Pyro-sequencing	"Funk, et. al., 2009"
	Isquemic stroke	1293 cases 4021 controls	European	No	GoldenGate genotyping assay	"Matarin, et. al., 2013"
	CAD	228	Turkish	No	PCR-RFLP	"Alp, et. al., 2009"
	Heart failure	169	Canadian	No	PCR-RFLP	"Zakrzewski-Jakubiak, et. al., 2007"
	T2D	24,309	American	No	PCR real time	"Conen, et.al., 2007"
	Oxidative stress	437	158,303	No	GoldenGate genotyping assay	"Sarah e Harris, et. al., 2007"
	Vascular stiffness	1157	American	No	PCR	"Mitchell, et. al., 2007"
	Hypertension	24,309	American	No	RT-PCR	"Conen, et.al., 2009"
	T1D e CVD	458 cases 319 controls	Finnish and Swedish	Yes	TaqMan, DNA sequencing	"Möllsten, et. al., 2009"
	Hypertension	1061 cases 1118 controls	Chinese	No	PCR-RFLP	"Xin, et. al., 2009"
	Hypoxic-ischemic encephalopathy	110 cases 128 controls	Croatian	No	RT-PCR	"Kuzmani_ _amija et. al., 2011"
	PE	230 cases 352 controls	Mexican	No	PCR	"Várquez, et. al., 2013"
Intron 4 (4b4a VNTR)	Hypertension	1061 cases 1118 controls	Chinese	No	PCR-RFLP	"Xin, et. al., 2009"
	PE	107 cases 98 controls	Brazilian	No	RT-PCR, chemiluminescence	"Sandrim, et. al., 2010"
	Gestational hypertension, PE	122 cases 295 controls	Brazilian	No	RT-PCR, chemiluminescence	"Muniz, et. al., 2012"
	Hypertension	1514	European	Yes	Illumina NGS	"Salvi, et. al., 2012"

rs3918226 (allele C/T)	Metabolic syndrome	20,806	American	No	TaqMan	"Goulart, et. al., 2009"
	Hypertension	18,738	American	No	Immobilized probe-based assay	"Conen, et. al., 2009"
	Hypertension	18,436	American	No	Immobilized probe-based assay	"Conen, et. al., 2008"
	T2D	24,309	American	No	Immobilized probe-based assay	"Conen, et. al., 2008"
	Hypertension	181	Brazilian	No	TaqMan	"Luizon, et. al., 2012"
	Vascular stiffness	1157	American	No	PCR	"Mitchell, et. al., 2007"
rs1800781 (allele G/A)	CAD	761	American	Yes	TaqMan	"Zhang, et. al., 2006"
rs1541861 (allele A/C)	Hypoxic-ischemic encephalopathy	110 cases 128 controls	Croatian	Yes	RT-PCR	"Kuzmani_ Samija, et. al., 2011"
rs3918227 (allele A/C)	Oxidative stress	560	Korean	Yes	TaqMan	"Kim, et. al., 2012"
rs2853796 (allele G/T)	Oxidative stress	437	158,303	No	GoldenGate genotyping assay	"Sarah e Harris, et. al., 2007"
rs3918232 (allele G/A)	T1D, CVD	458 cases 319 controls	Finnish and Swedish	Yes	TaqMan, DNA sequencing	"Möllsten, et. al., 2009"
rs743507 (allele A/G)	Heart attack	434	American	No	Mass spectrometry	"Podgoreanu, et. al., 2006"

CAD: coronary artery disease; PE: pre-eclampse; T1D: type I diabetes; T2D: type II diabetes; CVD: cardiovascular disease; RT-PCR: real tim PCR; PCR-

FLP: restriction fragment length polymorphism; NGS: next generation sequencing.

eNOSNet contains 51 nodes and 361 edges of interaction (Figure 1), representing the group of proteins and others molecules involved in the activation pathway of eNOS for NO production. A total of 13 proteins were enrolled in this pathway, together with 19 small molecules and 19 complexes for eNOS activation.

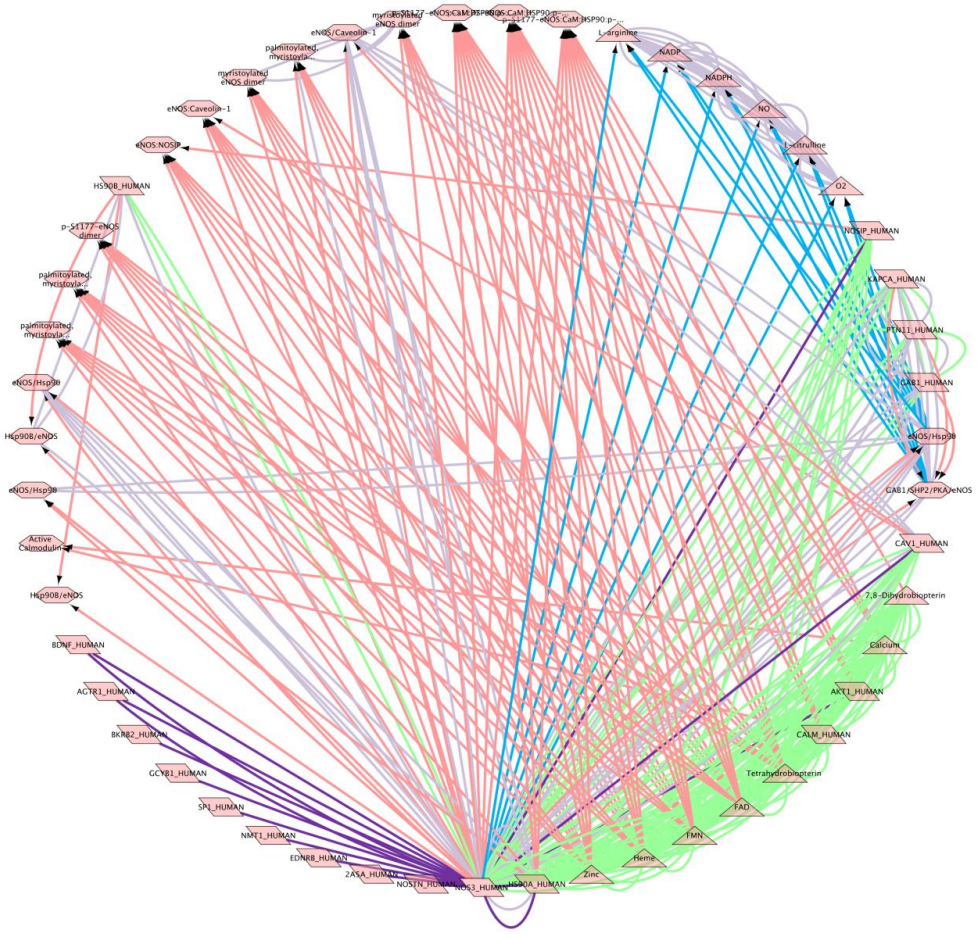

Figure 1. The proteins are shown in diamond, the complexes are in the form of a hexagon and small molecules are triangles. The edges in turn are differentiated according to their color: (1) light pink - "component" represents molecules that binds a protein and its complex; (2) purple - "interacts with" brand binding proteins that interact without forming complexes; (3) light green - "in the same component" represents components that are part of the same complex; and (4) lilac - “reacts with" means that interaction involves conversion of substrates and their products.

Each component establishes a different relationship between them, marked by an edge, which differed in color by its type. eNOSNetD had 98 interactions like "component of ", 148 indicating the interaction "in same component," 16 indicating "interacts with" 18 edges represent " metabolic catalysis" and 81 edges indicate the interaction "reacts with". The protein which showed the largest the network level was eNOS with 93 interactions, but the network also has molecules with a low degree of interaction as in the case of NOSTIN, NMT, 2A5A, NMT, SP1, GCYB1, BKRB2, EDNRB that relates only once eNOS.

The second network NeNOSeD (Figure 2) contains 27 nodes and 361 edges of interaction as a result of the analysis between proteins and diseases extracted from the literature. Cardiovascular disease showed the greatest number of interaction with proteins involved in eNOS network, being linked to 16 proteins due to the great number of studies conducted in this field. However, neurological and hormonal diseases also appear in this network, with 5 proteins linked in each one. Muscular diseases showed 7 proteins involved and neoplasms were linked to 11 proteins showing a promising area of analysis. Diseases related to dysfunction in lipid (11 proteins), protein (6 proteins) and carbohydrate (6 proteins) metabolism were also related to this network.

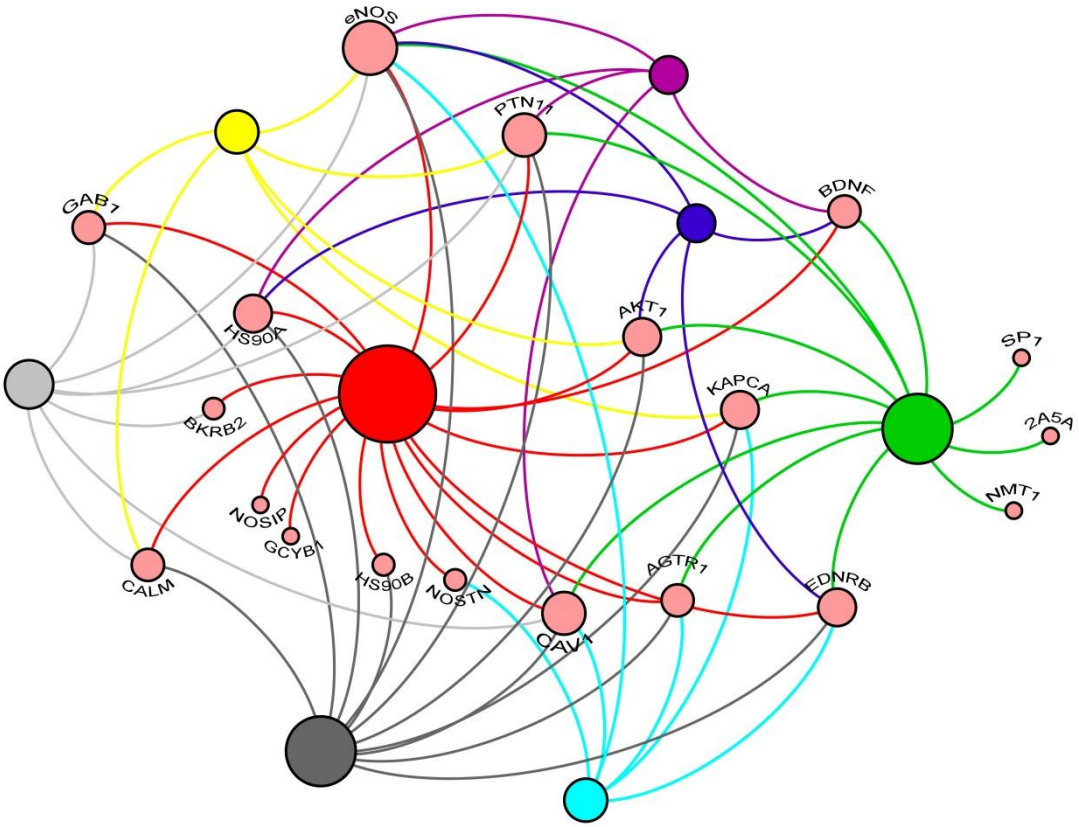

Figure 2. The distinct disorders are displayed in different colors and the sizes by the number of interactions generated by the network in which the proteins are involved. The size of the proteins and diseases are proportional to the amount of interactions that exist between them. Diseases are labeled by color as follow: red – cardiovascular diseases; dark grey – neoplasms; purple – hormonal diseases; dark blue – neurological diseases; light grey – muscular diseases; yellow – diseases related to carbohydrate metabolism; light blue – diseases related to protein metabolism; green – dyslipidemias.

Discussion

The result of systematic research on diseases related to polymorphisms in eNOS gene (Table 1) showed the relevance of the biological role of eNOS and its mutations. The different populations show different significance for the same pathology. It is possible to observe different associations in the general population, with increased number of data with different ethnicities. It may suggests that such heterogeneity of the disease, together with genetic and environmental factors (Gao et al., 2006), as well as heterogeneity of the case and control groups studied may influence the clinical correlations.

Each molecule in the network establishes a different relationship with eNOS according to the stimulation received from the cell membrane by specific receptors to post and co-translational modifications. These stimuli can be made by kinases, phosphatases and transferases, in addition to the molecules that form complexes in order to stabilize the molecule in its coupled state, allowing the passage of electrons.

eNOS molecule resides in a region of the plasma membrane called Caveolae, which is rich in cholesterol and sphingolipids. The enzyme undergoes co- and post-translational changes in order to become active. The co-translational myristoylation modification target in an anchor acyl N-terminal domain of eNOS catalyzed by a N-myristoyltransferase. The post-translational palmitoylation occurs at residues Cys15 and Cys26 (Fukata et al., 2007) to confer to eNOS three acyl anchors, allowing its attachment to the lipid bilayer caveolar. In the caveolae, eNOS forms an inhibitory complex with the caveolin 1 protein (Cav-1) and calmudolina (CaM), inhibiting the transfer of electrons from NADPH. However, the binding of calcium (Ca^{+2}) results in decoupling of Cav-1, so eNOS is sent to the cytoplasm for other regulatory processes. Then, intracellular calcium is critical for catalytic function of the enzyme for breaking inhibitory interaction with cav-1 (Davignon & Ganz, 2004; Bulotta et al., 2006).

This process recruits Akt kinase to phosphorylate the eNOS serine residue 1177 (ser1177), important for the activation process. The connection with BH4 stabilizes the molecule as it provides electrons for the iron complex to initiate the oxidation of L-arginine and its depletion can "uncoupling" the dimer eNOS leading to formation of superoxide (O2-) instead of NO (Davignon & Ganz, 2004; Vazquez-Vivar et al., 2002). Phosphorylation and dephosphorylation act as a complement to the post-translational modifications to eNOS activation. Serine and Threonine residues are regulatory, once phosphorylation of Ser1177, Ser635 and Ser617 are responsible for effects on increasing the activity of the enzyme, while phosphorylation at Ser116 and Thr497 are inhibitory processes. Kinases involved in this process include: Akt kinase, PKA (cyclic AMP-dependent protein kinase), AMP-activated protein kinase (AMPK), cyclic GMP-dependent protein kinase, and calcium / calmodulin-dependent protein kinase II (CaM kinase II) (Balligand et al., 2009; Dudzinski & Michel, 2007).

The dephosphorylation may activate or inhibit eNOS depending on the specific site. Phosphatases are not only serine-threonine protein phosphatase 1, serine-threonine 2A protein phosphatase, calcineurin participants of amino acid residues phosphorylation of eNOS, but also caveolin and calmudolina (Dudzinski et al 2006; Michel & Vanhoutte, 2010).

Proteins Partners

The caveolin, a membrane protein of 22 kDa ubiquitously expressed in their isoforms 1 and 2 in endothelial cells while isoform 3 is muscle-specific expressed in cardiomyocytes and skeletal muscle. Its connection with the eNOS prevents signal transduction by receptors attached to Caveolae (Gratton et al., 2004).

Hsp 90 chaperones act in the transport and folding for activation of eNOS, this binding stimulates the improvement of affinity binding with calmodulin balancing the production of

NO. Hsp90 interaction is required for interaction Akt/eNOS therefore increases the rate of phosphorylation of Akt (Sessa, 2004; Takahashi et al., 2003; Dudzinski & Michel, 2007).

The actin cytoskeleton is critical for translocation reversibly between plasmalemma and golgi. In addition to effects from shear stress transduces its effects via actin-based cellular architecture. The CAT-1 protein responsible for the transport of arginine regulates eNOS by actin filaments (Su et al., 2003).

NOSIP assists in the transport of eNOS from the membrane caveolar other internal membranes for binding to caboxi-terminal oxygenase domain. This association is inhibited by cav-1. NO release is decreased by overexpression of NOSIP, which possibly leads to uncoupling of eNOS from its attachments caveolares (Dedio et al., 2001; Dudzinski & Michel, 2007).

Mainly expressed in endothelial cells and in highly vascularised tissue, eNOS trafficking inducer protein (NOSTRIN) interacts with eNOS through its central domain and cav-1. This complex occurs at the plasma membrane and improves bonding of eNOS with other molecules. Overexpression of NOSTRIN promotes the displacement of eNOS to intracellular vesicles decreasing its activity (Zimmermann et al., 2002).

The proper function of eNOS is characterized as key step in the development of these pathologies. The regulations of these proteins in the activation of eNOS ensure the normal functioning of the various systems in which NO acts. Each protein has its role in the regulation of eNOS, inhibition, binding radicals acyl, phosphorylation and dephosphorylation the molecule, enabling the coupled state of the essential enzyme for NO production (Balligan et al., 2009). Endothelial dysfunction caused by decreased activity of eNOS has been a common point reported for these diseases. In this condition the endothelium have a reduced vasodilatory response to chemical mediators such as acetylcholine, bradikinin, endothelin, or angiotensin II and are followed by an elevated expression of adhesion molecules, enhanced vascular smooth muscle proliferation and the development of a hypercoagulatory state. Changes in endothelial

function disrupt homeostasis and presents itself as a predictor for pathologies represented here (Triggle at al., 2010).

The proteins are represented by their interactions with groups of pathologies expressed by the network. For example, Cav-1 is related to disorders in the negative regulation of eNOS leading to deregulation in response to hypoxia, vasoconstriction, vasculogenesis and blood coagulation, regulation of muscle contraction, lipid storage, response to stimulation by estrogen featuring its participation in different disease groups: cardiovascular, lipid and glucose metabolism and hormonal diseases (Bulotta et al., 2006; Kolluru et al., 2010; Garcia et al., 2012). Likewise, the AKT protein, which participates in phosphorylation of Ser1177 eNOS relates events in signaling pathway insulin-like growth factor receptor, glucose transport, maintenance of peripheral nervous system and cell proliferation being connected in the network to groups of the lipid and glucose metabolism, neoplasms, and neurologic diseases (Simoncini et al., 2002; Kolluru et al., 2010; Kashiwagi et al., 2013).

Another protein with a high degree of interaction is the PTN11, a tyrosine kinase phosphatase, has activities in the regulation of growth hormones, regulation of insulin secretion, metabolic process triglyceride, in the signaling pathway of epidermal growth factor, in the regulation of factor cell growth and development of the atrioventricular canal, justifying their interaction in groups of hormonal disorders, in lipid and glycidyl metabolism, muscular disorders, cancer and cardiovascular diseases. Thus, each protein indicates their importance in the homeostasis of each system in which acts eNOS (Butt et al., 2000; Ouchi et al., 2004; Kolluru et al., 2010).

Conclusions

The regulation of eNOS and its pathways is normally based on studies in endothelial cells, cardiac myocytes and non-cardiac cells. However, it is necessary more models to characterize and unifying genetic, proteomics, biochemical and pharmacological studies, fully delineating the functions of the partners in the eNOS in pathway normal physiological and disease states.

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Conclusões

A partir de nossas análises verificamos a importância da eNOS e do óxido nítrico na homeostase de diversos sistemas do organismo. A mutação T-786C na região promotora desse gene, identificadas por RFLP, forneceram a base para associações com moléculas como os lipídeos e a proteína C reativa, que pode alterar a regulação da eNOS, com desenvolvimento de patologias durante o período gestacional. As análises de bioinformática ampliaram a nossa visão sobre a influencia de polimorfismos no gene da eNOS e o desenvolvimento de outras doenças como as cardiovasculares e metabólicas. Estudos sobre o mecanismo de ação de SNPs nesse gene ainda precisam ser elucidados com a finalidade de se formatar diagnósticos e tratamentos mais precisos para essas doenças.

6. Anexos

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  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 20 June 2007

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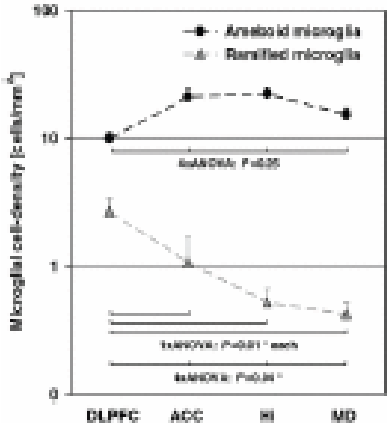

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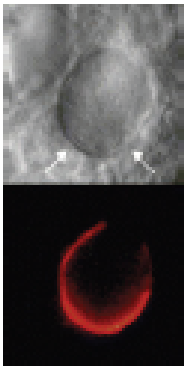

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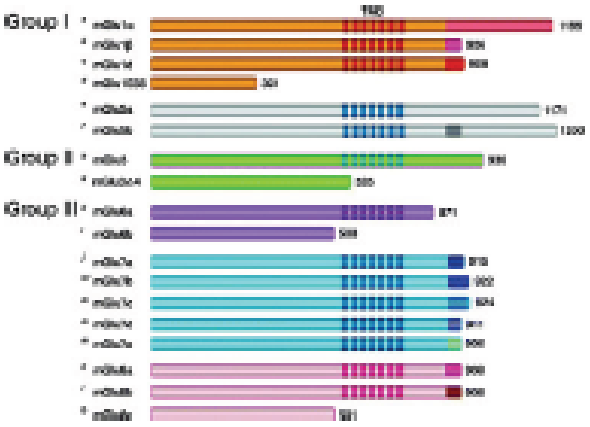

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