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MÁRCIA ANDRADE DA ROCHA

AVALIAÇÃO DAS CONCENTRAÇÕES DE VITAMINA D ASSOCIADAS A POLIMORFISMOS EM GENES LIGADOS A RESPOSTA IMUNE DURANTE A TUBERCULOSE PULMONAR ATIVA

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Tese apresentada ao Curso de Doutorado do Instituto de Pesquisa Clínica Evandro Chagas para obtenção do grau de doutor em Pesquisa Clínica em Doenças Infecciosas
Orientadora: Dra Maria da Glória Bonecini de Almeida

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Avaliação das concentrações de vitamina D associadas a polimorfismos em genes ligados a resposta imune durante a tuberculose pulmonar ativa

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Rocha, MA. **Avaliação das concentrações de vitamina D associadas a polimorfismos em genes ligados a resposta imune durante a tuberculose pulmonar ativa.** Rio de Janeiro, 2012. Tese [Doutorado em Pesquisa Clínica em Doenças Infecciosas]-Instituto de Pesquisa Clínica Evandro Chagas.

RESUMO

A tuberculose (TB) é uma doença multifatorial, onde fatores genéticos do hospedeiro e do *Mycobacterium tuberculosis (Mtb)*, bem como ambientais estão envolvidos. Assim, entre outros fatores, a predisposição genética para desenvolvimento da TB vem sendo estudada. Dessa forma, este estudo teve como objetivo avaliar se os marcadores genéticos e funcionais ligados aos genes do receptor de vitamina D (VDR*) (TaqI, FokI, BsmI e ApaI),* proteína transportadora da vitamina D (*VDBP ou Gc-globulin*) (*HaeIII e StyI*) *IFNG+874T/*A e *NOS2A-954G/C,* bem como, níveis séricos de vitamina D em pacientes com TB pulmonar ativa e controles saudáveis (HC), estão associados com a susceptibilidade a TB. Para tanto, polimorfismos dos genes que codificam componentes da via de vitamina D foram testados. Neste estudo avaliamos se polimorfismos dos genes que codificam o receptor da vitamina D (*VDR) e Gc-globulin* e os níveis séricos de 25(OH)D estariam associados com o desenvolvimento da TB. Foram incluídos 158 pacientes com tuberculose e 148 indivíduos sadios. A análise das concentrações de 25(OH)D foi estratificada por níveis de vitamina D e comparados com os genótipos e haplótipos. Em negros, o alelo *F* (*p*=0,03, OR=3,26) de *FokI* demonstrou um papel protetor para TB. De maneira similar, foi observada uma forte associação com resistência à TB nos carreadores do genótipo *bb* (*p*=0,01; OR=2,43) *e do alelo b* (*p*=0,04; OR=1,39) de *BsmI.* O fator de proteção foi mantido, em indivíduos não brancos, onde o genótipo *bb* (p=0.003; OR=0.21) e o alelo *b* (p=0.007; OR=1.89) foram associados a resistência à TB. Em indivíduos do grupo HC que apresentavam níveis séricos normais de 25(OH)D o genótipo *bb* em associação com o grupo Gc1-1 de *DBP*, teve a freqüência aumentada em 3 vezes quando comparamos a pacientes (*p*=0.009; OR=3.09) e HC. O haplótipo *tfBA* foi associado com adoecimento (*p*=0.03; OR=0.24) quando pacientes apresentavam os níveis de vitamina dentro da faixa de normalidade. Foram analisados polimorfismos dos genes de *IFNG+874T/*A e *NOS2A-954G/C e* foram incluídos 172 pacientes com TB pulmonar ativa e 179 indivíduos saudáveis. Não foi possível demonstrar associação com a susceptibilidade à TB entre pacientes e HC e o subgrupo de saudáveis PPD positivo (TB latente) em relação aos polimorfismos dos genes *IFNG+874T/A* e *NOS2-954G/C,* bem como demonstrar que estes genótipos estariam associados a produção/secreção de nitrito e nitrato. Os resultados descritos fornecem evidencias da associação entre os SNPs de *VDR* e *Gc-globulin* e os níveis séricos de 25(OH)D, com possíveis fatores de proteção contra a TB e também de suscetibilidade na população brasileira.

Rocha, MA. **Evaluation of vitamin D concentration associated with polymorphisms in genes related to immune response during active pulmonary tuberculosis.** Rio de Janeiro, 2012. Doctorate [Doctorate in Clinical Research in Infectious Diseases]-Clinical Research Institute Evandro Chagas.

ABSTRACT

Tuberculosis (TB) is genetically heterogeneous. Among other factors, genetic predisposition to developing TB has been studied. Thus, this study aimed to assess the genetic markers and functional genes linked to, vitamin D receptor (VDR*) (TaqI, FokI, BsmI e ApaI)* and protein carrier of vitamin D (*VDBP ou Gc-globulin*) (*HaeIII e StyI*) *IFNG+874T/A* and *NOS2A-954G/C* as well as, vitamin D serum concentration in patients with active pulmonary TB and healthy controls (HC), are associated with the immunopathogenesis of TB. Polymorphisms of genes encoding components of the pathway of vitamin D were tested. We evaluated whether polymorphisms of genes encoding *VDR* and *Gc-globuin* and serum of 25(OH)D are associated with the development of TB. The study includes 158 TB patients and 148 HC. The analysis of 25(OH)D concentrations was stratified by Vitamin D (VD) levels and genotypes and haplotypes were compared. In black Brazilian *F* allele (*p*=0.03, OR=3.26) of *FokI* showed to be a protective factor for TB. Similarly, the *bb* genotype (*p*=0.01; OR=2.43) and *b* allele (*p*=0.04; OR=1.39) were strongly associated with tuberculosis protection. On the other hand when the analysis were stratified by ethnicity the *bb* genotype (p= 0.003; OR=0.21) and *b* allele (p=0.007; OR=1.89) were linked with tuberculosis susceptibility in non-white Brazilian subjects. HC subjects with normal levels of 25(OH)D showed three-fold more frequency of *bb* genotype than tuberculosis patients (*p* = 0.009; OR=3.09) in a dependent way of Gc1-1 group. *tfBA* haplotype was associated with tuberculosis susceptibility (*p*=0.03; OR=0.24) when both group present normal levels of 25(OH)D. Were analyzed polymorphisms of genes of *IFNG+874T/A* and *NOS2A-954G/C* and we included 172 patients with active pulmonary TB and 179 HC. It was not possible to demonstrate any association with susceptibility to TB among patients and HC and the subgroup of HC TST positive (latent TB) in relation to polymorphisms of genes *IFNG+874T/A and NOS2A-954G/C* and the production of nitrite and nitrate. The results described provide evidence of the association between *VDR* SNPs and *Gc-globulin* and serum of 25(OH)D how possible protective factors against TB and also susceptibility in Brazilian population.

LISTA DE ABREVIATURAS

Mtb	*Mycobacterium tuberculosis*
TB	Tuberculose
OMS	Organização Mundial da Saúde
SMS	Secretaria Municipal de Saúde
TLR	Receptores toll
VDR	Receptor de Vitamina D
TLR 4	Receptor toll 4
TLR 2	Receptor toll 2
APC	Células apresentadoras de antígenos
Th1	Células auxiliares do tipo 1
IL	Interleucina
IFN-γ	Interferon gama
NK	Células matadoras profissionais
MHC	Complexo principal de histocompatibilidade
Th2	Células auxiliares do tipo 2
TNF-α	Fator de necrose tumoral-alfa
PPD	Derivado de proteína purificada
HIV	Vírus da Imunodeficiência humana
DBP	Gene *DBP*
VDBP	Proteína carreadora de vitamina D
NOS	Enzima óxido nítrico sintase
LD	Desequilíbrio de ligação
NRAMP-1	Proteína natural 1 associada a resistência em macrófagos
SNP	Polimorfismo de um único par de base
NO	Óxido nítrico
RNI	Radical intermediário de nitrogênio
IFNG	Gene *IFNG*
RFLP	Polimorfismo refratário de comprimento
MAF	Fator de ativação de macrófagos
Gc	Componente grupo específico do soro
CONEP	Comissão Nacional de Ética em Pesquisa
BAL	Lavado brônquio alveolar

SUMÁRIO

1 INTRODUÇÃO

1.1 EPIDEMIOLOGIA DA TUBERCULOSE

A tuberculose (TB) é a principal causa de morbi-mortalidade por doença infecciosa no mundo, principalmente em indivíduos HIV positivos. Ocorrem perto de 9,4 milhões de novos casos a cada ano e cerca de um terço da população mundial está infectada. Cerca de 2 milhões de mortes por TB ocorrem anualmente (WHO, 2009). O Brasil ocupa a 18ª posição com cerca de 6.000 óbitos ocorrendo anualmente. Apesar desses números alarmantes, as taxas de incidência e mortalidade apresentaram tendência decrescente nos últimos anos. Em 1990, taxa de incidência foi de 51,7 por grupo de 100 mil habitantes, decaindo para 38,3 por 100 mil habitantes em 2009. A taxa de mortalidade apresentou queda de 30% no mesmo período, variando de 3,6 para 2,5 por 100 mil habitantes (SVS, 2010). Em 2010 foram registrados 70.997 casos novos no país, sendo que as maiores taxas de incidência por 100 mil habitantes estão nos estados do Rio de Janeiro (69,5), Amazonas (65,8), Pará (46,0), Rio Grande do Sul (44,8) e São Paulo (39,5) (Sinan/SVS/MS, 2011). Com base nesses dados pode-se dizer que o curso da ampliação do problema pode ser reversível desde que tomadas medidas urgentes e inovadoras em várias frentes, principalmente por parte da comunidade científica em tentar compreender os possíveis mecanismos que levam ao adoecimento pela TB em somente 5 a 10% dos indivíduos infectados. Da mesma forma, novas estratégias para prevenção (melhor esclarecimento da população, vacinação e diagnóstico precoce) e diagnóstico (resultados mais apurados e rápidos) da TB são necessárias e isto requer uma melhor compreensão das interações entre patógeno e hospedeiro. No entanto, fatores relacionados à transmissão da doença, freqüentemente associadas à pobreza, mudaram o quadro para um cenário de eliminação da doença para apenas um quadro de uma doença curável, quando levado em consideração todas as medidas possíveis como: o não abandono ao tratamento, realização de exames periódicos que constatem a eliminação do bacilo, acompanhamento

médico regular e finalmente a ingestão adequada da medicação para evitar a resistência dos bacilos aos mesmos (Secretaria Munipal de Saúde, 2005).

1.2 IMUNOLOGIA DA TUBERCULOSE

Indivíduos imunocompetentes podem controlar o desenvolvimento da TB através de duas linhas de defesas específicas. A primeira linha de defesa do sistema imunológico contra microrganismos invasores é formada por células polimorfonucleares, monócitos e macrófagos. Quando o *Mycobacterium tuberculosis (Mtb)* atinge o sistema respiratório inferior, é fagocitado por estas células de defesa. Estudo recente descreve que a diferenciação de monócitos em macrófagos é alterada durante a infecção pelo *Mtb* alterando a capacidade de resposta destas células, a estímulos da imunidade inata e de células T (Castaño et al. 2011). Os macrófagos são os principais responsáveis na ativação de uma resposta imune específica contra a TB e quando estão infectados liberam interleucina (IL)-12 e 18, que estimulam predominantemente os linfócitos T CD4 a liberarem a citocina IFN-γ (Ellner, 1997), que por sua vez estimula a fagocitose do *Mtb* pelos macrófagos. Outro mecanismo antimicobacteriano dos macrófagos é a fusão do fagolisossoma. O lisossoma contém numerosas enzimas hidrolíticas e é uma organela de conteúdo ácido, assim sendo a fusão do lisossoma com o fagossoma contendo a bactéria ingerida é um mecanismo primário pelo qual o macrófago controla a infecção. Essa fusão é aumentada quando os macrófagos são ativados pelo IFN-γ ou demais citocinas (Mckinney et al. 2000). Além disso, macrófagos infectados são induzidos a entrar em apoptose pelo IFN-γ por uma via dependente de óxido nítrico (NO) (Herbst et al. 2011).

Células dendríticas (DCs) também estão envolvidas na proteção contra o *Mtb*, como principais apresentadoras de antígenos (Demangel et al. 1999). Durante a infecção as DCs localizadas nos pulmões migram para os linfonodos onde apresentam os antígenos para as células T e promovem a expansão de células T CD4 secretoras de IFN-γ. Além disso, o aumento de autofagia em DCs infectadas com BCG aumenta a produção de citocinas e a expressão de

moléculas co-estimulatórias, facilitando uma resposta imune Th1 mais efetora (Min et al. 2010).

Os receptores Toll Like (TLRs) são receptores de reconhecimento padrão capazes de iniciar uma resposta rápida contra o *Mtb* (Akira et al. 2006). Pelo menos 11 tipos desses receptores são descritos na literatura (Zhang et al. 2004). Os TLRs são receptores da imunidade inata e são expressos principalmente em células apresentadoras de antígenos (APCs) e células NK (natural killer). Estes receptores são responsáveis pela interligação com a imunidade adaptativa e são também expressos em linfócitos T CD4, CD8 e T regulatórios. Inúmeros TLRs estão envolvidos na resposta de células T à TB, tais como: TLR2, TLR5, e TLR7-TLR8 (TLR7/8) que são capazes de fornecer o segundo sinal de ativação de células T uma vez ativadas pela ligação com o receptor de células T (TCR) (Caron et al. 2005; Kabelitz et al. 2007; Lancioni et al. 2009; McCarron et al. 2009; Oberg et al. 2011). O reconhecimento do *Mtb* pelos TLRs (Salgame, 2005) resulta em ativação do fator de transcrição nuclear (NF)-κB e produção de citocinas pró-inflamatórias como o fator de necrose tumoral (TNF)-α, IL-1, IL-12, quimiocinas e óxido nítrico, através de duas vias, dependente ou independente da proteína de diferenciação mielóide primária (MyD88) (Yamamoto et al. 2003; Xu et al. 2007; Jo et al. 2008), de moléculas estimulatórias e do complexo principal de histocompatibilidade (MHCII) (Pecora et al. 2006; Harding et al; 2010). Monócitos e células T CD4 de fluido pleural de pacientes com TB, expressam mais TLR2 e 4, indicando o papel destes receptores na imunidade contra o *Mtb* (Prabha et al. 2008). Em macrófagos infectados pelo *Mtb* a cooperação entre os TLR2 e TLR4 resulta na sinalização que induz apoptose (Sánchez et al. 2010). O reconhecimento do *Mtb* através do TLR2 induz a secreção de citocinas e moléculas co-estimulatórias em células da imunidade inata, mas também da regulação da ação de linfócitos T de memória (Lancioni et al. 2011). Experimentos em camundongos TLR2 *knock-out*, demostraram má formação de granuloma, quando estes foram infectados com *Mtb* e apresentaram maior susceptibilidade à infecção quando comparados com camundongos selvagens (Reiling et al. 2002; Drennan et al. 2004). Além disso, esses animais apresentam dificuldades em controlar a infeção crônica pelo *Mtb* (Drennan et al. 2004). Outros estudos demonstraram que camundongos TLR2 *knock-out* apresentaram aumento na

progressão da infecção, quando infectados pelo *Mtb, Mbovis BCG* (Reiling et al. 2002; Heldwein et al. 2003; Sugawara et al. 2003; Drennan et al. 2004). Por outro lado, a sinalização prolongada através de TLR2, induzida por lipoproteínas do *Mtb*, inibe a expressão de moléculas do complexo principal de histocompatibilidade de clase II (MHCII) e apresentação de antígenos por macrófagos infectados (Banaiee et al. 2006). Além disso, induz a produção de IL-10 levando a resposta antiinflamatória, esse parece ser um mecanismo de *feed back* negativo que limita a extensão da resposta imune (Manicassamy et al. 2009). O modelo de infecção em camundongos utilizado para infecção aguda primária é inadequado para infecção assintomática persistente, encontrada em humanos, tornando difícil acessar o papel de TLR2 durante a infecção crônica. Por outro lado, o papel dos TLR2, TLR4 e MYD88 na infecção pelo *Mtb* vem sendo questionado (Holscher et al. 2008). Contudo, humanos com polimorfismos para o gene de TLR2 apresentam um aumento na susceptibilidade para TB (Bochud et al. 2003; Ben-Ali et al. 2004; Ogus et al. 2004), indicando que o TLR2 tem um importante papel na resposta imune contra a TB.

A segunda linha de defesa do sistema imunológico é composta pela resposta humoral específica e pela imunidade mediada por células. Esta resposta específica contra o bacilo é considerada o principal mecanismo de defesa contra organismos intracelulares como o *Mtb,* e os antígenos apresentados pelos macrófagos levam à estimulação tanto de linfócitos T CD4 como de linfócitos T CD8 (Saunders et al. 2002).

Antígenos do *Mtb* induzem a liberação do fator de necrose tumoral (TNF-α) por células T, que desempenha importante função no controle da TB através da formação dos granulomas, que é um processo de contenção do foco infeccioso, podendo gerar lesão pulmonar (Frieden et al. 2003). Assim sendo, o IFN-γ e TNF-α, representam o principal mecanismo através do qual são induzidas substâncias que controlam o crecimento intracelular micobacteriano, como o NO e radicais intermediários de oxigênio (ROIs). Essas substâncias são produzidas principalmente por macrófagos, células dendríticas e linfócitos T (Henderson et al. 1997; Ladel et al. 1997; Barnes et al. 1993). Bonecini-Almeida et al. (1998 a e b), demonstraram uma elevada expressão de NOS induzida (iNOS ou NOS2) em pacientes com TB pulmonar, bem como

associado à atividade micobactericida em macrófagos infectados com *Mtb* previamente estimulados com IFN-γ e contendo linfócitos do sangue periférico. A função relevante do NOS2 na TB também foi observada em estudos realizados em camundongos com deficiência desta enzima. Os animais exibiram maior susceptibilidade para a infecção aguda ou crônica pelo *Mtb,* quando comparados a camundogos selvagens (Chan et al. 1995). Lee et al (2009), descreveram o aumento da síntese de NO e da expressão de NOS2 em cultura de macrófagos infectados pelo *Mtb*, quando estas foram suplementadas com Vitamina D (VD) e IFN-γ, acarretando uma diminuição significante do crescimento da bactéria em macrófagos. Estes achados sugerem a importância de NO controle do *Mtb.*

As células T CD4 possuem o papel principal na contenção da infecção pelo *Mtb*. Os antígenos do *Mtb* são apresentados pelos macrófagos através do MHCII para células T CD4 que se encarregam de ativar linfócitos B e de os estimularem a sintetizar e liberar anticorpos. As células T CD4 realizam várias funções importantes, como controlar a infecção no granuloma, apoptose de macrófagos infectados, produção de citocinas e ativação de outras células do sistema imunológico (macrófagos ou células dendríticas) (Chan et al. 2004; Cooper, 2009). A resposta imune adaptativa contra o *Mtb* engloba a expansão de células T CD4 e CD8 efetoras e a geração células T de memória. (Kamath et al. 2006; Mueller et al. 2008). *In vitro*, macrófagos infectados desencadeam a expressão de granzima e perforina por células T CD8, dois compostos que são altamente ativos contra *Mtb* (Stegelmann et al. 2005). Células T CD4 são consideradas a principal fonte de IFN-γ, que é uma citocina importante para resposta contra TB em humanos e camundongos e são capazes de produzir mais IFN-γ do que as células TCD8, após a infecção pelo *Mtb* (Ngai et al. 2007). Camundongos deficientes destas células são altamente suscetíveis à infecção pelo *Mtb*, embora a produção de IFN-γ seja apenas transitoriamente diminuída (Caruso et al. 1999). No homem, o exemplo mais claro do papel fundamental destas células, ocorre durante a infecção pelo HIV-1, onde existe uma diminuição dos linfócitos T CD4, provocando uma grande susceptibilidade a primo-infecção ou à reativação de uma TB latente (Saunders & Britton, 2007). Estas evidências demonstram que, células T CD4 desempenham papel central na resistência contra a TB humana.

Vários estudos têm demonstrado que a infecção pelo *Mtb* inibe a apresentação de antígenos para as células T CD4, através do MHCII, esse fato contribui para a incapacidade do hospedeiro em eliminar a infecção persistente. No entanto, a incapacidade das células T CD4 para eliminar o *Mtb* pode ser devido à falta de reconhecimento ou ativação de macrófagos infectados (Gong et al. 1996; Rojas, 1999).

Durante muitos anos o foco das pesquisas em relação às células T, foram as células T CD4, porém nos últimos anos o papel das células T CD8 controle do *Mtb* foi reconhecido. As células T CD8 reconhecem os antígenos micobacterianos através do MHCI e esses antígenos são freqüentemente derivados do citoplasma dessas células. Estudos sugerem que o bacilo dentro dos vacúolos pode ter acesso ao citoplasma, através de poros encontrados nas membranas desses vacúolos (Mazzaccaro et al. 1996; Teitelbaum et al. 1999). A lise das células infectadas com o *Mtb,* pelas células T CD8 citotóxicas ocorre através da via perforina/granzima ou pela via Fas/FasL (Stenger et al. 1997). Essas células citotóxicas são estimuladas a provocar a lise de macrófagos infectados com micobactérias, exercendo uma ação importante na defesa contra a TB ao liberar os bacilos do meio intracelular para serem destruídos por outros macrófagos já ativados ou induzir a apoptose com a destruição da bactéria, evitando que possam infectar novas células. As células T CD8, além de produzirem IFN-γ e outras citocinas, são citotóxicas e capazes de destruir macrófagos infectados pelo *Mtb* via granzima, que facilita o controle da infecção na fase crônica (Grotzke et al. 2005; Cooper, 2009).

O importante papel das células T CD8 na proteção contra a TB foi sugerido através de diferentes experimentos. Células T CD8 antígeno específicas contra o *Mtb* encontradas nas vias aéreas de murinos proliferam continuamente e são mantidos mesmo na ausência de recrutamento de células T periféricas (Jeyanathan et al. 2010). A abundante presença de células T CD8 específicas contra o *Mtb* em indivíduos com infecção latente demonstrou que este grupo celular está envolvido no controle desta infecção*. A* depleção de células T CD8 ocasionou a reativação da infecção latente no modelo de Cornell (Van Pinxteren et al. 2000). Einarsdottir et al. (2009), demonstraram em murinos que células T CD8 de memória responderam mais rapidamente a infecção e estavam presentes em maior número nos pulmões do que T CD8

não ativadas. Contudo, essas células eram menos citotóxicas e secretavam menos IFN-γ do que células recém-ativadas, sugerindo que um fator que contribui para a persistência bacteriana pode ser diminuição da capacidade das T CD8 de memória de exercerem suas funções de maneira adequadas. Experimentos realizados em camundongos têm revelado a importância das células T CD8 no controle da infecção pelo *Mtb* (Flynn et al.1992; Behar et al. 1999). Por outro lado, pesquisadores têm reportado que células T CD8 não são necessárias para o controle da infecção pelo *Mtb.* Em camundongos manipulados genéticamente para não expressarem MHCII com conseqüente ausência de imunidade mediada por células T CD4, ocorreu um progressivo crescimento bacteriano e a média de sobrevida foi de 77 dias, por outro lado, na ausência de imunidade mediada por células T CD8 a média de sobrevida foi de 232 dias, sugerindo que este grupo celular não contribui efetivamente para o controle do crescimento bacteriano (Mogues et al. 2001).

Os linfócitos T CD4 e T CD8 humanos apresentam dicotomia quanto à produção de citocinas, similar ao modelo murino. Assim, as citocinas produzidas por linfócitos T CD4 e T CD8 do tipo Th1 secretam IFN-γ, IL-2, IL-3, e TNF-α, enquanto que linfócitos T CD4 e T CD8 do tipo Th2 secretam IL-4, IL-5, IL-6 e TNF-α. Inúmeras citocinas são produzidas em resposta à infecção pelo *Mtb* no hospedeiro humano e suas funções na tentativa de contenção da infecção ainda não são completamente compreendidas (Chacon-Salinas et al. 2005).

A atuação de cada um destes hormônios celulares resulta em diferentes perfis de ativação ou desativação celular. Foi demonstrado que o IFN-γ, mediador da ativação de macrófagos produzido inicialmente por células NK ou células T, as quais são estimuladas por IL-12 proveniente de macrófagos infectados, é capaz de induzir atividade micobactericida em macrófagos humanos (Bonecini-Almeida et al. 1998a). Humanos com mutações no gene IL-12 ou no seu receptor apresentam redução na produção de IFN-γ por células T sendo mais susceptível a infecção pelo *Mtb* (Saunders et al. 2002). Alguns autores sugerem que os níveis de IFN-γ são diminuídos em pacientes com TB ativa (Zhang et al. 1995). Por outro lado, foi demonstrado em população indiana que os níveis séricos de IFN-γ são mais elevados em pacientes com TB

pulmonar ativa do que em controles (Abhimanyu et al. 2011). Citocinas do tipo Th2, como a IL-10, atuam como potentes inibidores da atividade celular inflamatória, inibindo a produção de óxido nítrico, a produção de citocinas pró-inflamatórias (IL-1, TNF-α), o *burst* respiratório e a expressão de receptores para IL-1 e MHC II (Wallis et al. 1990; Ogawa et al. 1991). A TB humana ainda está sendo alvo de estudo para a determinação do padrão de citocinas que predomina durante a evolução desta doença. O balanço entre citocinas do tipo Th1, ditas como inibitórias (IFN-γ, TNF-α), ou do tipo Th2, ditas como aceleradoras do crescimento micobacteriano (IL-4, IL-10), pode ser importante na regulação da atividade micobactericida em macrófagos infectados pelo *Mtb*. Bonecini-Almeida et al. (1998b), demonstraram que células alveolares de pacientes com TB pulmonar ativa expressam citocinas Th1 (mRNA IL-2 e IFN-γ), em um ambiente também dito como Th2, pela presença de mRNA de IL-10 e IL-4. Winek et al. (2009), descreveram que não é o aumento de citocinas Th1, mas sim das citocinias Th2, que desempenham papel importante no desenvolvimento da TB. Recentemente, Tan et al. (2011), observaram que em infecções por cepas de *Mtb* multi drogas resistente (MDR), ocorre a diminuição da expressão de IFN-γ, IL-2 e IL-10 e aumento de IL-4, IL-6 e TNF-α, sugerindo que a alteração nos efeitos patogênicos e protetores induzidos pela imunossupressão das respostas Th1 e Th2 são características destas cepas, corroborando a importância do balanço de citocinas na TB.

A TB é uma doença complexa resultante de interações entre o hospedeiro (características genéticas), o patógeno e o ambiente. Um grande número de genes do hospedeiro já foram descritos como atuantes no desenvolvimento da TB (revisado por Moller et al., 2009). Remus et al. (2003), observaram que a concordância entre gêmeos para o desenvolvimento da TB foi de 65-85% para monozigóticos e 25-35% para dizigóticos. Desde então, várias associações têm sido feitas com genes relacionados principalmente ao sistema imune e a patogênese da TB, incluindo *NRAMP1, IL-1, HLA, IL-10, IFNG, NOS2, TLR1, TLR2, TLR4, TNFA, Gc-globulin e VDR,* dentre outros. Contudo, esta associação também é dependente da população estudada.

Entender a imunidade do hospedeiro humano para a TB torna-se importante por inúmeras razões. No entanto, devemos saber que somente

através do conhecimento de como a TB é reconhecida e controlada pelo sistema imune, poderemos desenhar e avaliar novas estratégias que objetivem a prevenção e tratamento da TB.

1.3 VITAMINA D

A vitamina D (VD) é um dos hormônios sintetizado pelas formas mais ancestrais de organismos, por mais de 750 milhões de anos. Fitoplanctions, zooplanctions e muitas formas de plantas e animais que são expostos a luz solar possuem a capacidade de sintetizar a VD.

A VD é uma substância lipossolúvel, chamada de secosteróide, pelo fato de possuir um dos anéis aromáticos clivados. São reconhecidos dois secosteróides naturais: ergocalciferol ou VD_2 (25(OH)D) e colecalciferol ou VD3 ($1,25(OH)_2D$) que são derivados da clivagem fotolítica dos anéis B do ergosterol e 7-dehidrocolecalciferol, na pele (Brown et al. 1999). A síntese cutânea da substância ativa a partir da pró-vitamina D_3 ocorre na epiderme, por irradiação de luz ultravioleta e depende de fatores como o tempo de exposição da pele ao sol, momento do dia em que ocorre a exposição, pigmentação da pele, idade, usa de filtro solar, estação do ano, poluição atmosférica, situação geográfica e capacidade de absorção intestinal (McKenna, 1992; Roncada, 2000; Douglas, 2002; Holick, 2003). Contudo, após a sua bioativação na pele, os precursores da VD, que são substâncias inertes, necessitam ainda serem submetidos a uma seqüência de hidroxilações para se transformarem na forma biologicamente ativa. O primeiro processo de hidroxilação ocorre no fígado e o segundo e mais importante ocorre nos rins. Essa segunda, hidroxilação pela enzima 1-α hidroxilase ou Cyp 27B1, dá origem a forma ativa da VD com maior atividade fisiológica ($1,25(OH)_2D$), (Roncada, 2000; Douglas, 2002). A forma ativa da VD, após ser sintetizada nos rins, é transportada para os tecidos alvos tal como ossos e intestino onde desempenham seu papel na homeostase de cálcio e fosfato, bem como atuam na proliferação, diferenciação celular (Tashiro et al. 2004) e na modulação da resposta imune (Piemonti et al. 2000) e no metabolismo ósseo pela absorção de cálcio (Sawada et al. 2001).

As necessidades diárias de VD podem ser obtidas a partir de quantidades apropriadas na alimentação. Como fontes de VD temos: a gema de ovo, arenque, cavala, salmão, sardinha, atum, derivados lipídicos do leite e óleo de fígado de bacalhau. Em geral, recomenda-se 5 a 30 minutos de exposição diária da pele ao sol para manter níveis satisfatórios de VD circulante (Holick, 2003).

É importante lembrar que atualmente alguns alimentos são industrialmente enriquecidos com VD (Roncada, 2000; Douglas, 2002; Holick, 2003). No Brasil, por exemplo, podem ser citados leite em pó, achocolatados, suplementos alimentares do tipo Sustagem®, Nutren® Active e similares, farinha láctea, farinhas de milho e de arroz e flocos de cereais.

Como preconizado, a quantidade diária necessária de VD recomendada em 1998 pelo "Food and Nutrition Board Institute of Medicine" é 200 IU para crianças, 400 IU adultos homens e mulheres até 51 anos. Para mulheres com mais de 51 anos e homens com mais de 70, a recomendação é de 600 IU/dia ou 800 IU/dia, respectivamente (Norman, 2000).

Para determinar o estado desta vitamina num indivíduo, a melhor maneira é dosar a concentração sérica de 25(OH)D cuja concentração normal é geralmente descrita entre 20 e 150 nmol/L que equivale manter-se entre 8 e 60 ng/mL (Holick, 1987). Contudo, Lips et al. (2004) descreveram que níveis de 50 nmol/L ou 20 ng/mL são considerados apropriados e classificam a deficiência de VD como: deficiência leve, quando a concentração de 25(OH)D se encontra entre 50 e 25nmol/L; deficiência moderada, quando a concentração se encontra abaixo de 25 nmol/L e deficiência grave, quando a concentração se mostra abaixo de 12,5 nmol/L. De maneira diferente, as faixas de referência de níveis séricos de VD utilizadas pelo Laboratório Sérgio Franco-Brasil, seguindo as referencias de Singh et al. (2006), são: 30 a 80 ng/mL ou ≥ 75 nmol/L (normal); <30 ng/mL ou 37-74 nmol/L (deficiência moderada); <15 ng/mL ou <37 nmol/L (deficiência severa) e > que 80 ng/mL (hipervitaminose). Esses níveis de concentração de VD são utilizados pelos médicos no Brasil para diagnosticar a deficiência ou suficiência e podem diferir entre os laboratórios de análise clínica, uma vez que não existe um padrão destas concentrações em população brasileira.

1.4 VITAMINA D E TUBERCULOSE

A possibilidade do envolvimento da VD na TB tem sido levantada há muito tempo. Em 1800, a terapia com VD na forma de óleo de fígado de bacalhau e exposição à luz solar eram usados para tratar pacientes com TB (Davies et al. 1987). Antes da descoberta das drogas anti-micobacterianas a VD foi empregada para “tratar” paciente com TB cutânea nos quais foram observados melhora do quadro clínico (Dowling & Prosser-Thomas, 1946), porém houve um gradativo declínio no uso da VD com o desenvolvimento de drogas anti-micobacterianas. O uso empírico da VD na TB foi somente identificado nos últimos anos pelas inúmeras evidências relatadas sobre a relação da VD e a resposta imune. O sistema imune é diretamente influenciado pelo padrão nutricional do indivíduo e a má nutrição ou a desnutrição afetam de maneira negativa a capacidade de estabelecer uma resposta imune adequada contra agentes infecciosos, acarretando em redução das imunidades celular e humoral (Chandra et al. 1994). Recentemente, o papel da VD na resistência do hospediero às infecções tem despertado grande interesse. Isso se deve ao fato de dois importantes achados: 1) a forma ativa da VD produzida pelo sistema imune é capaz de induzir a produção de catelicidina e 2) células do sistema imune são capazes de sintetizar a enzima capaz de converter a VD na sua forma inativa para ativa. Liu et al. (2006), descreveram que a catelicidina é capaz de inibir o crescimento do *Mtb in vitro.* Recentemente, estudos sobre a influência de níveis séricos de VD na susceptibilidade à TB, descreveram a ligação da deficiência de VD com o adoecimento (Ho-Pham et al. 2010; Talat et al. 2010; Gray et al. 2011; Martineua et al. 2011b; Arya et al. 2011; Banda et al. 2011).

Assim, a partir destes dados, os efeitos imunomodulatórios da VD vêm sendo intensamente estudados na TB. Em modelos animais foi observado que durante a infecção pelo *M. bovis* ocorre aumento da concentração de $1,25(OH)_2D$ no soro de gado (Rhodes et al. 2003). Por outro lado, o aumento da colonização nos pulmões e no tamanho das lesões, pode ser observado em camundongos com deficiência de VD (Waters et al. 2004).

Estudos que relacionam níveis de VD com células do sistema imune *in vitro*, demonstraram o importante papel desa vitamina. De acordo com Zittermann (2003), a VD é capaz de induzir a diferenciação de monócitos em macrófagos, que por sua vez, representam a primeira linha de defesa inespecífica do sistema imune contra o *Mtb.* A VD aumenta a atividade das enzimas lissossomais dos macrófagos e ainda facilita a atividade citolítica pelo aumento da fagocitose (Zittermann, 2003). Esse efeito tardio se dá pelo aumento da expressão de receptores de superficie Fc (Boltz-Nitulescu et al. 1995) e pela indução da enzima NOS2 (óxido nítrico sintase) e de radicais intermediários de oxigênio (ROI) (Rockett et al. 1998; Sly et al. 2001).

Macrófagos alveolares de pacientes com TB podem produzir grandes quantidades de $1,25(OH)_2D$ e a concentração desse metabólito nos granulomas pode ser suficiente para prevenir o crescimento bacteriano (Chandra, 2004). Além disso, pacientes com TB podem produzir $1,25(OH)_2D$ bem como outros metabólitos nos sítios de atividade da doença (Rook, 1988). Segundo Crowle et al. (1987), para haver uma proteção efetora da $1,25(OH)_2D$ sobre monócitos e macrófagos infectados com o *Mtb,* é necessário uma concentração de 4 µg/ml *in vitro*, sendo considerada maior do que o encontrado normalmente na circulação *in vivo*, contudo esta mesma concentração de VD é encontrada nos granulomas, sugerindo que as concentrações séricas e no sítio da infecção são diferentes. Assim, indivíduos com níveis séricos de VD dentro da normalidade, poderiam apresentar deficiência desta vitamina no sítio da infecção. Estudo *in vitro*, demostrou que células obtidas dos sítios granulomatosos convertem 25(OH)D em $1,25(OH)_2D$, bem como expressam o VDR (receptor de vitamina D) (Biyoudi-Vouenze et al. 1991). Estudos com células T alveolares de pacientes com TB, demonstraram a capacidade dessas células em converter 25(OH)D em $1,25(OH)_2D$ e expressar mais receptores de VD do que indivíduos controles (Davies et al. 1985; Cadranel et al. 1990). Segundo Khoo et al. (2011), em cultura de células infectadas pelo *Mtb* e suplementadas com $1,25(OH)_2D$, foi possível observar a diminuição na secreção de citocinas do tipo Th1, através da diminuição de expressão TLR2, TLR4 e receptor de manose. De modo interessante, foi observado que a VD é capaz de reduzir a transcrição e secreção de IFN-γ, IL-12 e TNF-α em células mononucleares e macrófagos infectados, indicando assim que a proteção

relacionada à suplementação da VD pode não estar associada somente a estas citocinas (Martineau et al. 2007b). Recentemente Selvaraj et al. (2011), observaram que a adição de VD em cultura de macrófagos levou ao aumento da expressão de catelicidina que poderia intensificar a imunidade contra TB pulmonar, sendo este um importante mecanismo de defesa contra o *Mtb*.

Outro estudo *in vitro* reportou que a citocina IFN-γ foi capaz de induzir em macrófagos uma via antibacteriana na presença de soro com níveis de VD considerados normais. Contudo, este achado não foi observado em suplementação com soro de afro-americanos que apresentaram menores níveis séricos de VD e são mais susceptíveis a TB (Fabri et al. 2011). Luong et al. (2011), observaram que a VD pode suprimir o crescimento do *Mtb in vitro*. A suplementação de VD em culturas celulares com insuficiência desta vitamina restaurou a expressão de peptídeos antibacterianos induzidos por IFN-γ, autofagia, fusão fagossoma-lisossoma e atividade antimicrobicida, sugerindo a importância de concentrações adequadas de VD para respostas inatas e adaptativas efetoras na TB. Todos estes trabalhos sugerem a importância da VD em níveis dentro da faixa de normalidade, para um funcionamento adequado do sistema imunológico.

Devido ao papel exercido pela VD na modulação das atividades do sistema imune, bem documentada em experimentos *in vitro*, estes estudos também foram realizados em pacientes com TB. Assim, como descreveram Sita-Lumsden et al. (2007), pacientes com TB apresentam baixos níveis séricos de 25(OH)D quando comparados com indivíduos saudáveis. Wilkinson et al. (2000), descreveram uma probabilidade dez vezes maior de desenvolver TB em asiáticos vegetarianos restritos, com baixos níveis séricos de VD vivendo em Londres, quando são pouco expostos à luz solar. Da mesma forma, a deficiência de VD tem sido associada com um risco cinco vezes maior de progressão de infecção latente para TB ativa em população paquistanesa (Talat et al. 2010). Em recente estudo, Martineau et al. (2011b), observaram associação entre níveis deficientes de VD no soro e o desenvolvimento da TB em indivíduos negros na cidade de Cape Town. Apesar de todos esses relatos, ainda hoje existem controvérsias em relação à produção de VD pelos pacientes com TB. Concentrações séricas de 1,25$(OH)_2$D em pacientes com TB são mais

baixas do que em indivíduos controles saudáveis em população da Inglaterra (Chan, 1999), no entanto não diferem estatisticamente entre a população da Indonésia (Grange et al. 1985). Foi observado em população indiana em pacientes com TB níveis de $1,25(OH)_2D$ aumentados e este fato pode estar relacionado a diminuição da expressão de VDR, resultando em uma sinalização deficiente e menor disponibilidade celular para a VD (Selvaraj et al. 2009). Assim, inúmeros estudos sugerem associação entre a deficiência de VD com a susceptibilidade e severidade da TB.

Recentemente o comitè do Instituto de Medicina-USA, publicou um documento, descrevendo a elaboração de um Plano Nutricional a ser implementado neste país. Assim, foi preconizado o uso clínico da suplementação com 25(OH)D para pacientes com câncer, diabetes, doenças ósseas, cardiovasculares, asma, autoimunes e infecciosas (TB e influenza) junto com o tramento convencional (Aloia et al. 2011). Hoje no Brasil ainda não existem parâmetros nacionais que determinem as taxas de normalidade para os níveis de 25(OH)D sérica, por isso, parâmetros internacionais são seguidos. Atualmente, não existe um consenso na comunidade cientifica brasileira em relação à dosagem adequada de VD que deve ser administrada nos pacientes com TB, uma vez que doses elevadas são tóxicas, assim, a suplementação com VD como profilaxia ou durante a infecção torna-se difícil de ser realizada.

Nos últimos anos, a suplementação com VD em pacientes com TB vem sendo cogitada como uma forma de diminuir a sintomatologia e redução da carga bacilar, apoiados em dados *in vitro* e em ensaios clínicos. Uma única dose de 2,5 mg de 25(OH)D intensifica significantemente a capacidade micobactericida *in vitro* de macrófagos obtidos de contatos de pacientes com TB, sem contudo apresentarem hipercalcemia e sem afetar a produção de IFN-γ (Martineau et al.2007b). Recentemente, Martineau et al. (2011a), reportaram que a suplementação de VD em pacientes que possuem o genótipo *tt* do polimorfismo *TaqI* do receptor de VD, produziu uma aceleração notável na eliminação do bacilo no escarro. A suplementação com VD também foi benéfica em paciente de origem afro-americana com deficiência de VD e falhas recorrentes no tratamento (Yamshchikov et al. 2009). A correção dos níveis séricos de VD em conjunto com a terapia anti-tuberculose resultou na melhora da paciente 13 meses depois do início do tratamento, indicando que o

tratamento com VD pode melhorar o prognóstico em pacientes com TB. Diferente destes resultados, Wejse et al. (2009), não encontraram diferença nos resultados clínicos ou na mortalidade de pacientes que receberam ou não suplementação oral com VD na população de Guinea-Bissau.

Inúmeros grupos de pesquisa estão trabalhando com a suplementação de VD no intuito de avaliar a possível melhora na resposta imune efetora ao *Mtb*. Dessa forma, podemos concluir que a suplementação de VD juntamente com os esquemas de tratamento da TB é uma nova perspectiva para o futuro.

1.5 POLIMORFISMO DA PROTEINA TRANSPORTADORA DE VITAMINA D (VDBP – "VITAMIN D BINDING PROTEIN" ou "Gc-GLOBULIN")

A forma inativa 25(OH)D e a forma ativa 1,25$(OH)_2$D da vitamina D são moléculas lipossolúveis. Devido a sua baixa solubilidade no meio aquoso do plasma, essas moléculas bem como todos os metabólitos oriundos da vitamina D são transportados na circulação ligados a proteínas do plasma, denominadas de proteínas transportadoras de vitamina D. A VDBP é uma glicoproteína monomérica com uma sequência de 458 aminoácidos com aproximadamente 58kDa. Ela é sintetizada no fígado e circula no plasma em concentrações 20 vezes maior do que o total de metabólitos circulantes de VD. Uma das principais funções da VDBP é se ligar, solubilizar e transportar os principais metabólitos da VD: 25(OH)D (o principal metabólito circulante) e 1,25 1,25$(OH)_2$D (o metabólito mais ativo). Esta proteína também é conhecida como componente grupo específico do soro ou grupo Gc-globulina. Apenas 5% do total de VDBP existente no plasma humano encontra-se ligada a VD. Apesar dessa pequena quantidade, a proteína VDBP se liga a 88% e a 85% de 25(OH)D (com alta afinidade) e 1,25$(OH)_2$D (com dez vezes menos afinidade) presentes no soro, respectivamente, podendo assim influenciar na farmacocinética desta vitamina (White & Cooke, 2000). Dessa forma, é possível preconizar que em condições fisiológicas normais, praticamente todos os compostos de VD estão ligados a VDBP, haja visto que esta vitamina em altas quantidades no soro se torna tóxica para o organismo. Logo, a proteína VDBP tem também um importante papel em resguardar o organismo contra a

intoxicação com a VD, capturando os metabólitos de VD livres no soro (Bouillon et al. 1981).

Tanto a VDBP livre ou àquela ligada aos metabólitos de VD são filtradas através dos glomérulos e reabsorvidas pelo receptor endocítico chamado megalin nas células dos túbulos proximais (White & Cooke, 2000). A endocitose da VDBP ligada à VD mediada pelo receptor megalin parece ser a principal rota para preservar os níveis de circulação de 25(OH)D e 1,25$(OH)_2$D. No entanto, em ratos nulos para megalin observa-se uma diminuição dos níveis do soro de 25(OH)D e 1,25$(OH)_2$D, sem evidências de deficiência biológica de VD com comprometimento ósseo (White & Cooke, 2000). Esta situação claramente indica que, na ausência de VDBP, a rápida captura dos metabólitos de 25(OH)D, bem como sua ativação em 1,25$(OH)_2$D ocorre por uma via independente da rota do receptor megalin. Contudo, quando os camundongos foram suprimidos para o gene que expressa o receptor megalin, observou-se que este fenótipo era inconsistente com a vida desses animais e que estes não sobreviviam para mensurar os níveis de 25(OH)D e 1,25$(OH)_2$D. Esses dados suportam a idéia de que, apesar de existir outra rota para a possível ativação de 25(OH)D em 1,25$(OH)_2$D, a rota através do receptor megalin deve ser a principal para ativação da VD. Além dessas observações, estudos com animais nocauteados para o gene *DBP*, demostraram a importância da expressão desta proteína por este gene em manter estáveis os estoques dos metabólitos de VD no soro, bem como modular suas bioviabilidades, suas ativações e suas atuações nos órgãos-tecidos alvos (Safadi et al. 1999). Estudos realizados por Lauridsen et al. (2005), também confirmaram que os níveis no soro de 1,25$(OH)_2$D estão diretamente relacionados com o gene *DBP*, que codifica a proteína VDBP, mas especificamente aos fenótipos determinados pelos polimorfismos do *DBP*.

Além de transportador de VD, a VDBP tem propriedades multifuncionais incluindo um importante papel como ativador de macrófagos a partir do fator de ativação de macrófagos (“macrophage-activating factor-MAF”). Estudos utilizando modelos *in vitro* identificaram a VDBP como uma molécula ativadora através de um sistema onde a VDBP presente no soro é convertida em MAF. Nesse processo a VDBP é sequencialmente deglicolizada pela β-galactosidase e sialidase presentes nos linfócitos B e T, respectivamente (Yamamoto &

Naraparaju, 1996). O papel de VDBP como ativador de macrófagos é independende da capacidade de ligação com a vitamina D (Yamamoto & Naraparaju, 1996). Vale salientar que o macrófago ativado desenvolve a capacidade de realizar tarefas específicas tais como quimiotaxia, fagocitose, lise de parasitas intracelulares, bem como a destruição de células tumorais (White & Cooke, 2000). Assim, variações nesta propriedade podem afetar o adequado funcionamento do sistema imune. Este mecanismo foi demonstrado por White & Cooke (2000), a partir de camundongos nocautedos para o gene *DBP*, que exibiram um prejuízo na resposta imune contra infecções bacterianas. O efeito da proteína VDBP na função como ativador de macrófago foi observada pela ação do IFN-γ. Esta citocina induz a produção de 25(OH)D1α-hidroxilase no pulmão e nos macrófagos da medula óssea que podem então converter 25(OH)D em $1,25(OH)_2D$ (Reichel et al. 1987). Macrófagos teciduais ativados de pacientes com doenças formadoras de granulomas, tal como TB, podem gerar suficientes excessos de $1,25(OH)_2D$ por esta rede a partir de MAF, causando hipercalcemia sistêmica. Em adição, a $1,25(OH)_2D$ é um potente agente diferenciador de monócitos, este efeito pode também ser atribuído pela habilidade em estimular a secreção de fator estimulador de colônia de macrófagos (M-CSF) (Roodman et al. 1985).

Sabe-se que o gene *DBP* é membro de um cluster multigênico que inclui albumina, α-fetoproteina e α-albumina/afamina localizado no cromossoma 4q11-q13 (Figura 8) e que pode conter três polimorfismos distintos: um no intron 8(TAAA)N e dois no éxon 11 - um no códon 416 com a substituição de T→G trocando assim o ácido aspártico pelo ácido glutâmico e um no códon 420 com a substituição de C→G que muda a treonina por lisina. O gene *DBP* é expresso por uma variedade enorme de tecidos, mas a vasta maioria da VDBP no soro é derivada da expressão gênica no fígado (McLeod & Cooke, 1989).

Variações das sequências nos códons 416 e 420 no éxon 11 do gene *DBP* ou *Gc* originam 3 principais variantes eletroforéticas da proteína VDBP chamadas de “Gc1 fast” (Gc1F), “Gc1 slow” (Gc1S) e Gc2 (Braun et al.1993; Ito et al. 2004). Essas variantes diferem por seqüência de aminoácidos bem como pelas estruturas de polissacarídeos anexados. Elas também diferem pela afinidade para $1,25(OH)_2D$ e seus metabólitos, no qual a variante *Gc2* apresenta menor afinidade e *Gc1F* maior afinidade (Pani et al. 2002).

Combinações das três variantes do gene *DBP* ou *Gc-globulin group* resultam em 6 fenótipos circulantes comuns: *Gc1F-Gc1F, Gc1F-Gc1S, Gc1S-Gc1S, Gc1F-Gc2, Gc1S-Gc2* e *Gc2-Gc2* (Ye et al. 2001). Em 2005, Lauridsen et al. comprovaram que a afinidade de ligação entre a $1,25(OH)_2D$ sérica e a VDBP é realmente dependente dos diferentes tipos de fenótipos. Desta forma, os portadores do grupo de fenótipos *Gc1-1* (*1S/1S, 1F/1F, 1S/1F)* possuem elevada afinidade, enquanto que os grupos de *DBP Gc1-2 (2/1S, 2/1F)* possuem afinidade intermediária e o grupo *Gc2-2 (2/2)* possui baixa afinidade para o calcitriol. Esta afinidade se relaciona a posterior disponibilidade de VDBP e os níveis séricos da forma ativa e inativa da VD. Foi observada uma diferença entre as concentrações plasmáticas de calcitriol entre os grupos, sendo aumentada no grupo *Gc1-1*, intermediária no grupo *Gc1-2* e diminuída no *Gc2-2*. Essa diferença também foi observada entre as concentrações plasmáticas de 25-hidroxicolecalciferol e entre o índice de 25-hidroxicolecalciferol (dividido pela VDBP plasmática) entre os grupos de *DBP*, sendo observada uma aumentada concentração no *Gc1-1*, intermediária concentração no fenótipo *Gc1-2* e uma diminuída concentração no grupo *Gc2-2*. Em recente trabalho, nosso grupo observou associação entre o grupo *Gc2-2* de *DBP* e susceptibilidade para TB em indivíduos provenientes da Ásia. Análises de níveis séricos de VD demonstraram que esta associação foi restrita aos participantes com profunda deficiência (Martineu et al; 2010). Chun et al. (2010), demonstraram que monócitos cultivados com soro humano de indivíduos com genótipo de baixa afinidade *Gc1-2* ou *Gc2-2,* apresentaram um aumento significativo na indução de catelicidina em relação à cultura de células de indivíduos com grupo de alta afinidade *Gc1F-1F .* Foi sugerido que mulheres com endometriose carreadoras do grupo *Gc2-2* têm diminuída a capacidade de ativar a função fagocítica de macrófagos (Faserl et al. 2010). Da mesma forma, foi possível verificar associação do grupo *Gc2-2* com a diminuição da concentração de VDBP no soro (Speeckaert et al. 2008). Gozdzik et al. (2011), demonstraram que as populações da Ásia e da Europa, *o Gc2-2* foi associado a baixas concentrações de 25(OH)D no plasma. Assim, pode-se entender que os níveis séricos de VD são influenciados por um conjunto de fatores complexos e heterogênicos entre grupos populacionais e que os diferentes

genótipos de *Gc-globulin* estão diretamente relacionados à concentração de VD no plasma.

A partir dos dados descritos acima, muitas questões a cerca das funções da proteína VDBP permanecem não compreendidas. No entanto é possivel verificar que o gene *DPB* é importante para determinar essas possíveis questões. Assim, a determinação de uma possível associação entre o gene *DBP,* a produção da proteína VDBP e sua associação com as diversas substâncias do organismo, principalmente em relação à afinidade com a VD, podem contribuir para a determinação de uma possível susceptibilidade a algumas doenças infecciosas, incluindo a TB.

1.6 POLIMORFISMO DO GENE QUE CODIFICA O RECEPTOR DE VITAMINA D–*VDR*

A VD exerce sua função no sistema imune via VDR e muitas de suas atividades biológicas são mediadas pela alta ou baixa afinidade com este receptor celular (Simboli-Campbell et al.1996). A atividade da VD através de seu receptor é capaz de regular a transcrição de outros genes envolvidos na regulação celular, desenvolvimento e imunidade (Slattery et al. 2007).

O VDR forma homodímeros ou heterodímeros com um dos receptors retinóides (RXRα, RXRβ, RXRγ) que permitem uma ligação específica com DNA de alta afinidade. A ligação da 1,25(OH)2D com o complexo VDR-RXR é seguida pela ligação deste complexo com o VDRE, iniciando a transcrição da região promotora dos genes alvo da VD. Os VDRE são sitios de ligação de DNA que podem ativar ou suprimir os genes alvo (Koszewski et al., 2010) e esses eventos são influenciados pelo tipo de receptor retinóide com os quais o VDR está associado. Este receptor pode se encontrado em vários tipos celulares, incluindo monócitos, macrófagos, células dendríticas, natural killer e linfócitos T e B ativados. Além disso, está envolvido na diferenciação e proliferação celular (Lin et al. 2004) e variações genéticas no VDR têm sido associadas com concentrações circulantes no soro de 25(OH)D3, bem como, com a densidade mineral óssea (Riggs,1997) e na variação dos níveis séricos de VD circulantes (Fujioka et al. 2000; Ikuyama et al. 2002).

O gene VDR está localizado no cromossoma 12 na região q12-14(13q1). Vários polimorfismos no gene *VDR* têm sido identificados, um no intron 8 (*Bsm* I e *Apa* I) e uma no éxon 9 (*Taq* I), bem como no éxon 2 (*Fok* I) mais próximo da região 5' e próximo à região promotora do gene *VDR* (Uitterlinden et al. 2004) e inúmeros SNPs têm sido identificados e podem influenciar no risco do desenvolvimento da TB. Assim, se a deficiência de VD é um fator de risco para a TB, conseqüentemente variações no gene *VDR* podem estar associados com o desenvolvimento da TB (Bellamy et al. 1999). Além desses polimorfismos descritos acima, mais de 25 polimorfismos diferentes são conhecidos no *locus VDR*, próximos da região final 5' do gene. No entanto, próximo dessa região e próximo a região promotora é reportado a substituição de uma timina por uma citocina no éxon 2 que cria um códon de iniciação *ATG* a três códons do real sítio de iniciação da transcrição. Assim, duas formas de proteínas VDR podem ser produzidas: uma de 427 e outra de 424 aminoácidos (Jurutka et al. 2000).

A presença do alelo *f* para o polimorfismo da enzima *FokI,* que reconhece essa especifica região do éxon 2, tem sido associado com susceptibilidade para TB pulmonar na população indiana residente em Londres (Wilkinson et al. 2000), uma vez que a transcrição deste alelo é 1 a 7 vezes menos eficiente que o alelo *F* (Arai et al. 1997). Foi descrito em nativos da América do Sul que os genótipos *FF* e *tt* estavam associados com proteção para TB (Wilbur et al. 2007). Similarmente, em população indiana, indivíduos com genótipo *FF* de *FokI* apresentaram uma diminuição no risco de desenvolver TB associado a cepas MDR, enquanto que carreadores do genótipo *Ff* e *tt* demonstraram elevado risco de desenvolver TB ligada a estas cepas (Privit et al. 2011).

Apesar dos genótipos *FF* de *FokI* e *Tt* de *TaqI* não estarem associados a resitência a TB na população do Peru, Roth et al. (2004), descreveram que portadores destes genótipos apresentaram rápida redução da carga bacilar no de escarro, estando então associados a um melhor prognóstico. Contudo, estes achados não foram confirmados posteriormete em pacientes com TB ativa em população sul africana (Babb et al. 2007). Na população da China foi encontrada associação do genótipo *ff* com TB espinhal (Zhang et al. 2010). Na Turquia foi reportada associação entre o alelo *B* de *BsmI* e susceptibilidade à TB, contudo, não foi encontrada associação entre *TaqI* e *FokI* e TB pulmonar

(Ates et al. 2010). Diferentes resultados foram encontrados em população iraniana. Merza et al. (2009) observaram que a combinação do alelo *b* do *BsmI* com o alelo *A* de *TNFA-308G/A* conferia susceptibilidade à TB. Apesar do polimorfismo no gene *BsmI* ser descrito como SNP silencioso e não alterar a quantidade, estrutura ou função da proteína VDR codificada, as variantes do gene *VDR* podem influenciar a estabilidade do RNA mensageiro (Jurutka et al. 2001; Uitterlinden et al. 2004). De maneira contrária aos trabalhos descritos acima, em população coreana, não foi encontrada associação com TB em relação aos polimorfismos de *TaqI, BsmI e FokI* (Kang et al. 2011), bem como em população chinesa, não foi possível observar associação com TB em carreadores das variantes de *TaqI* (Zhao et al. 2009).

No intuito de descrever a associação entre os diferentes SNPs do gene *VDR* e dessa maneira identificar haplótipos ligados a susceptibilidade à TB, foi realizado um grande estudo no oeste africano onde foi observada associação do haplótipo *F-A* com TB (Bornman et al. 2004), porém, em outro estudo na mesma população foi encontrada associação do haplótipo *F-b-A*-T com resistência à TB (Lombard et al. 2006).

Assim, é importante entender os mecanismos subjacentes as associações de haplótipos, descrever quais os polimorfismos do gene *VDR* são relevantes e finalmente determinar suas relações com a genômica funcional na determinação da susceptibilidade ou resistência a inúmeras doenças infecciosas, principalmente para a TB.

1.7 A CITOCINA IFN-γ E SEU POLIMORFISMO (*INFG +874T/A)*

Entre os genes descritos em associação com a TB, o *IFNG* tem sido intensamente estudado. O gene *IFNG* humano está localizado no cromossoma 12 (12q14) no intron 2 na posição *+874* deste gene (Etokebe et al, 2006) e a citocina codificada por este gene é importante no controle da infecção pelo *Mtb.* A citocina IFN-γ é produzida por células antígenos-específicas do tipo Th1 e células natural killers (NK), que por sua vez se liga ao complexo R1/R2 do receptor de IFN-γ, de 90 e 62 kDa respectivamente, na superfície do macrófago

(Ottenhoff et al. 2002). A proteína de 90 kDa se liga com maior afinidade ao IFN-γR1, ao passo que a proteína de 60 kDa possui um papel menos ativo na ligação com o IFN-γR2, mas é essencial para a transdução de sinal iniciado pelo IFN-γ (Axelrod et al. 1994). Essas proteínas são codificadas nos genes localizados no cromossoma 2 e 21, respectivamente. Experimentalmente, camundongos com deficiência desta citocina, foram altamente susceptíveis ao *Mtb* (Moreira et al. 2000). Indivíduos com deficiência parcial ou total no receptor de IFN-γ são mais susceptíveis a infecções com bactérias intracelulares (Hill et al. 1998; Ottenhoff et al. 1998). Pacientes com TB em população indiana apresentaram níveis de IFN-γ aumentado quando comparados com contatos/controles saudáveis, indicando neste caso, que apesar da presença de IFN-γ, esta citocina não é capaz de induzir os eventos necessários para uma resposta imune adequada contra o *Mtb* (Katti., 2011).

Polimorfismos localizados dentro de algumas regiões principalmente *SNPs* (polimorfismo de um único par de base) são capazes de afetar a transcrição gênica, causando variações individuais na produção de citocinas. Assim, possíveis predisposições genéticas para TB podem estar associadas a vários *loci* de citocinas que codificam suas diferentes estruturas protéicas e que conseqüentemente podem contribuir com maior ou menor susceptibilidade para esta doença (Bulat-Kardum et al. 2006). A seqüência especifica do alelo *T* deste polimorfismo, fornece um sítio de ligação para o fator de transcrição nuclear-β (NF-kβ) (Pravica et al. 1999; Bream et al. 2002), que induz a expressão de IFN-γ, por isso, o alelo *T* está relacionado ao aumento da produção de IFN-γ e o alelo *A* com baixa produção (Pravica et al. 2000). Outra característica importante é que baixos níveis de secreção de IFN-γ (devido à ausência do alelo *T* no sítio *+874*) poderiam influenciar na replicação do *Mtb,* influenciando no tempo de conversão da baciloscopia (Etokebe et al. 2006). Atualmente pouco se sabe sobre a influência deste polimorfismo na reposta ao tratamento da TB. Sundaram et al. (2009) e Shibasaki et al. (2009), demonstraram em população japonesa, forte associação do genótipo *AA* do *IFNG+874T/A* com demora na conversão da cultura de escarro, sugerindo um efeito quantitativo de IFN-γ no tratamento da TB. Embora alguns estudos demonstrem que a freqüência do genótipo *AA* do *IFNG+874T/A* seja

significantemente expressa entre pacientes com TB quando comparados com controles (Lio et al. 2002; Lopez-Maderuelo et al. 2003; Rossouw et al. 2003; Tso et al. 2005), outros estudos não encontraram os mesmos resultados (Fitness et al. 2004; Henao et al. 2006). Diversos trabalhos vêm associando ou não este polimorfimo à susceptibilidade à TB em diferentes populações. Na população chinesa (Ding et al. 2007; Wang et al. 2010) e tunisiana (Ben Selma et al. 2011), o homozigoto *AA* de *IFNG+874* foi associado com TB pulmonar. Estudo em população indiana, ao relacionar as variantes de *IFNG+874 e os níveis de* IFN-γ no soro não conseguiu demonstrar associação entre pacientes com TB e controles saudáveis*,* apesar de encontrar níveis de citocina aumentados nos pacientes (Abhimanyu et al. 2011). Lio et al. (2002), observaram em pacientes com TB na Sicília, que a alta capacidade secretora de IFN-γ deve proteger da TB. Esses dados não foram confirmados por Etokebe et al. (2006), quando estudaram pacientes com TB da Croácia, onde estes pacientes possuíam freqüência de genótipos *TT* de *IFNG+874* similares ao encontrado nos indivíduos controles.

Poucos estudos vêm sendo conduzidos na população brasileira, no entanto, Amim et al. (2008), demonstraram associação do genótipo *AA* de *IFNG +874T/A,* em pacientes do Rio de Janeiro, com a ocorrência da TB ativa independente do resultado do PPD. Outro estudo em população brasileira descreveu que as concentrações da citocina IFN-γ no plasma de pacientes com TB são baixas nos carreadores do genótipo *AA* (Vallinoto et al. 2010).

Dessa forma, é possível verificar que a susceptibilidade para a TB surge de múltiplos fatores hereditários que provavelmente poderiam influenciar diferentes populações (Casanova & Abel, 2002). Logo, determinar possíveis associações deste gene com a susceptibilidade para TB na população brasileira seria de extrema relevância, haja visto que esta doença acomete parte desta população.

1.8 POLIMORFISMO DO GENE *NOS2-954G/C* (QUE CODIFICA A ENZIMA ÓXIDO NÍTRICO SINTASE INDUZIDA)

O óxido nítrico (NO) é um gás que funciona como um radical livre lipossolúvel que medeia a resistência do organismo do hospedeiro contra infecções (Nathan, 1997). O NO é produzido por três diferentes enzimas óxido nítrico sintases (NOS), NOS1, NOS2 e NOS3 em humanos (Bredt & Snyder, 1994), via conversão da L-arginina em L-citrulina na presença de várias co-substâncias (Kun et al. 2001). A enzima NOS2 produz NO em resposta a patógenos e a estímulos do sistema imune (Nathan, 1997). A produção desta enzima representa uma importante rede para a produção de NO e é regulada primariamente a nível transcripcional pela ação de citocinas inflamatórias e toxinas (Nathan, 1997). Estudos imunohistoquímicos têm demonstrado uma expressão abundante da proteína NOS2 (Flynn et al. 1998), bem como uma produção *in situ* de RNI em granulomas, nos pulmões de camundongos e de humanos infectados pelo *Mtb* (Scanga et al. 2000; Choi et al. 2002). Camundongos com deficiência de NOS2 apresentam carência de NO e são altamente suscetíveis a infecção pelo *Mtb* (Yang et al. 2009). Além disso, em modelos murino o NO induz a apoptose de macrófagos infectados, contribuindo para a morte do *Mtb* (Chan et al. 2001). Recentemente, Liu et al. (2011), observaram que macrófagos infectados com *Mtb H37Ra* produzem mais NO, H_2 e O_2 e intensificam a secreção de IL-12 e TNF-α, indicando que esta cepa de *Mtb*, induz maior produção de citocinas importantes na resposta do hospedeiro contra a TB (Liu et al. 2011). Segundo Rich et al. (1997), a expressão de NOS2 não tem conferido atividade micobactericida, talvez pela atividade da NOS2 não ter sido alta o suficiente ou pelo fato da bactéria ser resistente. Desta forma, acredita-se que os RNI, principalmente pela ação de NOS2, ajudem no controle do crescimento micobacteriano no interior dos macrófagos. Outro dado importante é que foi possível demonstrar em macrófagos humanos infectados pelo *Mtb,* que a síntese de NO é modulada pela ação da VD combinada com IFN-γ levando a ativação NFκβ, essencial para a expressão do gene *NOS2A* nestas células (Lee et al. 2009).

A expressão de NOS2 é transcripcionalmente regulada e quatro polimorfismos são descritos na região promotora do gene *NOS2*: uma repetição na região microsatélite localizada 2.5 kb *upstream* do sítio de iniciação da transcrição (CCTTTn) (Burgner et al. 1998) e três SNPs, um na posição -954 com a substituição de uma guanina por uma citosina (G-954C) (Kun et al. 1998), um na posição -1173 com a substituição de uma citosina por uma timina (C-1173T) (Hobbs et al. 2002) e o último na região -1659 com a substituição de uma adenina por uma guanina (A-1659G) (Burgner et al. 2003). Sabe-se que macrófagos pulmonares de pacientes com TB expressam NOS2 (Wang et al. 1998) e pode usá-la para matar a micobactéria *in vitro* (Nozaki et al. 1997). Velez et al. (2009), demonstraram associação do gene *NOS2A-954G/C* e susceptibilidade à TB, particularmente em afro descendentes. Em estudo realizado em população da Tanzania e do Kenia foi encontrada associação com o polimorfismo na posição -1173 e proteção contra malária celebral, além disso, esta variante foi associada com o aumento da produção de NO (Hobbs et al. 2002). De maneira diferente, estes achados não foram confirmados em população africana do Gabon com malária em relação ao polimosfimo na posição -954G/C (Mombo et al. 2003).

Assim, tentar identificar possíveis polimorfismos que alteram a expressão de NO podem ser de grande valia para o entendimento da patologia causada pela TB.

2 JUSTIFICATIVA

Segundo a Organização Mundial de Saúde (OMS) aproximadamente um terço da população mundial está infectada com o *Mtb*. Apesar de apenas 10% desses indivíduos infectados desenvolverem a doença clínica (ativa), o número de óbitos por ano é alarmante, de 2 a 3 milhões (WHO, 2010). Neste contexto, é importante entender quais fatores poderiam estar associados ao desenvolvimento da forma ativa da doença ou a resistência. Há muito tempo sabe-se que fatores ambientais como: condições sócio-econômicas, deficiência nutricional, *stress,* aspectos culturais, virulência do bacilo, acesso a saúde, medicação, bem como outras doenças possuem um papel fundamental na determinação da susceptibilidade à tuberculose (TB) na população humana (revisado por Leandro et al. 2009). No entanto, acredita-se que fatores genéticos de ambos, tanto do *Mtb* quanto do homem, possuem um grande impacto na determinação da resposta imune contra o *Mtb* (Möller et al. 2009). A interação e o impacto entre esses diferentes fatores no desenvolvimento da doença clínica ainda não é bem determinada na literatura. Inúmeros polimorfismos estão sendo associados com o desenvolvimento da TB (Goldfeld, 2004). Logo, foi proposto um estudo multigênico para avaliar se polimorfismos em genes que codificam *DBP* ou *Gc-globulin, VDR, IFNG* e *NOS2* poderiam estar contribuindo para o desenvolvimento da TB. Assim, o maior desafio na atualidade é entender o porquê da manutenção da infecção latente na maioria dos indivíduos expostos ao *Mtb* e do desenvolvimento da TB ativa em somente 10% dos expostos. Desta forma, seria de grande importância, compreender o papel dos fatores genéticos humanos relacionados com os diferentes mecanismos biológicos que modulam o sistema imune na determinação de resistência ou susceptibilidade ao desenvolvimento da TB, a fim de fornecer bases científicas para o desenvolvimento de novos métodos terapêuticos e preventivos contra a TB, bem como para o desenvolvimento da chamada terapia gênica.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar em estudo caso-controle a importância de marcadores genéticos e funcionais ligados aos genes da resposta imune inata e adaptativa e a susceptibilidade à TB.

3.2 OBJETIVOS ESPECÍFICOS

- Descrever as freqüências de polimorfismos de base única e associá-los a susceptibilidade à TB pulmonar.
- Comparar as análises funcionais do sistema imune às análises genotípicas com o desenvolvimento da tuberculose pulmonar ativa.

1- No gene *DBP*, nas posições 416 e 420, determinadas respectivamente pela endonucleases *HaeIII* e *StyI* e relacionar com os níveis séricos de VD.
2- No gene *VDR*, determinadas pelas endonucleases *TakI, FokI, BsmI e ApaI* e relacionar com os níveis séricos de vitamina D.
3- No gene *IFNG* na posição *+874T/A*.
4- No gene *NOS2 na posição -954G/C,* determinada pela endonuclease *BsaI* e relacionar com produção de radicais de nitrogênio.

4 ARTIGOS

A metodologia empregada neste estudo e os resultados obtidos serão apresentados no formato de artigos científicos. Nesta seção encontram-se quatro artigos, sendo um deles de revisão, com resultados relacionados ao projeto.

4.1 GENETIC POLYMORPHISMS IN *VITAMIN D RECEPTOR, VITAMIN D-BINDING PROTEIN, TOLL-LIKE RECEPTOR 2, NITRIC OXIDE SYNTHASE 2*, AND *INTERFERON-γ* GENES AND ITS ASSOCIATION WITH SUSCEPTIBILITY TO TUBERCULOSIS

Artigo publicado no *Brazilian Journal of Medical and Biological Research* (2009)

Uma extensa revisão de literatura foi realizada resultando no artigo de revisão. Diversos trabalhos descrevem a existência de fatores genéticos do hospedeiro relacionados à susceptibilidade à TB. Assim, neste artigo foram revisados trabalhos que descreveram as freqüências de diferentes polimofirmos, dentre os quais, VDR, *DBP, TLR2, NOS2 e IFNG*, em indivíduos com TB em diferentes populações. Estes polimorfismos e suas complexas interações, bem como, a deficiência de VD, vêm sendo estudados na tentativa de entender o envolvimento destes genes e a função imunomodulatória desta vitamina na resposta imune contra o *Mtbs.*

4.1.1 Artigo publicado no *Brazilian Journal of Medical and Biological Research (2009)*

Brazilian Journal of Medical and Biological Research (2009) 42: 312-322
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Genetic polymorphisms in vitamin D receptor, vitamin D-binding protein, Toll-like receptor 2, nitric oxide synthase 2, and interferon-γ genes and its association with susceptibility to tuberculosis

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Mycobacterium tuberculosis kills more people than any other single pathogen, with an estimated one-third of the world's population being infected. Among those infected, only 10% will develop the disease. There are several demonstrations that susceptibility to tuberculosis is linked to host genetic factors in twins, family and associated-based case control studies. In the past years, there has been dramatic improvement in our understanding of the role of innate and adaptive immunity in the human host defense to tuberculosis. To date, attention has been paid to the role of genetic host and parasitic factors in tuberculosis pathogenesis mainly regarding innate and adaptive immune responses and their complex interactions. Many studies have focused on the candidate genes for tuberculosis susceptibility ranging from those expressed in several cells from the innate or adaptive immune system such as Toll-like receptors, cytokines (TNF-α, TGF-β, IFN-γ, IL-1b, IL-1RA, IL-12, IL-10), nitric oxide synthase and vitamin D, both nuclear receptors and their carrier, the vitamin D-binding protein (VDBP). The identification of possible genes that can promote resistance or susceptibility to tuberculosis could be the first step to understanding disease pathogenesis and can help to identify new tools for treatment and vaccine development. Thus, in this mini-review, we summarize the current state of investigation on some of the genetic determinants, such as the candidate polymorphisms of vitamin D, VDBP, Toll-like receptor, nitric oxide synthase 2 and interferon-γ genes, to generate resistance or susceptibility to *M. tuberculosis* infection.

Key words: Tuberculosis; Vitamin D; Vitamin D-binding protein; Toll-like receptor; Nitric oxide synthase 2; IFN-γ

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Introduction

Tuberculosis, primarily caused by *Mycobacterium tuberculosis*, continues to be an important public health problem despite the existence of national and international tuberculosis control programs. Recent data from the World Health Organization show that about 8-10 million new cases arise annually and eventually 2-3 million die of the disease every year. Tuberculosis is one of the major infections that cause disease and death worldwide. It is estimated that one-third of the World's population is infected with *M. tuberculosis*, but that only one in ten (10%) of those infected ever develop clinical disease.

The central question is if tuberculosis patients are

inherently susceptible to the disease or if disease development is caused by specific environmental factors. Clearly, environmental factors such as poor economic conditions, malnutrition, stress, and overcrowding play a role in determining the susceptibility to tuberculosis in human populations. It is known that genetic and non-genetic factors of both the bacterium and the host have impact on the immune response to *M. tuberculosis*. The interaction between these different factors is unknown as well as the impact on disease development. Thus, the increasing interest in understanding the role of human genetic factors in the immune system controlling susceptibility/resistance to infectious diseases is of great importance for tuberculosis research because this will allow a genetic dissection of anti-mycobacterial immunity and should open new possibilities for preventive and therapeutic measures.

Vitamin D

When skin is exposed to sufficient UVB light (290 to 320 nm), the hormonal cascade for the endogenous production of vitamin D is activated. The prohormone (7-dehydrocholesterol) is converted into pre-vitamin D_3 (25-hydroxy-VD3 or $25(OH)_2D_3$) and then to 1,25 dihydroxy-D3 ($1,25(OH)_2D_3$ or vitamin D_3) within keratinocytes. Sunlight also induces the expression of vitamin D receptors (VDR). Circulating $25(OH)_2D_3$ is the major form of vitamin D, with concentrations 1000 times higher than $1,25(OH)_2D_3$, the active form of vitamin D. The conversion of $25(OH)_2D_3$ to $1,25(OH)_2D_3$ is controlled mainly by parathyroid hormone, calcium and phosphate levels. The extent of this conversion regulates the effect of vitamin D on bone, gut and kidney. The importance of sunlight has been observed throughout the history of tuberculosis treatment as an alternative treatment of pulmonary and cutaneous tuberculosis since as early as 1849. During the 13th Workshop consensus for vitamin D nutritional guidelines, held in Victoria, British Columbia in 2007, it was pointed out that half of the elderly in North America and two-thirds of the elderly in the rest of the world do not have enough circulating vitamin D to maintain healthy bone density through calcium metabolism, thus increasing their risk of fractures, major cancers, problems of tooth attachment, and of reduced muscle strength. Research reported at that meeting showed that adults must maintain a much higher blood vitamin D level than children, i.e., approximately 75 nM/L. Low levels of circulating vitamin D were reported for an elderly Brazilian population, which has a deficiency (25 nM/L; 15.4%) or insufficiency (25-50 nM/L; 41.9%) of vitamin D (1). To date, there are no reports regarding the serum level of vitamin D in the Brazilian population comparing age, gender, sun exposure, food intake, and related disease. However, the general consensus is that a desirable serum concentration of vitamin D is 50 nM (or 20 ng/mL). A recent nutritional analysis of food intake demonstrated vitamin D deficiency in all major geographic regions of Brazil that could result in up to a 6-fold decrease in presumable normal serum levels of vitamin D, independent of socio-economic level (2). The correlation between serum level of vitamin D and risk for tuberculosis latent infection or disease development has been described in African immigrants living in Australia (3). Lower mean vitamin D levels were observed in latent infection compared with individuals with no *M. tuberculosis* infection and lower in tuberculosis patients. Higher vitamin D levels were associated with lower probability of any *M. tuberculosis* infection (4). These results and other studies in sunny countries have shown that the existence of vitamin D deficiencies might be related to some risk factors, such as inadequate food source, sunshine exposure, sun block use, religion, pollution and, finally, skin color (ethnicity). These factors explained how it was possible in sunny countries to detect a vitamin D deficiency (4). This deficiency associated with the existent environmental conditions and parasitic infection seemed to explain the increased risk for tuberculosis development (4).

Vitamin D metabolites are transported in the blood by vitamin D-binding protein (VDBP) and are cleared from the blood by the liver, where they are converted into 25-hydroxyvitamin D_3 (calcidiol) and released back into the blood as the major circulating form of vitamin D. $25(OH)_2D_3$ is taken up by the kidneys, where it undergoes further conversion into the most active hormonal form of vitamin D ($1,25(OH)_2D_3$), and is released back into the blood.

Recently, the role of vitamin D has been emphasized not only in bone mineralization and in anti-tumor activity but also, a large body of work has accumulated supporting an important role for vitamin D in multiple organ systems as well as physiologic and molecular processes. VDR are distributed ubiquitously, and by binding with its receptor, vitamin D initiates a series of events that can affect cellular proliferation and differentiation, inflammation, the immune and endocrine systems, insulin resistance, and lipid metabolism (5). Vitamin D metabolites are important immunomodulatory hormones, which activate monocytes and suppress lymphocyte proliferation, immunoglobulin production, and cytokine synthesis. Moreover, this hormone has been shown to act as both an up-regulating agent during natural immunity via the enhancement of phagocytosis by monocyte/macrophage populations and a down-regulator during acquired immune response via an inhibitory effect on MHC class II expression by professional antigen-pre-

senting cells (6). Apparently $25(OH)_2D_3$ has no direct antimycobacterial action, but its active metabolite $1,25(OH)_2D_3$ is able to block the maturation of myeloid dendritic cells (DCs) by reducing the expression of co-stimulatory molecules. This inhibits secretion of IL-12 and increases IL-10 production as well as inhibits the secretion interferon-γ (IFN-γ) and IL-2 by T cells and stimulates Th2 cell development with increased production of IL-4, IL-5, and IL-10 (7). Thus, these data indicate that the $1,25(OH)_2D_3$ does not mediate protection via those cytokines. It is known that the modulation of $1,25(OH)_2D_3$ by binding to nuclear VDR upregulates protective innate host responses, including the induction of nitric oxide synthase (NOS2). However, the mechanisms that regulate the expression of NOS2 and the role of $1,25(OH)_2D_3$ in tuberculosis infection are not known. Some investigators have reported that the human macrophage-like cell line acquires the ability to produce substantial amounts of nitric oxide (NO) on stimulation with lipopolysaccharide or $1,25(OH)_2D_3$ in the absence of activating factors such as IFN-γ. This suggests that $1,25(OH)_2D_3$ acts to suppress the growth of *M. tuberculosis* in these cells and that vitamin D-induced NO production may play a role in the host defense against human tuberculosis (8). In addition to the NO production induced by $1,25(OH)_2D_3$, this metabolite also up-regulates the expression of 1α-hydroxylase, the enzyme that metabolizes $25(OH)_2D_3$ to $1,25(OH)_2D_3$ due to ligation of Toll-like receptor (TLR)2/1 in macrophages stimulated by *M. tuberculosis* antigens and up-regulates the cathelicidin gene. $1,25(OH)_2D_3$ regulates the expression and activation of antimicrobial peptides in keratinocytes and monocytes in the epidermis and provides a stimulus for rapid production of a chemical antimicrobial shield with the expression of LL-37, a human antimicrobial peptide belonging to the cathelicidin family (9). Cathelicidins are relevant to defense against microbes including Gram-positive and -negative bacteria as well as fungi and certain viruses and it is increased with increasing concentrations of $1,25(OH)_2D_3$ (9). The addition of a VDR antagonist VAZ (ZK159222) inhibited the induction of cathelicidin mRNA by greater than 80%, and consequently the antimicrobial activity was reduced by about 70% (10). The increase in TLR2/1 enabled the cells to respond to microbial stimulation and also amplify the synthesis of $1,25(OH)_2D_3$ (Figure 1). The active metabolite of vitamin D, $1,25(OH)_2D_3$, suppresses growth of *M. tuberculosis in vitro* and this effect may be facilitated by TLR *in vivo* (10). Thus, there is an elegant system of control of innate immunity by $1,25(OH)_2D_3$. The most reasonable explanation suggests that vitamin D stimulates the immune cells to resist *M. tuberculosis* infection by mediating protection against tuberculosis by 'nonclassical' mechanisms.

Vitamin D receptor

Vitamin D exerts its action through VDR, a nuclear hormone receptor when activated by its ligand $1,25(OH)_2D_3$, that is present on monocytes and activated T and B lymphocytes (Figure 1) (5). Extensive data indicate that there are two mechanisms of action. One involves the activation of nuclear VDR and transcriptional regulation of many vitamin D-responsive genes. The other involves activation of non-genomic signal transduction pathways in target cells. This second mechanism is likely to involve a membrane VDR. A candidate form of VDR that is a 64.5-kDa protein from chick epithelium has been described recently, which specifically binds $1,25(OH)_2D_3$ and is responsible for some of the rapid cellular actions of $1,25(OH)_2D_3$ (11). Is it possible that this membrane receptor for $1,25(OH)_2D_3$ acts with ligand-binding properties that are different from those of the nuclear/cytosol receptor? Studies suggest that the ligand-receptor complex mediates the signal transduction of the hormone via opening of voltage-gated Ca^{21} channels to initiate the biological response(s) (12).

The *VDR* gene is located on the long arm of chromosome 12 (12q12-14) and is composed of 10 exons, the first of which is not transcribed (13). The *VDR* gene is a candidate locus for susceptibility to different diseases, such as prostate cancer, inflammatory bowel disease, osteoporosis and tuberculosis, due to allelic variation that affects the activity of the receptor and subsequent downstream vitamin D-mediated effects such as calcium absorption, excretion, and modulation of cellular proliferation and differentiation. The variation in the ability to synthesize vitamin D, including polymorphisms in the *VDR* gene, may be a contributing factor to increase tuberculosis susceptibility. Four of these *VDR* polymorphisms can be distinguished by digestion with restriction enzymes. Three of them do not change the translated protein (*Taq*I, *Apa*I and *Bsm*I). The polymorphisms recognized by *Bsm*I (*BB*, *Bb* and *bb*) and *Apa*I (*AA*, *Aa* and *aa*) are located in intron 8 of the *VDR* gene and the one recognized by *Taq*I (*TT*, *Tt* and *tt*) is located in exon 9, that leads to a silent codon change, with ATT and ATC, both coding for isoleucine and has been associated with increased *VDR* mRNA stability. The genotype *tt*, detected with the *Taq*I restriction enzyme, is associated with decreased risk of tuberculosis. The fourth VDR polymorphism, recognized by *Fok* I restriction enzyme (*FF*, *Ff* and *ff*), results from a C→T transition that creates an alternative initiation codon (ATG), three codons from the downstream start site. The *VDR* encoded by the *f* allele from the *Fok*I restriction enzyme recognition is increased in length by three amino acids, and the transcription of this allele is 1-7 times less efficient than the *F* allele and can

alter the amount of VDR produced (13).

The association or not of *VDR* polymorphism with tuberculosis in several geographic areas was discussed by Lewis et al. (14). They evaluated *VDR* gene polymorphism in different ethnic population studies published prior to August 2004. The *tt* genotype was underrepresented in tuberculosis patients from Gambia (15) and India (16), but in patients from Peru (17) the *Tt* and *FF* genotypes were associated with faster sputum culture conversion after initiation of therapy. The same was seen in China (18), where the *FF* genotype was associated with resistance to tuberculosis. After this meta-analysis description other results appeared describing *VDR* polymorphism in Chinese Tibetans (19) in which *Fok*I polymorphism of the *VDR* gene might be associated with tuberculosis, but there was no evidence that the *Taq*I polymorphism was associated with the disease in this population. A larger study conducted by Babb et al. (20) with a South African population reported that the *VDR* genotypes might affect the severity of disease or the ability of the treated cases to recover (positive sputum conversion or smear stain to negative), but no association was demonstrable between tuberculosis and *VDR* polymorphisms. When they used diplotypes and haplotypes to analyze the results, only a weak association was detect between the *VDR* haplotypes and tuberculosis susceptibility. They found that the *Fok*I-*Apa*I-*Taq*I haplotype ('FAT') tended to be associated with tuberculosis and may be a risk factor, whereas the 'FaT' haplotype is probably protective. Regarding the role of *VDR* polymorphisms in disease caused by *Mycobacterium leprae*, Goulart et al. (21) reported that there was a higher risk of leprosy when individuals carry the combination of *tt* genotype from *Taq*I polymorphisms in the *VDR* gene and negative Matsuda test, demonstrating a possible synergistic role of these two variables in leprosy susceptibility via effects on cellular immunity in a Brazilian population.

While there is evidence that vitamin D promotes macrophage killing of *M. tuberculosis*, the effector mechanisms are not clear, and the association of *VDR* polymorphisms with susceptibility to tuberculosis remains unproved. As described in Lewis et al. (14), larger studies are required to determine whether *VDR* polymorphisms play a role in genetic susceptibility to tuberculosis worldwide. A sample size of more than 2000 cases and controls is required to demonstrate if *VDR* polymorphism is associated with tuberculosis. This large sample size would be necessary to have enough power to detect specific polymorphisms related to tuberculosis, because any association will be diluted due to the extended linkage disequilibrium with the specific functional allele. Most studies conducted until now were in ethnically and geographically different populations, thus, they were underpowered and impossible to reach any conclusions by examining the alleles separately. The *VDR* polymorphisms contain many more single nucleotide polymorphisms (SNPs) than the four normally measured and it is necessary to conduct family-based studies that will allow research to construct reliable haplotypes and eliminate the possibility of population stratification and other risk factors such as gender, HIV status, occupation, and environment issues.

Vitamin D-binding protein

VDBP is a multifunctional, highly expressed, polymorphic serum protein that was initially named the group-specific component of serum (Gc-globulin). VDBP, a 458-amino acid polymorphic serum protein, is the major plasma carrier of vitamin D_3 and its metabolites and ensures that vitamin D is transported to the liver, $25(OH)_2D_3$ to the kidney, and $1,25(OH)_2D_3$ to target cells and organs. VDBP is encoded by the *Gc* gene, a member of a multigene cluster that includes albumin and α-fetoprotein genes, located at chromosome 4q11-q13. Sequence variations at codons 416 and 420 in exon 11 of the *Gc* gene give rise to major electrophoretic variants of VDBP, termed Gc1 fast (Gc1F), Gc1 slow (Gc1S) and Gc2 (22). These variants differ by amino-acid sequence, as well as by attached polysaccharides. Combinations of the three VDBP or Gc variants result in six common circulating phenotypes: Gc1F/Gc1F, Gc1F/Gc1S, Gc1S/Gc1S, Gc1F/Gc2, Gc1S/Gc2, and Gc2/Gc2 (23). *DBP* polymorphism (Gc phenotype) is related to the VDBP concentration and vitamin D status, as described by Lauridsen et al. (23). These authors showed a strong correlation of higher, intermediate and lower circulating levels of $25(OH)_2D_3$ and $1,25(OH)_2D_3$ with Gc1-1, Gc1-2 and Gc2-2 phenotypes, respectively, in Danish Caucasian postmenopausal women population. In addition to storing and transporting vitamin D_3 hormones, VDBP also affects their pharmacokinetics (24) and plays a role in the sustained effective conversion of cutaneously derived vitamin D_3 into $25(OH)_2D_3$. In addition to transporting vitamin D_3, VDBP has multifunctional properties, including an important role in macrophage activation that is distinct from its vitamin D-binding ability. Variations in this property could affect the functioning of the immune system, as shown for *DBP* knockout mice that exhibited an impaired immune response to bacterial infections (24). VDBP has been shown to enhance the leukocyte chemotactic activity of activated complement peptides, which are the precursor of the macrophage-activator factor (MAF). Indeed Gc-MAF influences macrophage chemotaxis (24).

A role of *DBP* polymorphism in autoimmune diabetes

mellitus and infectious disease in Polynesia and Japan (25) has been suggested. Until now, only one study evaluated the *DBP* phenotype in tuberculosis patients and no differences were seen among patients and the control group. In that study, a 33% frequency of Gc2 in tuberculosis patients was slightly but not significantly higher than in the control group (26%), and this elevation was at the expense of both Gc1F and Gc1S alleles (26). Further studies are necessary to understand the physiological role of VDBP and its phenotypes on susceptibility to tuberculosis and other diseases.

The Toll-like receptors

The TLRs represent a group of single-pass transmembrane receptors observed from *Drosophila* to mammals. They are expressed on innate immune cells and are the key sensors for a broad spectrum of pathogen-derived molecules, and are critical in shaping host-pathogen interactions (Figure 1). All bacteria contain ligands for many TLRs and the ligation of TLRs by these pathogen-specific ligands initiates a signal transduction pathway in the host cell that culminates in the activation of nuclear factor-kappa B (NF-κB) and the induction of cytokines and chemokines (27), which are crucial for eliciting the adaptive immune response against pathogens. The production of IL-12, TNF-α and NO is induced mostly by macrophages soon after innate recognition of mycobacteria through TLRs (28). TLRs have been recognized to be the principal mediators of the pro-inflammatory signal, TNF-α, induced by whole *M. tuberculosis* (28). Indeed, TLR2 has been shown to be involved in response to mycobacteria, specifically to the 19-kDa lipoprotein and lipoarabinomannan from *M. tuberculosis*. Interestingly, the triacylated mycobacterial lipopeptide is recognized by dimmers of TLR2 and TLR1, whereas similar diacylated compounds are recognized by dimmers of TLR2 and TLR6 (29). However, recent data point to the deactivation of macrophage cytokine induction by *M. tuberculosis* proteins. ESAT-6, a member of a unique family of proteins present in *M. tuberculosis*, probably attenuates the innate immune response by dampening the production of the IL-12 p40, TNF-α and NO (30). Recently, it has been shown that ESAT-6 and some peptides were able to dampen TLR2 signaling by preventing assembly of the cytosolic MyD88-dependent signaling scaffold (31). However, other members of the TLR family could be activated by *M. tuberculosis*, with or without TLR2 interaction. DNA from mycobacteria contains stimulatory CpG motifs that activate TLR9 (31) and up-regulate Th1 response.

The role of TLR in resistance to *M. tuberculosis* was suggested initially by the fact that MyD88-deficient mice are more susceptible to *M. tuberculosis* infection (31) and by the observation that TRL2/TLR1 reduced the viability of intracellular *M. tuberculosis* in human monocytes and macrophages, but not in monocyte-derived DCs (10). Liu et al. (10) also reported that TLR induces up-regulation of the *VDR*, 1α-vitamin D hydroxylase (enzyme that converts inactive to active vitamin D) and *CYP27B1* gene expression in monocytes and macrophages. These actions permit the macrophages to internalize serum VDBP-bound $25(OH)_2D_3$ from the extracellular fluid by facilitated endocytosis and use that $25(OH)_2D_3$ as substrate for the upregulated CYP27B1 (expression gene of 1α-vitamin D hydroxylase). Because VDR is functional when exogenous $1,25(OH)_2D_3$ is added, it was hypothesized that TLR2/1 induction of CYP27B1 and the conversion of $25(OH)_2D_3$ to $1,25(OH)_2D_3$ by 1α-vitamin D hydroxylase could represent a key step of the TLR pathway. Because TLR affects the interaction of innate and adaptive immune response against *M. tuberculosis*, a TLR2 agonist has been used as "natural adjuvant" to enhance Th1-induced response, inducing pulmonary IFN-γ secreting-$CD4^+$ T cell (32), or in prime-boost vaccine, inducing antigen specific IFN-γ and IL-2 responses in mice (33). The structural integrity of TLR signaling components is essential for immunological protection from infection. Changes in structure of TLR signaling molecules that result from SNPs are often associated with susceptibility to various infectious diseases. The fact that TLR2-deficient mice are highly susceptible to *M. tuberculosis* suggests that polymorphisms that affect TLR2 expression or structure may impair the susceptibility to tuberculosis in the human host. Approximately 89 SNPs have been reported in the TLR2 gene; 26 of them are in the 5'-untranslated region, 17 are in the 3'-untranslated region, 29 are located in intronic parts of the gene, and 17 modify bases of the third exon of TLR2. Six non-synonymous SNPs of the *TLR2* gene change amino acids in the cytosolic part of this receptor. However, only two have been linked to reducing NF-κB activation and increasing risk of infection. The first consists in a change of C to T replacing arginine (Arg; R) with tryptophan (Trp, W) at position 677, abolishing the binding with MyD88 with TLR2. This specific polymorphism located within the *bb* loop of *TLR2* (Arg677Trp) abolishes activation of NF-κB in response to *M. tuberculosis* (34), resulting in decreased IL-12 serum level production by 677W carriers. The second functional *TLR2* variant consists of a G to A substitution, which substitutes an arginine for glutamine at position 753. The *TLR2* 753Q seems to be associated with an increased risk of developing tuberculosis for carriers the AA and AG genotypes (35). Recently, Thuong et al. (36) described a

strong association of SNP T597C *TLR2* with susceptibility to military tuberculosis patients from Vietnam. Further association was described among Koreans regarding the microsatellite polymorphisms in intron II or *TLR2* (37). In addition, *TLR1* polymorphism in a non-synonymous region (I602S) could be associated with TLR1/2 heterodimer binding sites to mycobacterial lipopeptide, since individuals with 602II genotype produced substantially more IL-6 than those with the 602SS variant (38). However, these preliminary studies need to be repeated on a large scale in distinct populations. Currently, the polymorphism in *TLR2* might be an important risk factor for disease progression, and that the functional consequences of different alleles may explain some of the variation in cytokines and NO

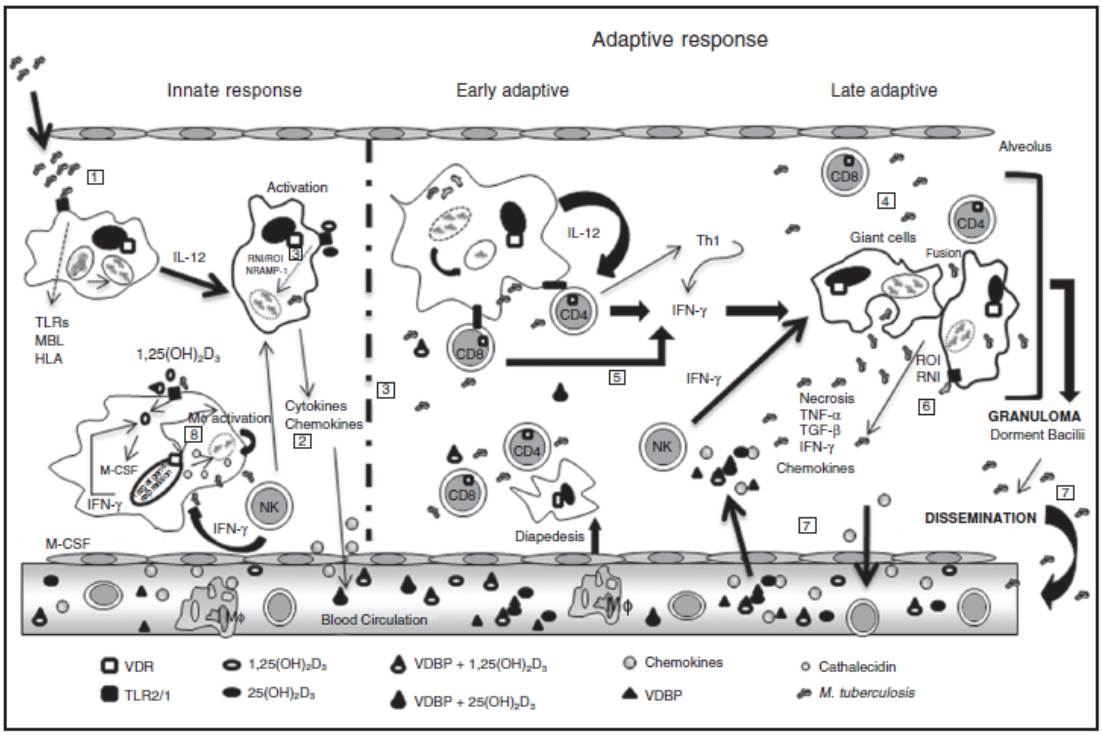

Figure 1. Scheme of the innate and adaptive immune response phases against mycobacteria. The figure identifies all polymorphic proteins involved during and after entrance of mycobacteria into the cell. The first step achieved by the mycobacteria is entering the macrophages (Mϕ) (1). After this step, the Mϕ are activated by cytokines (IFN-γ) to kill the mycobacteria (2). This phase is marked by important polymorphisms like nitric oxide production inside the phagocyte (3). If the Mϕ survives, the adaptive immune system is activated. This system establishes the granuloma, an important structure formed to contain the dissemination of the mycobacteria. The diagram illustrates the Mϕ, giant cells (GC-epithelial cells fusion), CD4, CD8 T and natural killer cells (NK) harboring intracellular mycobacteria from the centre of granuloma (4 and 5). The IFN-γ activates monocytes from circulation to Mϕ, which starts to kill the intracellular mycobacteria via reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (6). The Mϕ associated with the other antigen presenting cells (GC) present antigens to T cells and activate them to produce a variety of cytokines and chemokines. The chemokines recruit additional cells from circulating to kill the mycobacteria. Granulomas are a result of CD-mediated delayed type hypersensitivity reaction within parenchymal tissues (7). Accordingly, the same system that is responsible for bacterial growth decrease (host defense) is also intrinsically associated with tissue damage through granuloma formation and necrosis. If you have any disturbance in this system, you could be more susceptible to tuberculosis development. $1,25(OH)_2D_3$ and the vitamin D receptors (VDR) then together induce the expression of the gene encoding the human antimicrobial peptide LL-37. Circulating monocytes are activated by TLR2/1 agonists present on specific microbes. The genes encoding VDR and CYP27B1 are induced. CYP27B1 converts $25(OH)_2D_3$ from the circulation to $1,25(OH)_2D_3$, joins with VDR and activates the gene encoding LL-37, leading to an increase in cellular LL-37 and enhanced microbicidal activity of the phagocyte (8). IL = interleukin; IFN-γ = interferon-γ; TNF-α = tumor necrosis factor α; TLR = Toll-like receptor; MBL = mannose binding lectin; HLA = human leukocyte antigen; Th1 = T helper 1; TGF-β = tumor growth factor β; VDBP = vitamin D-binding protein; NRAMP-1 = natural resistance-associate macrophage protein 1. M-CSF = macrophage colony-stimulating factor.

expression and clinical forms of the disease. Understanding gained from knowledge of the effects of different alleles can contribute to the design of new therapeutic strategies including vaccines.

Nitric oxide synthase 2

NO is a free radical and second messenger that has been shown to be important in the development of several diseases, including tuberculosis. NO plays a major role in the pulmonary host-defense mechanism in response to infections and is implicated in bacteriostatic as well as bactericidal processes. NO is formed when the guanine group of the essential amino acid L-arginine is split forming NO and L-citrulline. The reaction is catalyzed by a class of enzymes called NO synthases (NOS). NO is produced by three different NOS: NOS1, NOS2 and NOS3. NOS1 is constitutively expressed due to fluctuations of Ca^{2+} and produces relatively small amounts of NO. The alveolar macrophages induced by mycobacteria are capable of producing TNF-α, IL-1β. These cytokines along with IFN-γ produced by T-cells can induce NO via action of NOS2 (Figure 1). Thus, the NO and radical nitrogen intermediate can kill and/or inhibit intracellular pathogens like mycobacteria. In contrast to murine models of tuberculosis, there is greater controversy about the role of NO killing or limiting the growth of *M. tuberculosis* in humans. It has been proposed that NO produced by tuberculosis-infected human macrophages and by epithelial cells is also antimycobacterial against *M. tuberculosis* (10). Nevertheless, a report from our group indicates that the alveolar macrophages from the lungs of patients with tuberculosis express NOS2 in potentially mycobactericidal amounts and that this NOS2 can kill mycobacteria *in vitro* (39). NOS2 expression is not usually detected in human macrophages from cell lines or macrophages derived *in vitro* from blood monocytes of health humans. However, alveolar and tissue macrophages from the lungs of tuberculosis patients show high levels of functional NOS2. Thus, NOS2 may represent a pivotal mechanism that protects against tuberculosis. Investigation is hampered by difficulty in estimating *in vivo* production of NO, but genetic studies provide a potential means of examining the relationship between NOS2 expression and disease outcome. NOS2 is encoded by a polymorphic gene known as *NOS2A* at chromosome 17q11.2-q12. Several SNPs and microsatellite polymorphisms have been described. *NOS2* gene is located in a region that has been linked to susceptibility to tuberculosis. It is known how important the biological and genetic action of NOS2 is in the immune system, because of that; the SNPs in this gene have been reported in many populations around the world. The SNPs in the promoter region of the encoding gene (-954G→C, -1173C→T, -1659 A→T) have been shown to increase NO synthesis (40). This region in the human gene is situated from -0.7 to -2.6 kb upstream of the transcription start and contains important DNA motifs for binding of NF-κB, activator protein 1, signal transducer and activator of transcription protein 1, and NF-κB repressing factor (41). Another important region lies between -2.5 and -950 kb upstream, and is characterized by a number of polymorphic elements like SNPs and repeat regions. It is this region in which various mutations are associated with *NOS2* expression *in vivo* (40). The -954G→C variant was originally reported in a highly endemic African malaria area (42), suggesting that this mutation originated as a consequence of selective pressure of *Plasmodium*. The G allele has been shown to be absent from Caucasian populations (42) as well as from the Peruvian population (43). In Mexicans, in whom the frequency of G allele was ≈5%, this SNP was not associated with tuberculosis (44). There are no further reports regarding NOS2 polymorphism frequency in Brazilian populations. Further work is in progress to determine if *NOS2A* gene polymorphism is associated with tuberculosis in this population.

Interferon-γ

IFN-γ is essential to a relationship between innate and adaptive immunity in combating *M. tuberculosis* infection (Figure 1). Active tuberculosis is characterized by lower peripheral blood mononuclear cell production of IFN-γ than latent infection, and local and systemic IFN-γ levels correlate with the severity of disease. Perhaps the most relevant mechanism is the activation of mononuclear phagocytes by the cytokines, particularly by IFN-γ. This cytokine is produced as a consequence of antigenic activation by natural killer (NK) and T cells, especially T helper 1 (Th1), and its production is increased by antigen-presenting cell-derived cytokines such as IL-12 and -18. IFN-γ has regulatory effects in both innate and adaptive immune responses, which include activation of mononuclear phagocytes and NK cells, increased expression of major histocompatibility complex I and II, induction of CD8+ lymphocyte maturation into cytotoxic cells, and promotion of the immunoglobulin isotype switch in B cells towards immunoglobulin G1 and G3 in humans (45).

Production of IFN-γ is genetically controlled, and there are two well-known polymorphisms in the *IFN-γ* gene. It has been reported that the 12 CA repeat microsatellite allele in the noncoding region of the first intron is associated with a high level of *in vitro* cytokine production (46). The same group has also reported complete linkage disequilibrium between the 12 CA repeat allele and the pres-

ence of the T allele at position +874 T/A *IFN-γ* from the translation start site. This polymorphism lies within a binding site for the transcription factor NF-κB, and electrophoretic mobility shift assays showed specific binding of NF-κB to the allele sequence containing the +874 *IFN-γ* T allele (47). As this transcription factor induces IFN-γ expression, +874T *IFN-γ* T and A alleles probably correlate with high and low IFN-γ expression, respectively (48). In addition, Ottenhoff et al. (49) discovered several families with Mendelian susceptibility to mycobacterial disease that has mutations in one of two subunits of the *IFN-γ* receptor gene (*IFN-γ R1* and *IFN-γ R2*). More recent data suggested that a more common polymorphism at position +874 is associated with risk of tuberculosis in different populations (48,50). IFN-γ acts as a regulator of gene expression through activation by a receptor complex comprising two subunits, each encoded by a different gene: *IFN-γ R1* on chromosome 6, region q23-24 and *IFN-γ R2* on chromosome 21 region q22.1-22.2. Homodimers of IFN-γ interact with both receptor proteins, leading to receptor dimerization. None of the three molecules (two receptor proteins and the cytokine itself) play a redundant role in ligand-activated receptor signaling.

The AA genotype in +874T/A region is associated with increased tuberculosis susceptibility in Spanish and South African populations (48,50), but not in Croatian and North American populations (51,52). Few studies have been conducted in the Brazilian admixed population regarding *IFN-γ* polymorphisms at position +874 and its correlation, if any with infectious diseases. Amim et al. (53) showed an important association in +874 AA genotype with the occurrence of active tuberculosis disease, independent of tuberculosis skin test status. However, the selection of these tuberculosis cases (from the Southwest region of Brazil) and healthy controls (from the Central-west region of Brazil) may cause a misunderstanding, because the Brazilian population is considered admixed. The genetic background between these two geographic areas should be different and may interfere in the genetic background analysis and results.

Perhaps lower IFN-γ secretion (inferred from the lack of T at +874 position) might influence replication of *M. tuberculosis*, thus influencing the outcome of the tuberculosis diagnosis (51) and disease. The association of *IFN-γ* genotype +874 AA with disease in several populations confirms a significant role of genetic variation at the *IFN-γ* locus and provides more detailed understanding of the genetic mechanisms underlying the association with disease. The absence of association with *IFN-γ R2* polymorphism in the African population was interesting, but firm conclusions cannot be drawn without additional studies. Furthermore, the association of disease with *IFN-γ R1* polymorphisms in the same population was novel, and together, these findings support the hypothesis that a genetically determined variation in both IFN-γ production and responsiveness influences the disease. Thus, despite the remarkable importance of IFN-γ production in *M. tuberculosis* growth control, published studies can only suggest that polymorphism in this gene might influence susceptibility to tuberculosis in some populations.

On the other hand, an important IFN-γ inducer may interfere in the multigenic tuberculosis resistance or susceptibility. IL-12, a heterodimeric pro-inflammatory cytokine produced by monocytes, macrophages, DCs and B lymphocytes, modulates Th1 differentiation, having a clear interface in IFN-γ production. The importance of IL-12 as an IFN-γ inducer lies not only in its high efficacy at low concentration but also in its synergism with many others activating stimuli. IL-12-induced IFN-γ production requires the presence of low concentrations of TNF-α and IL-1, which are usually produced in an autocrine manner by T and NK cells. The gene for the IL-12 p40 subunit is located on chromosome 5q 31.1-33.1. The SNP in the gene responsible to express this subunit was first described by Hall et al. (54). They showed a frequency of 60, 36.9, and 3.1% for genotypes AA, AB and BB, respectively, in a Caucasian population in the United Kingdom. Recently, several reports have described the *IL-12B* polymorphism in black and white North American populations with no tuberculosis susceptibility correlation. However, four SNPs described at positions 641 A-G, 684 C-T, 1094 T-C, and 1132 G-C can cause three missense variants (Q214R, M365T and G378R) and one synonymous substitution in the extracellular domain of the *IL-12Rβ1* gene (55). These investigators reported that the association of R214-T365-R378 allele (allele 2) is over-expressed in Japanese tuberculosis patients with the homozygosis for R214 - T365 - R378 (the 2/2 allele) being significantly associated with tuberculosis. In this manner, *IFN-γ* and *IL-12* gene polymorphisms and further protein expression may play an important role during the innate and adaptive immune response to *M. tuberculosis* infection and disease, driving a protective long life latent infection or disease installation. Further investigations are necessary to identify the haplotype distribution in *IFN-γ* and *IL-12* gene polymorphism.

Cross talk between *TLR2*, *NOS2*, *IFN-γ*, *VDR* and *DBP* gene expression and immunity to *M. tuberculosis*

It is known that genetic defects can lead to increased risk of mycobacterial infections (56). Thus, the identification of the genes where mutations lead to extreme suscep-

tibility will help us to identify essential components of the innate and/or adaptive human immune defense to *M. tuberculosis*. Several SNPs or defined haplotypes have been studied to identify possible candidate(s) allele(s) that may cause subtle changes in genetic function that could account for individual variation in susceptibility to tuberculosis.

For this reason, many studies described in this review have looked for an association of tuberculosis susceptibility with polymorphisms in genes encoding specific elements of the immune system thought to be important in controlling mycobacterial infection.

One of the first lines of defense of the immune response is the recognition and uptake of microorganisms by phagocytes and several different pattern recognition receptors in these cells including the TLR. Pathogen binding to specific TLRs or combinations of TLRs may recruit different adaptor proteins allowing a specific signaling cascade and gene activation programs. A central function of TLRs is to directly activate acute anti-microbial defense systems (57). These receptors, after recognition, can stimulate signalizing pathways that activate the innate immune response, cytokine production and the process of adaptive immunity. Activation of TLRs from specific mycobacterial lipoproteins can stimulate macrophages to express inflammatory cytokines (TNF-α, IL-6, IL-12 and IFN-γ) and induction of anti-microbial genes, such as the expression of *NOS2* and final production of NO (57). The activation of TLRs can also up-regulate the expression of vitamin D receptor and vitamin D-1-hydroxylase genes (enzyme that metabolizes $25(OH)_2D_3$ to $1,25(OH)_2D_3$), leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular *M. tuberculosis* (8). In addition, $1,25(OH)_2D_3$, by itself, can modulate the immune response by its nuclear receptor (VDR), where it up-regulates protective immune host defense by induction of NOS (58) and cathelicidin (9) as well as down-regulation of *IFN-γ* gene expression by down-regulating its promoter (59). It is known that $1,25(OH)_2D_3$ is present 1000-fold less in peripheral blood than $25(OH)_2D_3$, and the release of these forms in the granuloma space by VDBP may modulate T cell responses. The induction of $1,25(OH)_2D_3$ can inhibit T cell proliferation and suppression of B cell immunoglobulin production initially by inhibition of IL-2, GM-CSF and IFN-γ (7). However, $1,25(OH)_2D_3$ suppresses Th1 cytokine profile favoring Th2 cells and can activate regulatory T cells (7), modulating the host response to *M. tuberculosis* infection. On the other hand, the TLR signaling in macrophages and DC leads to secretion of IL-12 that skews the resultant T-cell response towards Th1 phenotype. Th1 cells secrete IFN-γ, which has multiple functions in activating macrophages and enhances their phagocytic and microbicidal abilities (60). IFN-γ is, also, critical for further expression and production of NOS2 in adaptive response (39) (Figure 1). In addition, IFN-γ secreted by T cells inside the granuloma potentiates the up-regulation of 1α-hydroxylase and inhibits the key enzyme in $1,25(OH)_2D_3$ inactivation (24-hydroxylase) (58).

Therefore, it is possible to suggest a link between TLR, vitamin D (receptor and transporter) and specific cytokines expressed in the immune response, such as IL-12 and IFN-γ. Consequentially, possible defects in these molecules caused by specific polymorphisms might be associated with susceptibility to tuberculosis (34,35,37).

Conclusions

Several gene polymorphisms are being described in association with human tuberculosis susceptibility; however, few of them are clearly described in a genetically distinct population. Tuberculosis incidence, socio-economic status, nutritional condition, exposition grade, cultural aspects, bacilli virulence, *M. tuberculosis* strain distribution, access to healthcare and medication should also be considered. There is always a need for better understanding of the immunopathology of tuberculosis through the knowledge of the precise genetic mechanisms controlling human infectious diseases. Furthermore, SNP description alone will not be sufficient to describe susceptibility to tuberculosis in a broad diverse population, and thus, functional gene studies need to be done. A real challenge is to associate candidate genes with a biologically plausible mechanism that explains the epidemiological data for tuberculosis in which only 10% of the infected individuals will develop tuberculosis. We believe that from the genes involved in *M. tuberculosis* control, *TLR2*, *NOS2*, *IFN-γ* and vitamin D, their carrier and their receptor may generate a potent resistant haplotype and could be highlighted as powerful tools for understanding the interplay between innate and adaptive host resistance. The relationship between *M. tuberculosis* virulence strains and host genetic variability will further help us to design new vaccines and immunotherapy.

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4.1.2 Conclusões referentes ao artigo publicado no *Brazilian Journal of Medical and Biological Research* (2009)

1- Apenas estudos genéticos não são o suficiente para elucidar os mecanismos de desenvolvimento de TB ativa.
2- Os diferentes resultados descritos na literatura, nos estudos genéticos associados ao adoecimento ou resistência à TB, podem estar relacionados as diferenças nas frequências genotípicas em cada população estudada.
3- No estudo da TB o maior desafio é entender a genética funcional do indivíduo, elucidando como as variantes dos genes podem influenciar na resposta imune contra o *Mtb*.
4- Para entender as diferentes combinações destes polimorfismos com eventos que não estão relacionados ao perfil genético do hospedeiro, novos estudos são necessários.

4.2 ASSOCIATION BETWEEN *GC* GENOTYPE AND SUSCEPTIBILITY TO TB IS DEPENDENT ON VITAMIN D STATUS

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Entre os anos de 2004 e 2007, foram selecionados pacientes com tuberculose pulmonar e controles saudáveis. Como objeto deste estudo, foram analisados os polimorfismos de *DBP* ou *GC-globulin* (gene que expressa a proteína carreadora de vitamina D) em três populações distintas (asiática, africana e brasileira), através da técnica de PCR-RFLP. Além disso, realizou-se a dosagem de VD sérica e da citocina IFN-γ *in vitro*, apenas em população proveniente da Ásia (Gujarati). Os dados obtidos foram comparados entre pacientes e controles das diferentes populações. Foi possível demonstrar associação entre pacientes com TB em relação ao genótipo Gc2-2 de *GC* e níveis séricos de vitamina D e de IFN-γ *in vitro* em população asiática.

Os resultados obtidos respondem parcialmente ao objetivo nº1 desta tese de doutorado. A dosagem de VD na população proveniente do Rio de Janeiro será descrita no artigo a seguir (item 4.3.1, página 62), respondendo totalmente a este objetivo.

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Association between Gc genotype and susceptibility to TB is dependent on vitamin D status

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Abstract

Gc variants of vitamin D binding protein differ in their affinity for vitamin D metabolites that modulate antimycobacterial immunity. We conducted studies to determine whether Gc genotype associates with susceptibility to tuberculosis.

123 adult tuberculosis patients and 140 controls of Gujarati Asian ethnic origin in the United Kingdom, 130 adult tuberculosis patients and 78 controls in Brazil, and 281 children with tuberculosis and 182 controls in South Africa were recruited to case-control studies. Gc genotypes were determined and their frequency was compared between cases vs. controls. Serum 25-hydroxyvitamin

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D (25[OH]D) concentrations were obtained retrospectively for 139 Gujarati Asians, and case-control analysis was stratified by vitamin D status. Interferon-γ release assays were also performed on 36 Gujarati tuberculosis contacts.

The Gc2/2 genotype was strongly associated with susceptibility to active tuberculosis in Gujarati Asians, compared with Gc1/1 genotype (OR 2.81, 95% CI 1.19 to 6.66, P=0.009). This association was preserved if serum 25(OH)D was <20 nmol/l (P=0.01), but not if serum 25(OH)D was ≥20 nmol/l (P=0.36). Carriage of the Gc2 allele associated with increased PPD-stimulated Interferon-γ release in Gujarati Asian tuberculosis contacts (P = 0.02). No association between Gc genotype and susceptibility to tuberculosis was observed in other ethnic groups studied.

Introduction

Tuberculosis (TB) is a leading global cause of death. Vitamin D deficiency associates with susceptibility to active TB in numerous settings [1] and vitamin D supplementation enhances antimycobacterial immunity [2]. Vitamin D is synthesized in the skin during exposure to ultra-violet light and is metabolized by the liver to form 25-hydroxyvitamin D (25(OH)D), the major circulating vitamin D metabolite and accepted measure of vitamin D status. 25(OH)D then undergoes a further hydroxylation step to form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the immunomodulatory metabolite which enhances antimycobacterial activity by pleiotropic mechanisms including the induction of antimicrobial peptides with antituberculous activity [3,4] and the suppression of matrix metalloproteinase enzymes implicated in degradation of pulmonary extracellular matrix [5].

Vitamin D metabolites in the circulation are bound to vitamin D binding protein (DBP), a highly expressed multifunctional 58 kDa serum glycoprotein encoded on chromosome 4. The DBP locus is among the most polymorphic known [6]. Two common polymorphisms at codons 416 (GAT→GAG, Asp→Glu) and 420 (ACG→AAG, Thr→Lys) of exon 11 of the DBP gene (defined by the presence of restriction endonuclease sites for HaeIII and StyI, respectively) give rise to the three major electrophoretic variants of DBP, termed group-specific component 1 fast (Gc1F), Gc1 slow (Gc1S) and Gc2. These variants differ in their functional characteristics: the Gc1F and Gc1S variants have been reported to have greater affinity for 25(OH)D than the Gc2 variant [7], potentially leading to more efficient delivery of 25(OH)D to the target tissues, while the Gc2 variant is associated with decreased circulating concentrations of 25(OH)D, $1,25(OH)_2D$ and DBP [8,9]. We therefore reasoned that possession of the Gc2 variant of DBP might associate with susceptibility to TB, and conducted case control studies in three different settings to test this hypothesis. We also conducted functional studies to determine whether antigen-stimulated release of interferon-gamma (IFN-γ) from whole blood of healthy TB contacts varied according to Gc genotype.

Methods

Populations studied

Case-control study participants were recruited at 3 sites (Table 1). One hundred and twenty-three adult TB patients and 140 healthy adult TB contacts, all of Gujarati ethnic origin, were recruited at Northwick Park Hospital, London, UK from 1993 to 2004; 281 children with TB (210 Xhosa, 71 of Cape Coloured ethnic origin) and 182 healthy child TB contacts (163 Xhosa, 19 Cape Coloured ethnic origin) were recruited at Red Cross Children's Hospital, Cape Town, South Africa, from 2000 to 2003; and 130 adult TB patients (55 white, 44 mixed, 31 black) and 78 healthy adult controls (49 white, 18 mixed and 11 black) were recruited in Instituto de Pesquisa Clínica Evandro Chagas (IPEC) at Fiocruz and in Municipal Health Centres, Rio de Janeiro, Brazil, from 2004 to 2007. Diagnosis of TB was established on the basis of smear

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positive for acid-fast bacilli and/or culture positive for *M. tuberculosis* in all adult cases and in 33% of paediatric cases; for remaining paediatric cases, diagnosis was based on WHO criteria for diagnosis of TB in children [10] with 42% classified as having probable TB, and 25% classified as having possible TB. Patients with known HIV infection or taking immunosuppressant drugs were excluded. Adult TB contacts were recruited at participating TB clinics and health centres in London and Rio de Janeiro on the basis of a history of household contact with a TB patient, absence of symptoms of active TB and genetic unrelatedness to any patient recruited as a TB case in the study. Child TB contacts were recruited by locating children living three houses adjacent to a household in which a childhood TB case was identified. Where possible, it was ensured that controls were unrelated to TB cases. All adult participants gave informed consent for inclusion, as did parents of child participants. Permission to conduct the case-control study was obtained from research ethics committees in the UK (Harrow REC ref. 1646), South Africa (University of Cape Town REC ref. 013/2000) and Brazil (IPEC REC ref. 0008.0.009.000-04 and Rio de Janeiro Municipal Health Centre REC ref. S/CRH/DRH/DIC3).

Functional studies were also conducted in a group of 36 healthy TB contacts recruited to a clinical trial of vitamin D supplementation at Northwick Park Hospital (n=26) and Newham Chest Clinic (n=8), London, UK from 2002 to 2005. Characteristics of the whole study population have been reported elsewhere [11]. The sub-group of 36 participants whose results are presented here was selected on the basis of self-assigned Gujarati ethnicity and availability of a result of a whole blood IFN-γ release assay (IGRA) performed prior to randomisation. The study was approved by the research ethics committees of North East London and Harrow (REC refs. P/02/146 and EC 2759, respectively) with written, informed consent to take part in the study obtained from all participants.

Genotyping

Genomic DNA was extracted from peripheral blood samples using the DNA Midi kit (Qiagen, Lewes). A 483-bp fragment of exon 11 of the Gc gene was amplified by polymerase chain reaction (PCR). Reaction mix included 2 μl DNA at 50 μl/ml, 11.1 μl H_2O, 2 μl 10× PCR buffer, 0.4 μl 25 mM $MgCl_2$, 0.4 μl 10 mM deoxynucleotide triphosphates (dNTP), 0.1 μl HotStar Taq polymerase (Qiagen) and 2 μl of 20 μM primers. Primer sequences were 5′-AAATAATGAGCAAATGAAAGAAGAC-3′ (forward) and 5′-CAATAACAGCAAAGAAATGAGTAGA-3′ (reverse). Cycling conditions were 95°C for 15 minutes, followed by 35 cycles at 94°C for 45 seconds, 51°C for 45 seconds, 72°C for 45 seconds, then a final 7 minutes at 72°C. PCR products were digested separately with restriction enzymes HaeIII (for 4h at 37°C) and Sty I (overnight at 37°C) (New England Biolabs, Mississauga, Ontario). Digestion with HaeIII produces 297- and 186-bp fragments in the presence of the h allele and digestion with StyI produces 305- and 178-bp fragments in the presence of the s allele. Digested products were visualised on 2% agarose gels stained with ethidium bromide. The presence of restriction sites was assigned by the lower case (h for HaeIII, s for StyI), and absence was assigned by the upper case (H for HaeIII and S for StyI). Gc genotype was assigned as per Table 2.

Determination of 25(OH)D concentrations

Serum 25(OH)D concentrations of Gujarati participants recruited in London were determined by radio-immunoassay (DiaSorin, Stillwater, MN) in a clinical biochemistry laboratory that participates in the international Vitamin D external quality assessment program (http://www.deqas.org/). For participants with active TB, serum 25(OH)D concentrations were determined at diagnosis. Vitamin D deficiency was defined as serum 25(OH)D < 20 nmol/l. [12]

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Interferon-gamma Release Assay (IGRA)

The IGRA used in this study has been described elsewhere [13]. Triplicate samples of venous blood diluted 1:10 with RPMI-1640 (Life Technologies, Paisley, UK) were cultured with 1000 U/ml PPD (Statens Serum Institute, Denmark), 2.5 μg/ml recombinant ESAT-6 or 5 μg/ml CFP-10 (Lionex, Braunschweig, Germany) at 37°C in 5% CO_2. Supernatants were aspirated at 96 hours for determination of IFN-γ concentration by ELISA.

Statistical analysis

Due to similar functional characteristics [7] Gc1F and Gc1S allele carriers were combined to produce a total of 3 genotypes: Gc1/1, Gc2/1 and Gc2/2. Contingency tables were analyzed using chi-square tests, unless more than 20% of cells in a table had an expected frequency of <5, when Fisher's exact tests were employed. Linkage disequilibrium was evaluated by calculating estimating predicted haplotypes frequencies based on random assortment of HaeIII and StyI alleles, and comparing these with observed frequencies by a chi square test; D′ was calculated using Lewontin's equation [14]. Mean ages were compared between groups using unpaired t-tests and one-way ANOVA, and median serum 25(OH)D concentrations were compared between different groups using Kruskal-Wallis tests. Chi square tests were performed to test for association between genotype and susceptibility to TB, and binary logistic regression analysis was conducted to adjust odds ratios (OR) for age and sex. Data were analyzed using SPSS (version 12.0.1, 2003) and GraphPad Prism (version 4.03, 2005) software packages.

Results

Participant characteristics: case-control study

The characteristics of case-control study participants are presented in Table 3. Gujarati Asian cases and controls did not differ with respect to age or sex distribution. Males were over-represented in cases vs. controls among participants recruited in Cape Town (Xhosa: 55.2% vs. 41.1% male, P = 0.007; Cape Coloured: 47.9% vs. 21.1% male, P = 0.04) and Rio (black: 61.3% vs. 27.3% male, P = 0.01; mixed: 63.6% vs. 38.9% male, P = 0.02). The mean age of cases was lower than that of controls among Xhosa participants in Cape Town (5.2 years vs. 6.1 years, P=0.002) and higher than that of controls among white participants in Rio (38.7 years vs. 29.6 years, P < 0.001).

DBP allele frequency varies between ethnic groups

DBP allele frequency varied significantly between different ethnic groups (Table 4, P<0.0001). Frequency of the Gc2 allele was highest among Gujarati participants in London and white participants in Rio (35.9% and 33.2% respectively) and lowest among black participants in Rio and Xhosa participants in Cape Town (11.9% and 4.6% respectively). A similar ethnic distribution was observed for the 1S allele (47.9% in London Gujaratis, 41.8% in Rio whites, 19% in Rio blacks and 7.9% in Cape Town Xhosa). The opposite ethnic distribution was observed for the 1F allele, which was most common among Xhosa participants in Cape Town and black participants in Rio (87.5% and 69.0% respectively), and least common among white participants in Rio and Gujarati participants in London (25.0% and 16.2% respectively). Intermediate frequencies of all three alleles were found among ethnically admixed participants recruited in Rio and Cape Town. Both loci were in Hardy-Weinberg equilibrium in all populations studied. The loci were in linkage disequilibrium in Gujaratis (D′=0.52, P < 0.0001), in white participants in Rio (D′=0.36, P < 0.0001) and in ethnically admixed populations in Rio (D′=0.15, P < 0.0001) and Cape Town (D′=0.06, P=0.0002), but not in Xhosa participants in Cape Town (P=0.08) or black participants in Rio (P=0.10).

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Carriage of the Gc2 allele associates with susceptibility to active TB in Gujarati Asians

Given that the allele frequency varied by ethnic group, case-control analysis of DBP variant frequency was stratified by ethnic group. Among Gujarati Asian participants, the Gc genotype was associated with active TB (Table 5; P=0.04). The unadjusted OR for Gc2/2 compared with Gc1/1 was 2.81 (95% CI 1.19 to 6.66, P=0.009), while the unadjusted OR for Gc2/1 compared with Gc1/1 was 1.69 (95% CI 0.96 to 2.96, P=0.052). The unadjusted OR for genotypes Gc2/1 and Gc2/2 combined vs. Gc1/1 was 1.89 (95% CI 1.11 to 3.22, P=0.012). These associations were unaltered by adjustment for age and sex (adjusted OR for Gc2/2 vs. Gc1/1 was 2.83, 95% CI 1.27 to 6.31, P=0.01; adjusted OR for Gc2/1 vs. Gc1/1 was 1.73, 95% CI 1.01 to 2.96, P = 0.045; adjusted OR for Gc2/1 and Gc2/2 combined vs. Gc1/1 was 1.94, 95% CI 1.17 to 3.23, P=0.01). No statistically significant variation in frequency of DBP genotype was observed between cases and controls among any of the populations studied in Cape Town or Rio, either before or after adjustment for age and sex.

Vitamin D status does not vary with DBP variant in Gujarati Asians

We have previously shown that vitamin D deficiency associates with susceptibility to TB in Gujarati Asians [15]. Since then, others have reported that serum 25(OH)D concentrations vary with Gc genotype, being lowest in carriers of the Gc-2 allele [8,9]. We were interested to determine whether serum 25(OH)D concentrations varied according to Gc genotype in Gujarati Asians. Since serum samples were not collected prospectively for this purpose, we conducted a retrospective search for results of routinely performed assays of serum 25(OH)D concentration for all Gujarati Asian participants. Results were available for 84/123 cases and 55/140 controls. Vitamin D deficiency (serum 25(OH)D < 20 nmol/l) was more common among TB cases vs. controls (affecting 60/84 vs. 31/55) but this difference did not attain statistical significance (P=0.07). No significant differences in median serum 25(OH)D concentration or prevalence of vitamin D deficiency were observed between individuals with different genotype (median serum 25(OH)D, 17.0 vs. 12.0 vs. 10.0 nmol/l for Gc1/1, Gc1/2 and Gc2/2 genotypes respectively, P=0.28; prevalence of vitamin D deficiency, 35/58 vs. 43/63 vs. 13/18 deficient for Gc1/1, Gc1/2 and Gc2/2 genotypes respectively, P=0.53).

The association between Gc genotype and susceptibility to TB in Gujarati Asians varies according to vitamin D status

We next investigated whether association between Gc genotype and susceptibility to TB in Gujarati Asians varied by vitamin D status by performing separate contingency analyses for vitamin D deficient participants and non-deficient participants (Table 6). Among vitamin D deficient Gujarati Asians we found that Gc genotype was strongly associated with susceptibility to TB, with genotype 2/2 present in 13/60 vitamin D deficient cases vs. 0/31 vitamin D deficient controls (P=0.01). In contrast, no association between Gc genotype and susceptibility to TB was seen among Gujarati Asians with serum 25(OH)D ≥ 20 nmol/l (P=0.36). Statistical analysis to determine whether Gc genotype and vitamin D status interact to influence susceptibility to TB was indeterminate, as the zero value for vitamin D deficient controls with Gc2/2 genotype in the contingency table (Table 6) resulted in an undefined odds ratio.

Carriage of the Gc2 allele associates with increased IFN-γ release from PPD-stimulated whole blood of Gujarati Asian TB contacts

Finally we investigated whether IFN-γ responses to mycobacterial antigens varied according to Gc genotype in a group of 36 Gujarati Asian TB contacts whose characteristics are presented in Table 7. No significant differences in sex ratio, BCG status, tuberculin skin test reactivity, site of exposure or vitamin D status were observed between participants of different Gc genotype, although a statistically significant difference in mean age was observed between participants of different genotype (55.3 yr vs. 41.3 yr vs. 34.9 yr for genotypes Gc2/2, Gc2/1

and Gc1/1 respectively, P = 0.03). Median PPD-stimulated IFN-γ concentration was higher among TB contacts of Gc2/2 and Gc2/1 genotype vs. those of Gc1/1 genotype (790.8 pg/ml vs. 663.5 pg/ml vs. 60.5 pg/ml respectively, P=0.02; Figure); no difference in ESAT-6 or CFP-10-stimulated IFN-γ concentration was observed between TB contacts of different genotype (Table 7).

Discussion

We have demonstrated an association between the Gc genotype of vitamin D binding protein and susceptibility to TB in Gujarati Asians living in London. Stratification of this analysis by vitamin D status revealed that this association was restricted to participants with profound vitamin D deficiency.

No association between carriage of the Gc2 allele and susceptibility to TB was observed among populations studied in Rio and Cape Town. Gc2 allele frequency in these populations was lower than in Gujarati Asians, and absolute numbers of Gc2 carriers were therefore small (Table 4); our study may have been underpowered to detect an association in these populations. An alternative explanation for the lack of association seen in Rio and Cape Town is that populations in Rio (latitude 22°S) and Cape Town (latitude 33°S) are likely to have significantly more exposure to UVB than those living in London (latitude 51°N). Profound vitamin D deficiency is therefore likely to be much less common in these settings. If the association between Gc2 allele and susceptibility to TB is restricted to individuals with profound vitamin D deficiency, as we postulate, then low prevalence of vitamin D deficiency in Rio and Cape Town may explain non-replication of the association in these settings. This explanation may also account for lack of association between Gc genotype and susceptibility to TB in other populations previously studied in Kuwait[16] and India [17].

Individuals carrying the Gc2 allele have previously been reported to have lower circulating concentrations of both DBP and 25(OH)D [8,9,18], phenomena that have been attributed to the fact that the gene product of Gc2 allele is metabolized faster than that of Gc1 alleles [19]. Vitamin D deficient carriers of the Gc2 allele may therefore have particularly low circulating concentrations of 25(OH)D-DBP complex. Receptor-mediated endocytosis of 25(OH)D-DBP complex has previously been shown to be essential for induction of vitamin D-mediated biological activity in both renal and mammary cells [20,21]. If 25(OH)D-DBP complex is similarly required for initiation of vitamin D-inducible antimycobacterial responses then reduced circulating concentrations of this complex in vitamin D deficient carriers of the Gc2 allele could explain the association that we report. Further studies are required to establish whether circulating concentrations of 25(OH)D-DBP complex vary according to Gc allele, and to investigate whether receptor-mediated endocytosis of 25(OH)D-DBP complex is essential for induction of vitamin D-inducible antimycobacterial responses.

Our finding of striking ethnic variation in Gc allele frequency is in keeping with published literature reporting that populations with deeply pigmented skin have higher frequencies of the 1F allele [22]. Reports that cutaneous penetration of UVB is decreased in individuals with pigmented skin [23] and that the Gc1 variants of DBP have greater affinity for 25-hydroxyvitamin D than the Gc2 variant [24] raise the possibility that Gc1 variants may carry a survival advantage in persons with pigmented skin due to their superior delivery of 25(OH) D to the target tissues. If this is the case, then the observation of increasing frequency of the Gc2 allele in Caucasian populations implies that possession of this allele confers a survival benefit in conditions where solar radiation is limited. Consistent with this hypothesis, possession of the Gc2 allele has been associated with decreased risk of several other pathologies including breast cancer [9], chronic obstructive pulmonary disease [25] and fractures [26]. We also observed that alleles of the loci at codons 416 and 420 were in linkage disequilibrium in

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Gujaratis, whites and ethnically admixed populations, but not in Xhosa or black participants. This phenomenon has been demonstrated for other coding single nucleotide polymorphisms in multiethnic populations, and may be attributed to a 'bottleneck' experienced by Asian and European populations migrating from Africa 800 to 1,600 generations ago [27].

Our functional study revealed that TB contacts carrying the Gc2 allele had significantly higher PPD-stimulated IFN-γ responses than Gc1 homozygotes, who demonstrated low or undetectable IFN-γ responses to PPD; all TB contacts had low or undetectable IFN-γ responses to RD-1-encoded antigens. ESAT-6 and CFP-10 are secreted early in infection, and reversion of IFN-γ responses to these antigens with persistence of tuberculin reactivity has been previously reported in longitudinal studies of TB contacts, and may represent latent TB infection [28]. Our data may therefore indicate that Gc1 homozygotes are relatively resistant to acquisition of latent TB infection, and that Gc2 allele carriers are more susceptible.

Our study has some limitations. Gujarati Asian ethnicity was self-assigned, and although the community is genetically homogenous [15] the possibility of ethnic admixture cannot be excluded. Serum 25(OH)D concentrations were only available for 68% of Gujarati cases vs. 39% of Gujarati controls. This difference presumably arose because clinicians had a lower threshold for testing vitamin D status in TB cases than in TB contacts. Since the Gc2/2 genotype was less common among controls than cases, the absolute number of Gc2/2 controls with known vitamin D status was small (n=2). However, the proportion of participants for whom 25(OH)D was available did not differ by Gc genotype, and the results of this analysis cannot therefore be attributed to selection bias. Larger studies with prospective evaluation of circulating concentrations of 25(OH)D should be performed in populations at risk of vitamin D deficiency to determine whether profound vitamin D deficiency and Gc2 genotype interact to increase susceptibility to TB.

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Martineau *et al*, Figure

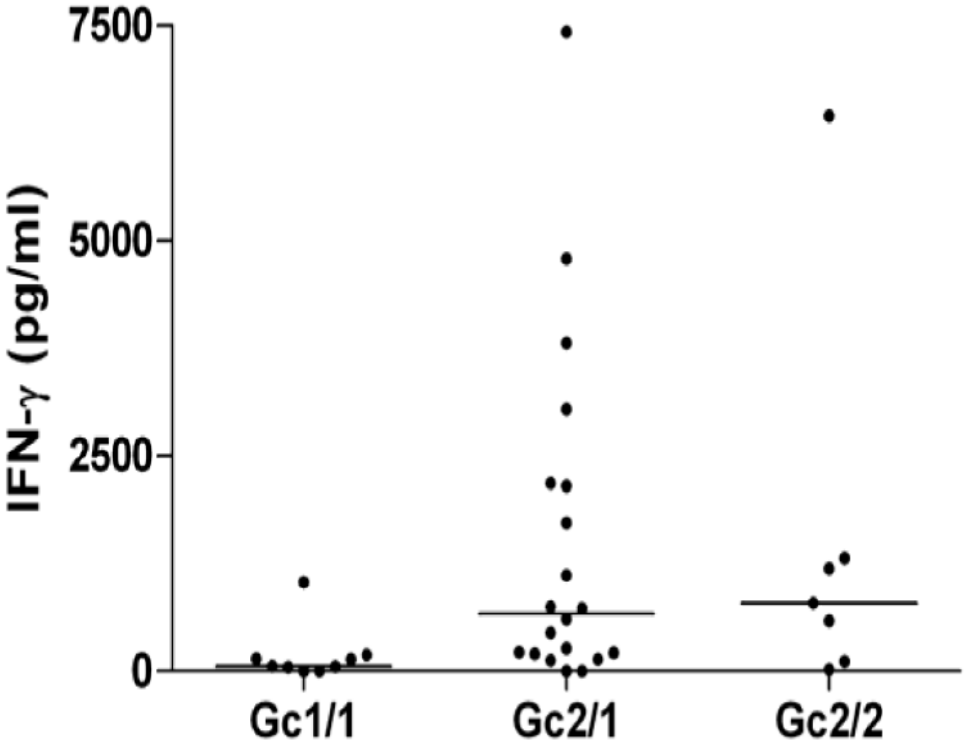

Figure.
PPD-stimulated Interferon-γ release in functional study participants by Gc genotype

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Table 1

Study populations

City, Country	Ethnic group	Cases	Controls
London, UK	Gujarati Asian	Adults (n=123)	Adults (n=140)
Cape Town, South Africa	Xhosa	Children (n=210)	Children (n=163)
	Cape Coloured	Children (n=71)	Children (n=19)
Rio de Janeiro, Brazil	White	Adults (n=55)	Adults (n=49)
	Mixed	Adults (n=44)	Adults (n=18)
	Black	Adults (n=31)	Adults (n=11)

Table 2

Deduction of DBP genotype from HaeIII and StyI genotypes

HaeIII/StyI Genotype	Potential HaeIII/StyI haplotypes	Deduced HaeIII/StyI haplotypes	Corresponding DBP genotype
HH SS	HS/HS	HS/HS	Gc1F/ Gc1F
HH Ss	HS/Hs	HS/Hs	Gc1F/Gc2
HH ss	Hs/Hs	Hs/Hs	Gc2/Gc2
Hh SS	HS/hS	HS/hS	Gc1F/Gc1S
Hh Ss	Hs/hS or HS/hs*	Hs/hS	Gc2/Gc1S
hh SS	hS/hS	hS/hS	Gc1S/Gc1S
hh Ss	hS/hs*	Gc1s/Not deduced	Not assigned

*frequency of the hs haplotype is extremely low due to linkage disequilibrium between loci; therefore subjects heterozygous at both loci were assumed to carry Hs/hS haplotypes, and subjects with potential haplotype hs were not assigned a DBP genotype.

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Table 3

Characteristics of TB cases and controls

Ethnic group, City		Cases	Controls	P
Gujarati, London	Mean age, yr, (s.d.)	43.8 (16.2)	41.9 (13.3)	0.3
	Male sex, n (%)	47 (38.2)	67 (47.9)	0.12
Xhosa, Cape Town	Mean age, yr, (s.d.)	5.2 (3.9)	6.1 (3.6)	0.002
	Male sex, n (%)	116 (55.2)	67 (41.1)	0.007
Cape Coloured, Cape Town	Mean age, yr, (s.d.)	4.4 (3.9)	5.5 (4.1)	0.16
	Male sex, n (%)	34 (47.9)	4 (21.1)	0.04
White, Rio	Mean age, yr, (s.d.)	38.7 (13.8)	29.6 (10.3)	<0.001
	Male sex, n (%)	37 (67.3)	16 (32.7)	0.2
Mixed, Rio	Mean age, yr, (s.d.)	36.6 (11.9)	34.9 (13.6)	0.63
	Male sex, n (%)	28 (63.6)	7 (38.9)	0.02
Black, Rio	Mean age, yr, (s.d.)	38.0 (13.7)	33.6 (14.8)	0.37
	Male sex, n (%)	19 (61.3)	3 (27.3)	0.01

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Table 4

Frequency of DBP alleles by ethnic group

Ethnic group, City	DBP allele, n (%)			P
	Gc2	Gc1F	Gc1S	
Gujarati, London	189 (35.9)	85 (16.2)	252 (47.9)	<0.0001
Xhosa, Cape Town	34 (4.6)	653 (87.5)	59 (7.9)	
Cape Coloured, Cape Town	24 (13.3)	108 (60.0)	48 (26.7)	
White, Rio	69 (33.2)	52 (25.0)	87 (41.8)	
Mixed, Rio*	26 (21.3)	52 (42.6)	45 (36.3)	
Black, Rio	10 (11.9)	58 (69.0)	16 (19.0)	

*Allele not assigned for one individual (haplotype hs)

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Table 5

Frequency of DBP genotypes: TB cases vs. controls

Ethnic group, City	Cases / Controls	DBP genotype, n (%)			P
		Ge2/2	Ge2/1	Ge1/1	
Gujarati, London	Cases (n=123)	22 (17.9)	60 (48.7)	41 (33.3)	0.04
	Controls (n=140)	13 (9.3)	59 (42.1)	68 (48.5)	
Xhosa, Cape Town	Cases (n=210)	0 (0)	20 (9.5)	190 (90.5)	0.86
	Controls (n=163)	0 (0)	14 (8.6)	149 (91.4)	
Cape Coloured, Cape Town	Cases (n=71)	3 (4.2)	14 (19.7)	54 (76.1)	<0.99
	Controls (n=19)	0 (0)	4 (21.1)	15 (78.9)	
White, Rio	Cases (n=55)	8 (14.5)	20 (36.4)	27 (49.1)	0.56
	Controls (n=49)	5 (10.2)	23 (46.9)	21 (42.9)	
Mixed, Rio	Cases (n=44)*	2 (4.5)	12 (27.3)	29 (65.9)	0.64
	Controls (n=18)	2 (11.1)	6 (33.3)	10 (55.6)	
Black, Rio	Cases (n=31)	1 (3.2)	5 (16.1)	25 (80.6)	0.75
	Controls (n=11)	0 (0)	3 (27.3)	8 (72.7)	

* DBP genotype not assigned for one case (Hae III genotype hh, Sty I genotype Ss)

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Table 6

Frequency of DBP genotypes, Gujarati TB cases vs. controls, stratified by vitamin D status

Serum 25(OH)D		DBP genotype, n (%)			P
		Gc2/2	Gc2/1	Gc1/1	
<20 nmol/l	Cases (n=60)	13 (21.7)	26 (43.3)	21 (35.0)	0.01
	Controls (n=31)	0 (0)	17 (54.8)	14 (45.2)	
≥20 nmol/l	Cases (n=24)	3 (12.5)	12 (50)	9 (37.5)	0.36
	Controls (n=24)	2 (8.3)	8 (33.3)	14 (58.3)	

Table 7

Characteristics of functional study participants by Gc genotype

Variable	Gc2/2 (n=7)	Gc2/1 (n=20)	Gc1/1 (n=9)	P
Mean age, years (s.d.)	55.3 (7.8)	41.3 (15.0)	34.9 (16.7)	0.03
Male sex, no. (%)	3 (42.9)	11 (55.0)	3 (33.3)	0.54
BCG scar present, no. (%)	6 (85.7)	15 (75.0)	8 (88.9)	0.63
UK born, no. (%)	3 (42.9)	6 (30.0)	3 (33.3)	0.35
Tuberculin skin test reactive, proportion (%)*	0/5 (0)	5/13 (38.5)	0/5 (0)	0.06
Household exposure, no. (%)	4 (57.1)	17 (85.0)	6 (66.6)	0.27
25(OH)D < 20 nmol/L, no. (%)	3 (42.9)	12 (60.0)	5 (55.6)	0.73
Median serum 25(OH)D, nmol/l (IQ range)	25.0 (13.2 to 43.0)	18.4 (9.5 to 27.9)	17.5 (7.1 to 37.0)	0.63
Median PPD-stimulated IFN-γ, pg/ml (IQ range)	790.8 (118.6 to 1311.0)	663.5 (208.3 to 2162.0)	60.5 (22.7 to 170.4)	0.02
Median ESAT-6-stimulated IFN-γ, pg/ml (IQ range)	16.6 (0.0 to 324.9)	53.2 (20.7 to 233.7)	52.9 (0.0 to 154.3)	0.60
Median CFP-10-stimulated IFN-γ, pg/ml (IQ range)	0.0 (0.0 to 23.6)	53.1 (0.0 to 263.1)	0.0 (0.0 to 62.8)	0.29

*Reactive tuberculin status defined as Heaf grade 3 or 4, or Heaf grade 2 in the absence of a BCG scar; negative tuberculin status defined as Heaf grade 0 or 1, or Heaf grade 2 in the presence of a BCG scar. Tuberculin skin test results were available for 5/7 Gc2/2, 13/20 Gc2/1 and 5/9 Gc1/1 participants.

4.2.2 Conclusões referentes ao artigo publicado no *European Respiratory Journal* (2010)

1- O fato do genótipo *Gc2-2* estar ligado à susceptibilidade à TB apenas nos pacientes com deficiência de VD sugere a importância de concentração de VD sérica dentro da normalidade, uma vez que em indivíduos com suficiência de VD, este genótipo não está influenciando no risco de adoecimento.

2- Foi encontrada associação entre as variantes de *GC-globulin* em população asiática. Contudo, não foi encontrada associação em população proveniente do Rio de Janeiro e de Cape Town. Este resultado poderia estar relacionado à distribuição das freqüências deste genótipo nestas populações, que diferem da população de asiáticos.

3- Foi observado um aumento significativo no nível de secreção de IFN-γ nos carreadores do genótipo *Gc2-2,* entre os contatos de pacientes de TB, apenas em população de asiáticos. Este resultado poderia estar relacionado, a uma resposta imune diferenciada ao PPD de acordo com os polimorfismos no *GC-globulin*, sugerindo que as variantes neste gene podem modular a produção desta citocina.

4.3 ASSOCIATION BETWEEN *VITAMIN D RECEPTOR* AND *GC-GLOBULIN* GENE POLYMORPHISMS WITH TUBERCULOSIS DEVELOPMENT IN BRAZILIAN POPULATION IS DEPENDENT OF VITAMIN D LEVELS.

Artigo submetido ao *Tuberculosis Journal (2012)*

Entre os anos de 2004 e 2007, foram coletados amostras de sangue periférico em pacientes com tuberculose e controles saudáveis. Para realização deste estudo, foram analisados os polimorfismos de *VDR* e *DBP* ou *GC-globulin* (gene que expressa a proteína carreadora de vitamina D), através da técnica de PCR-RFLP. A dosagem das concentrações de VD e as freqüências das variantes dos genes foram comparadas entre os grupos estudados. Foi possível demonstrar diferença entre pacientes com TB e controles em relação ao genótipo de *VDR* e *GC-globulin* e níveis séricos de vitamina D.

Os resultados obtidos respondem os objetivos de nº 1 e 2 da tese de doutorado.

4.3.1 Artigo submetido *Tuberculosis Journal (2012)*

Title page

Association between *Vitamin D-receptor* and *Gc-globulin* gene polymorphisms with pulmonary tuberculosis among Brazilian population is dependent of serum vitamin D concentration

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Concise Title. *VDR* and *GC* polymorphism and vitamin D levels in tuberculosis

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Abstract

Polymorphisms in genes encoding components of the vitamin D pathway variably associates with tuberculosis. We tested whether polymorphism in genes encoding for vitamin D receptor (*VDR)*, Gc-globulin (*GC*) and 25(OH)D serum concentration were associated with tuberculosis in Brazil. Serum 25(OH)D was determined and stratified as normal or deficiency and related to genotypic and haplotypic profiles. There was no statistical difference in 25(OH)D serum concentration and *FokI, TaqI*, and *ApaI* genotype and allele distribution between tuberculosis and HC subjects. However, *F* allele frequency was associated with protection in black subjects (p=0.03). The *bb* variant of *BsmI* genotype (p=0.01) and *b* allele (p=0.04) were associated with protection. When the analysis was stratified by ethnicity the *bb* genotype (p=0.003) and *b* allele (p=0.007) were more frequent in non-white HC, indicating a protective role for *BsmI* in these subgroups. Interestingly, *bb* genotype (p=0.009) tuberculosis protection in subjects with normal serum 25(OH)D concentration. *VDR* haplotype *ftBA* and serum of 25(OH)D concentration were associated with tuberculosis susceptibility when normal serum concentration was identified (p=0.03). Gc-globulin groups did not show association with tuberculosis susceptibility in our population. We tested whether a combined Gc-globulin group and *VDR* SNPs might influence tuberculosis susceptibility. Carries of both Gc1-1 group and *bb* genotype were more frequent in the HC group than in patients (p=0.03). No statistical difference was observed when Gc1-2 and in Gc2-2 groups and *VDR* genotypes were combined. The results provide evidence of ethnicity dependent association between *VDR* and *GC* variants, serum levels of 25(OH)D and tuberculosis in Brazil.

Keywords. Tuberculosis, vitamin D serum concentration, *Gc-globulin*, *VDR*

INTRODUCTION

One third of the world population is estimated to be latently infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (WHO 2010). Non-genetic and genetic factors in both bacterium and host impact the immune response to *M. tuberculosis* (Bellamy 2005; Schurr 2007; Schurr 2011). At the present, the evidence for a human genetic component in susceptibility to tuberculosis is incontrovertible (Yim et al. 2010).

The active form of Vitamin D, 1-α,25-dihydroxyvitamin D [1,25$(OH)_2$D] mediates its biological actions via the vitamin D receptor (VDR). The *VDR* gene is located on the long arm of chromosome 12 (12q12-14) and is composed of 10 exons, the first of which is not transcribed. Four single nucleotide polymorphisms (SNPs) are defined by restriction sites for *FokI, TaqI*, *ApaI,* and *BsmI,* have been associated with susceptibility to pulmonary tuberculosis (Bornmam et al. 2004; Roth et al. 2004; Lombard et al. 2006; Olesen et al. 2007; Larcombe et al. 2008; Merza et al. 2009; Selvaraj et al. 2009; Wilkinson et al. 2000; Ates et al. 2010; Banoei et al. 2010; Gao et al. 2010; Andraos et al. 2011; Sharma et al. 2011). However, there are inconsistent results with respect to *VDR* polymorphisms and their association with tuberculosis in some populations. In recent meta-analysis, Gao et al. (2010) showed that VDR polymorphisms and vitamin D deficiency might play a role as risk factor during the development of tuberculosis.

1,25$(OH)_2D_3$ has pleitropic actions including modulation of growth, differentiation status and function of cells of the immune system (reviewed by Baeke et al. 2010). 1,25$(OH)_2D_3$ targets different components of the innate and adaptive immune compartment. In humans, 1,25$(OH)_2D_3$ stimulates the chemotactic and phagocytic responses of macrophages (Helming et al. 2005) and mediates the antimicrobial response triggered by Toll-like receptors (TLRs), leading to the induction of the antimicrobial peptide cathelicidin and restriction of growth of intracellular *M. tuberculosis* (Liu et al. 2006). Moreover, Martineau et al. (2007a) showed that a moderate concentration of cathelicidin induces superior suppression of *M. tuberculosis* growth in cultured peripheral blood mononuclear cells. The addition or induction of cathelicidin and vitamin D increases killing efficiency of macrophages through direct effect or by alternatively facilitating ability of mycobacterial phagosomes to fuse with

lysosomes (Sonawane et al. 2011). Conversely $1,25(OH)_2D_3$ inhibits the surface expression of MHC-II-complexed antigen, co-stimulatory molecules (Almerighi et al. 2009), proinflammatory cytokine expression by inhibition of TLR2/4 (Khoo et al. 2011) and stimulates Th2 inflammatory cytokine production (Penna et al. 2000; Van Halteren et al. 2002; Almerighi et al. 2009). *In vitro* studies with human CD4+ T cells showed conflicting data regarding to the effects on Th2 cells. Some studies showed an increase in IL-4 producing CD4+ T cells or IL-4 secretion by CD4+ T cells cultured in the presence of $1,25(OH)_2D$ (Boonstra et al. 2001; Imazeki et al. 2006; Bartels et al. 2007; Jeffery et al. 2009), while others observed no effect (Correale et al. 2009). In addition, $1,25(OH)_2D_3$ favors T regulatory cells development via modulation of dendritic cells and by directly targeting T cells (Pedersen et al. 2011).

$1,25(OH)_2D_3$ can modulate both macrophage and lymphocyte response to live *M. tuberculosis* or culture filtrate antigens (Chandra et al. 2004), is able to induce autophagy and mediates co-localization of *M. tuberculosis* and antimicrobial peptides within autophagolysosomes, thereby facilitating the destruction of these bacteria (Yuk et al. 2009). $1,25(OH)_2D_3$ also upregulates inducible nitric oxide synthase (NOS2) in a human macrophage-like cell line (Rockett et al.1998; Chang et al. 2004).

Vitamin D is transported with high affinity in the plasma by a carrier protein called vitamin D – binding protein (VDBP) or Gc-globulin (White & Cooke 2000). Besides the transport of inactive 25-hydroxyvitamin D [25(OH)D] and active form $1,25(OH)_2D$ of vitamin D, VDBP has other properties, including an important role as a macrophage activating factor (MAF, White & Cooke, 2000). The *GC* gene encoding VDBP has two common polymorphisms at codons 416 (GAT→GAG, Asp→Glu) and 420 (ACG→AAG, Thr→Lys) of exon 11 (defined by the presence of restriction endonuclease sites for *HaeIII* and *StyI*, respectively) resulting in three major electrophoretic variants of Gc-globulin, termed component group-specific (Gc) 1 fast (Gc1F), Gc1 slow (Gc1S) and Gc2. The combination of *GC* alleles result in different genotypes *GC1S-1S (hS/hS), GC1S-1F (HS/hS), GC1F-1F (HS/HS), GC2-1S (Hs/hS), GC2-1F (HS/Hs),* and *GC2-2 (Hs/Hs).* Due to similar functional characteristics (Yamamoto et al. 1996) *GC1F*, *GC1S* and *GC2* allele carriers may be combined

to produce a total of 3 phenotypes: Gc1-1, Gc1-2 and Gc2-2 (Martineau et al. 2010).

There is association between *GC2-2* genotype and susceptibility to active tuberculosis in Gujarati Asian people with profound vitamin D deficiency (Martineau et al. 2010). In addition, VDBP plays a pivotal role in regulating the bioavailability of 25(OH)D to monocytes. Vitamin D-dependent antimicrobial responses are therefore likely to be strongly influenced by *GC* polymorphisms (Martineau et al. 2010). Our goal was to investigate the association of serum 25(OH)D concentrations and *VDR* and *GC* polymorphisms from pulmonary tuberculosis patients from a high endemic area in Brazil.

Materials and methods

Study population

Patients and healthy controls (HC) were recruited from Evandro Chagas Clinical Research Institute at Fiocruz and from Municipal Health Centers, Rio de Janeiro, Brazil, between 2004 and 2009. All volunteers included in this study lived on the metropolitan area of Rio de Janeiro City (State of Rio de Janeiro, Brazil), were greater 18 years and provided written informed consent. Cases and HC were matched by age, socio-economic class and area of residence. HC were excluded if they had a history of prior anti-tuberculosis therapy, signs and symptoms of suggestive active tuberculosis. The diagnostic criteria for tuberculosis were defined as the presence of a positive smear for acid fast bacilli (WHO 2007) and/or culture positivity for *M. tuberculosis* in a sample from sputum and/or bronchial lavage and/or other clinical specimens according to Brazilian National Guidelines (2010). HIV-infected people and those taking immunosuppressant drugs were excluded from participation in the study. The protocol was approved by the Research Ethics Committees in Brazil (IPEC REC ref. 0008.0.009.000-04) and Rio de Janeiro Municipal Health Centre (REC ref. S/CRH/DRH/DIC3). The current Brazilian population is one of the most heterogeneous in the world, descending from an admixture of Europeans, Amerindians and Africans during the last five centuries (Lins et al. 2010). Thus, to analyze the genetic profile in association with different ethnicities in Brazilian

population, TB patients and HC were stratified by classical self-definition as: white, mixed or black and using two categories as: white and non-white (where were included people colored as mixed and black).

Determination of 25(OH)D concentrations

Serum 25(OH)D concentrations were determined by high performance liquid chromatography (Chromsystems Diagnostics) in Sérgio Franco Diagnostic Laboratory Center, Rio de Janeiro, Brazil. Recommended vitamin D serum concentration is not clearly defined in Brazil and four categories have been used in the clinical practice, following International Standards. We defined insufficiency and deficiency as serum 25(OH)D concentrations of 37-74 nmol/L and 37 nmol/L respectively. Serum 25(OH)D concentrations of ≥ 75 nmol/L and ≥200 nmol/L as categorized as normal and hypervitaminosis, respectively (Singh et al. 2006). For further analysis two categories were used to determine deficiency (< 75 nmol/L) and normal (≥ 75 nmol/L) serum 25(OH)D levels.

Genotyping *VDR* and *GC*

The genotyping of *VDR* polymorphisms. *BsmI* (Lee et al. 2001), *ApaI* (Stefanic et al. 2005). *TaqI* and *FokI* (Wilkinson et al. 2000), and *GC* polymorphisms *HaeIII* and *StyI* (Martineau et al. 2010) was as previously described. Amplified DNA was digested with the respective *FokI, TaqI, ApaI and BsmI* restriction enzymes of *VDR* polymorphisms (New England Biolabs, USA) and *HaeIII* and *StyI* restriction enzymes (New England Biolabs, USA) of *GC* polymorphisms in according to manufacturer's instructions. The digestion products were separated on agarose gel and viewed using ethidium bromide. Capital letters define the absence of restriction sites for enzymes and small letters define their presence (Martineau et al. 2010). Gc-globulin group is defined by the combination of *HaeIII* and *StyI* genotypes as previously described (Martineau et al. 2010).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium for the genetic variants was assessed by the chi-square test (χ^2) in both case and control groups. We used the χ^2 test to compare the differences in each genotype, allele and haplotype of *VDR* and *GC* polymorphism frequencies between TB patients and HC subjects. Additionally, we used unconditional univariate and multivariate logistic regression analyses to examine association between the selected SNPs and tuberculosis risk by estimating odds ratios (ORs) and 95% confidence intervals (CIs) with and without adjustment for gender and ethnicity between tuberculosis and HC subjects. All statistical tests were two-sided, a *p* value of ≤ 0.05 was considered significant, and analyses were performed using Epi Info 6 (Version 6.04, July 1996, CDC, Atlanta GA, USA), SNPStats (http://bioinfo.iconcologia.net/SNPstats) and SPSS (Version 16, September 2007).

Results

Study population

A total of 148 pulmonary tuberculosis patients; 91 males (61.5%, mean age 38.5±13.5) and 57 females (38.5%, mean age 35.8±12.1) and 158 HC volunteers; 58 males (36.7%, mean age 34.1±11.1) and 100 females (63.3%, mean age 35.1±12.0) were enrolled. Volunteers were asked to define their own ethnic group as white (Caucasian), black (of African descent) or mixed. Table 1 describes the characteristics of TB cases and HC.

Serum 25(OH)D concentrations in Brazilian tuberculosis patients and healthy control subjects

The mean 25(OH)D level was slightly higher in tuberculosis patients than HC (108.2±58.9 nmol/L and 96.5±47.7 nmol/L) respectively, p=0.05. Of these 148 tuberculosis patients, only 3 (2.0%) were vitamin D deficient (serum 25(OH)D< 20 nmol/L) and 42 (28.4%) were vitamin D insufficient (serum 25(OH)D< 75 nmol/L). In the HC group 1 of 158 (0.6%) and 59 of 158 (37.3%) were respectively deficient and insufficient. The mean serum concentration of vitamin D was higher in male tuberculosis patients (108.3±54.5 nmol/L, p=0.002) when compared with male HC subjects (83.3±35.9 nmol/L). The vitamin D concentration was not different when compared male and female in tuberculosis patients and HC subjects (p>0.05). Vitamin D deficiency was not associated with age and ethnicity on univariable analysis. In further *VDR* and *GC* polymorphism we combined both deficiency and insufficiency categories for serum 25(OH)D concentration (Table 2).

VDR genotype and allele distribution and association with tuberculosis

Genotype frequencies for *FokI, TaqI and ApaI* were in Hardy-Weinberg equilibrium (H-WE) in cases and controls. However, *BsmI* genotype was not in H-WE in tuberculosis patients (p=0.0002; χ^2=*13.40*). When H-WE was re-calculated according to ethnic differences in tuberculosis group, the deviation

was not significant after correction among white (p=0.13, χ^2=2.24), black (p=0.07, χ^2=3.27) and mixed (p=0.07, χ^2=3.38) subjects. However, when non-white (mixed + black) individuals were analyzed together deviation from H-WE was observed (p=0.01, χ^2 =6.61). The genotype and allele distribution of *FokI, TaqI and ApaI* were similar in both groups (Table 3). Univariable analysis showed no association with age, gender and ethnicity within tuberculosis and HC groups. Multivariable model not showed statistical significance (p>0.05), when the analysis were adjusted by gender and ethnicity for *FokI, TaqI,* and *ApaI* (Table 3). No association was identified in *FF* genotype frequency in black subjects, however, when *F* allele frequency in black tuberculosis patients and black HC (72.2% and 86.1%, respectively) were compared we showed a 3.2-fold increase in *F* allele (p=0.03, OR=3.26, 95%CI=1.1-9.69) carriers, showing a protective role in tuberculosis.

A significant difference was observed in the *bb* genotype (TB 12.2% and HC 25.3%, p=0.01; OR=2.43, 95%CI=1.17-5.07) and *b* allele (TB 44.3% and HC 52.5%,p=0.04; OR=0.72, 95%CI=0.52-0.99) frequency between groups, indicating a protective role against tuberculosis (Table 3). Univariable analysis showed no association with age, gender when white tuberculosis patients and white-HC groups were compared. Interestingly in the non-white population the *bb* genotype (TB 10.1% and HC 33.3%, p= 0.003; OR=0.21, 95%CI=0.07-0.62) and *b* allele (TB 42.1% and HC 59.3%, p=0.007; OR=0.53, 95%CI=0.33-0.84) were more frequent in HC, indicating a protective role for *BsmI* in these subgroups (Table 4). The *bb* genotype was 2-fold more prevalent in HC group than in TB patients, when adjusted by gender and ethnicity (p=0.005, OR=3.33, 95%CI=1.44-7.69), indicating a protective association of this genotype in non-white males (Table 3).

VDR haplotype distribution and association with tuberculosis

Pairwise Linkage Disequilibrium (D`) between different gene polymorphisms was calculated and haplotype blocks were constructed using R 2.13.1 Statistical Data Analysis Program. The LD pattern in different study groups is shown in Figure 1. We observed weak LD between *Fok-Apa* (D' = 0.005, p=0.956) and

between *Taq-Fok* (D' 0.07, *p*=0.267). Moderate LD was identified between *Fok-Bsm* (D' = 0.19, *p*=0.04). Strong LD between *Taq-Bsm* (D' = 0.613, $p=1.44^{-10}$), *Taq-Apa* (D' = 0.657, $p=3.8^{-9}$) and *Bsm-Apa* (D' = 0.408, $p=1.8^{-9}$) in TB patients was observed. In HC group there was weak LD between *Taq-Fok* (D' = 0.080, *p*=0.22), *Fok-Bsm* (D' = 0.117, *p*=0.17) and *Fok-Apa* (D' = 0.088, *p*=0.42). Strong LD was observed between *Taq-Bsm* (D' = 0.619, $p=2.0^{-16}$), *Taq-Apa* (D' = 0.640, $p=9.31^{-12}$), and *Bsm-Apa* (D' = 0.477, $p=5.01^{-11}$ Figure 1). Haplotype frequencies were compared between tuberculosis and HC groups for each of the genes (Table 5). The haplotype most frequent was *FTab* in both tuberculosis (*f*=0.1711) and HC groups (*f*=0.2034). In addition, *FtaB* was observed in low frequency (*f*=0.0255) in tuberculosis and *fTAB* (*f*=0.0336) in HC groups (Table 5). There was no statistical difference in the distribution of *VDR* haplotypes.

GC genotype and allele distribution and association with tuberculosis

The *GC* genotype distributions were in HW-E in tuberculosis ($p>0.05$, $\chi^2=0.34$) patients and in the HC group ($p>0.05$, $\chi^2=0.41$). Genotypes and alleles distributions of *HaeIII* and *StyI* were similar in both groups and no statistical differences were found. The most frequent genotypes observed in our study were the *HH/Hh* and *SS* (42.6% and 62.8%) in tuberculosis, and *Hh* and SS (44.3% and 53.8%) in HC group. Less frequent were *hh* and *ss* (14.8% and 6.8%) in tuberculosis and in HC (16.5% and 8.2%). The most frequent alleles were *H* and *S* (63.9% and 78.1% in tuberculosis and 61.4% and 72.8% in HC) as seen in Table 6. Univariable and multivariable analysis showed no association with gender and ethnicity between tuberculosis and HC groups (p>0.05). Analyses were stratified by Gc-globulin groups 1-1, 1-2 and 2-2 and the most frequent group observed was Gc1-1 in tuberculosis (64.2%) and HC (53.9%), the last frequent being Gc2-2 in tuberculosis (7.4%) and HC (8.2%). No statistically difference was seen (Table 6). As Gc-globulin groups have distinct vitamin D affinity and did not show association with tuberculosis susceptibility in our population, we tested the hypothesis that a combined Gc-globulin group and *VDR* SNPs might influence tuberculosis susceptibility. Carries of both Gc1-1 group and *bb BsmI* genotype were more frequent in the

HC group (24.7%) than in patients (11.6%, *p*=0.03; OR=1.82, 95%CI= 0.70-4.71). No statistical difference was observed when Gc1-2 and in Gc2-2 groups and VDR genotypes were combined (Table 7). When *VDR* haplotypes were combined with Gc1-1, Gc1-2 or Gc2-2 no statistical difference was identified (p>0.05).

Serum 25(OH)D concentration in relation with VDR and *GC* gene polymorphism

We investigated whether associations between *VDR and GC* genotypes and susceptibility to tuberculosis were modified by vitamin D serum concentration. Serum 25(OH)D concentration was stratified as normal or deficient. HC subjects (31.9%) with normal levels of 25(OH)D showed a two-fold greater frequency of *bb BsmI* genotype than tuberculosis patients (13.2%, *p*=0.009; OR=3.09, 95%CI=1.22-7.79). When subjects with deficient serum levels of 25(OH)D were analyzed, no further association between *VDR* genotypes and alleles was identified (Figure 2). No statistical difference was observed when *GC* genotypes (*HH, Hh* and *hh*; *SS*, *Ss* and *ss*), alleles (*H,h,S* and *s*) and Gc-globulin groups were combined with serum 25(OH)D concentration (p>0.05) (data not shown).

Serum 25(OH)D concentrations in relation to *VDR* haplotypes

The *VDR* haplotype *ftBA* was more frequent in tuberculosis patients (*f*=0.1219, *p*=0.03; OR=0.24, 95%CI= 0.06-0.87) than in HC (*f*=0.0467) in subjects with normal serum 25(OH)D concentration. Subjects with 25(OH)D deficiency did not show any difference in *VDR* haplotype among tuberculosis and HC groups (*p*>0,05) (data not shown).

Discussion

The role of *VDR* polymorphism in susceptibility to tuberculosis remains controversial. Some studies have proposed that ethnic factors are more important than socioeconomic determinants of susceptibility to tuberculosis (Doherty et al. 1995) and *VDR* polymorphism could contribute to the difference in the immune response between ethnicities (Davies et al. 2001). Therefore, we analyzed *VDR* polymorphisms to better understand genetic risk factors for tuberculosis. No association between *TaqI, FokI, and ApaI* VDR genotype, allele and haplotype polymorphism with tuberculosis was observed in this study. The least frequent genotype in our study was *ff of FokI* (8.2%). In black Brazilian subjects the *F allele* of *FokI* was associated with protection against tuberculosis (3.2-fold). There are only two descriptions of VDR polymorphism in South America, in Peruvian and Paraguayans population and the *TaqI* and *FokI* genotype and allelic frequency were equally distributed. However, Wilbur et al. (2007) described a protective association for *TT* and *FF* genotypes against active tuberculosis and infection, respectively. The *FF* genotype was also associated with faster smear conversion during TB treatment (Roth et al. 2004). The *f* allele of *FokI* is more frequent in Caucasians (Uitterlinden et al. 2004). However, in healthy pregnant Brazilian women the distribution of *VDR* genotypes and alleles for *3 VDR* gene polymorphisms showed no interethnic difference, including *F* and *f* alleles (Rezende et al. 2007). Polymorphism at the *FokI* site has been associated in changes in number of amino acid present in human VDR isoforms. The *F* allele encodes a shorter protein more able to associate with the transcriptional factor TFIIB (Jurutka et al. 2000). Distinct population possess different frequencies of *FokI* variant, as seen in India population where the *FF* genotype was associated with spinal tuberculosis (Selvaraj et al. 2004), whereas the *ff* genotype was associated with spinal tuberculosis in China (Zhang et al. 2010) and *FF* genotype correlated with extrapulmonary tuberculosis in North America (Motsinger-Reif et al. 2010).

When comparing with other studies in Brazil the *BB* (ranging from 10.2% to 20.9%) genotype is less frequent than *bb (*38.5% to 41.5%) genotype (Lazaretti-Castro. 1997; Hauache et al. 1998; Mory et al. 2009), in accordance with our results. As described previously in India (Selvaraj et al. 2004; Sharma

et al. 2011) and Turkey (Ates et al. 2011) the frequency of *bb* is also decreased in our tuberculosis patients, suggesting a protective role in active tuberculosis. However, the *b* allele has also been associated with risk of tuberculosis in Iranians (Merza et al. 2009).

VDR haplotypes have been associated with disease resistance in South Africa (*FbAT*; Lombard et al. 2006), Iran (*fTAb*, Marashian et al. 2010) and India (*BAtf*, Selvaraj et al. 2004) or with susceptibility to tuberculosis in West Africa (*F-A,* Bornman et al. 2004), India (*batF*, Selvaraj et al. 2004; Alagarasu et al. 2009). The most frequent haplotype in tuberculosis patient and HC subjects in our study was *FTab* (*f*=0.1711 and 0.2034), with no association with tuberculosis. This haplotype was identified as most frequent either in Africa population (Bornman et al. 2004), with no association. Our haplotype distribution was slightly different from another Brazilian study (Rezende et al. 2007, 2010), with respect to the distribution of *FAB, FAb* and *fab* haplotypes on *VDR* polymorphism.

A previous study described the association between ethnicity and the production of vitamin D, showing that the 25(OH)D serum concentration of African Americans was markedly lower than those from Caucasians due to increased production of melanin in the skin. This decreases the production of vitamin D, which consequently results in a decrease in cathelicidin or LL-37, a potent antimicrobial peptide that can restrict the growth of *M. tuberculosis* (Liu et al. 2006). Our patients and HC did not show any difference in serum concentration of 25(OH)D, irrespective of ethnicity. Rio de Janeiro has around 200 sunny days per year and sun exposure is very frequent and the discrepancy from Liu et al. (2006) may also be related to dietary factors.

The classical function of VDBP is to store and prolong the half-life of circulating vitamin D metabolites (White & Cooke., 2000). Therefore, we analyzed the correlation between *GC* variants and tuberculosis. No significant association between *GC* genotypes and alleles carriages with tuberculosis was observed. The some profile was seen between Gc-globulin groups and tuberculosis. However, the Gc1-1 group was most prevalent in non-white tuberculosis patients (73.9%) indicating that black/mixed tuberculosis patients Gc1-1 carriers might have a predisposition to illness. Even Gc1-1 group is responsible for higher vitamin D affinity (Lauridsen et al. 2005), this group

induced 2.4 to 2.5 fold less cathelicidin than other groups (Gc1-2 and Gc2-2, respectively), indicating a less efficient *M. tuberculosis* control (Chun et al. 2010). We recently showed that the *Gc2* allele was associated with tuberculosis susceptibility in Gujarati Asians population, but not in Brazilian subjects. The association in Asian subjects carrying *Gc2-2* genotype was most pronounced in those with 25(OH)D deficiency (Martineau et al. 2010). No association with 25(OH)D serum concentration was associated with Gc-globulin groups in the present study. The difference between Asians and Brazilians populations could be explained by the dietary habit and the greater sun exposure in Brazilian people, especially in Rio de Janeiro, where people perform many outdoor activities. Another study in Brazilian population (in São Paulo State), found age, season and ethnicity dependent variation in vitamin D levels (Unger et al. 2010). The difference between our results might be related with cultural habits, life style, air pollution, outdoor activities work and dietary factors.

We tested whether a combined Gc-globulin group and *VDR* SNPs might influence tuberculosis susceptibility. To our knowledge this is the first study to correlate both genes and 25(OH)D serum concentration with tuberculosis. The protective influence of both genes was identified only in those carrying both *bb* genotype of *BsmI* and Gc1-1 group (p=0.03), indicating that the low cathelicidin environment induced by Gc1-1 group might be sufficient to control tuberculosis bacilli growth in those individuals carrying the higher VDR protein expressing *bb* of *BsmI VDR* gene (Selvaraj et al. 2009). In our population serum concentration of 25(OH)D is normal in two-third of tuberculosis patients and directly influence the *VDR* and *GC* gene expression. Nutritional status is more important than the genetic background in our population.

In summary, our results suggest the importance of maintaining physiological serum levels of vitamin D may decrease the risk of active tuberculosis in higher incidence countries, independent of *VDR* and *GC* genotypes, alleles and haplotypes. Vitamin D dietary intake or supplementation could be take in account in countries where both tuberculosis and malnutrition incidence is higher to driven a decrease in active tuberculosis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. Clinical data from Brazilian tuberculosis patients and healthy control subjects

		Tuberculosis Patients N=148[a]	Healthy Controls N=158[a]
Sex, N (%)	Male	91(61.5)	58 (36.7)
	Female	57 (38.5)	100 (63.3)
Age (years)[b]		36.0±13.1	32.0±13.1
Ethinicity	white	47 (40.2)	71 (48.6)
	black	29 (24.8)	18 (12.3)
	mixed	41 (35.0)	57 (39.1)
	white	47 (40.2)	71 (48.6)
	non-white	70 (59.8)	75 (47.5)

[a]Values are N (%)
[b]Mean of age between tuberculosis patients and healthy control

Table 2. 25(OH)D serum concentration in Brazilian tuberculosis patients and healthy control subjects

Vitamin D range	Tuberculosis Patients n=136 (%)	Healthy Controls n=153 (%)	*P Value*[c]	OR (95% CI)
Normal (reference)[a] (≥ 75 to ≥200 nmol/L)	91 (66.9)	94 (61.4)	0.33	1.27 (0.64-1.55)
Deficiency[b] (≥ 37 to 74 nmol/L)	45 (33.1)	59 (38.6)		

Serum 25(OH)D concentration was identified as ([a]) normal or ([b]) deficiency in tuberculosis and healthy controls
[c]*p*-value for 2x2 chi test for tuberculosis patients and healthy controls comparison
p-value considered < 0.05 (confidence interval of 95%); OR, odds ratio.

Table 3. Genotype and allele frequencies in *VDR* gene polymorphisms in Brazilian tuberculosis patients and healthy control subjects

	Genotypes / Alleles	Tuberculosis Patients n=148 (%)	Healthy Controls n=158 (%)	*P* Value[a/*]	Crude Model[b] OR (95% CI)	Adjusted Model[c] OR (95%CI)	*P* Value[c]
FokI	*FF*	65 (43.9)	82 (51.9)	0.37	1.00	1.00	0.59
10735810	*Ff*	68 (45.9)	63 (39.9)		0.73 (0.46-1.18)	0.76 (0.44-1.29)	
	ff	15 (10.2)	13 (8.2)		0.69 (0.31-1.55)	0.82 (0.32-2.09)	
	F	198 (69.9)	227 (71.8)	0.19*	0.79 (0.55-1.13)	-	
	f	98 (33.1)	89 (28.2)			-	
TaqI	*TT*	67(45.3)	63 (39.9)	0.63	1.00	1.00	0.66
731236	*Tt*	68 (45.9)	79 (50.0)		1.24 (0.77-1.98)	1.15 (0.67-1.98)	
	tt	13 (8.8)	16 (10.1)		1.31 (0.58-2.94)	1.49 (0.60-3.67)	
	T	202 (68.2)	205 (64.9)	0.38*	1.16 (0.82-1.65)	-	
	t	94 (31.8)	111 (35.1)			-	
ApaI	*AA*	57 (38.5)	53 (33.5)	0.66	1.00	1.00	0.54
7975232	*Aa*	73 (49.3)	84 (53.2)		1.24 (0.76-2.02)	1.02 (0.59-1.78)	
	aa	18 (12.2)	21 (13.3)		1.25 (0.60-2.61)	1.63 (0.65-4.08)	
	A	187 (63.2)	190 (60.1)	0.44*	1.14 (0.81-1.60)	-	
	a	109 (36.8)	126 (39.9)			-	
BsmI	*BB*	35 (23.6)	32 (20.3)	0.01	1.00	1.00	0.005
1544410	*Bb*	95 (64.2)	86 (54.4)		0.99 (0.56-1.74)	1.16 (0.61-2.19)	
	bb	18 (12.2)	40 (25.3)		2.43 (1.17-5.07)	3.33 (1.44-7.69)	
	B	165 (56.7)	150 (44.5)	0.04*	1.39 (0.97-1.89)	-	
	b	131 (44.3)	166 (52.5)			-	

[a]*p*-value of 3x2 chi test for trend for genotype frequencies between tuberculosis patients and healthy controls; [*]*p*-value of 2x2 chi test for overall allelic frequencies comparison between tuberculosis patients and healthy controls; [b]Odds ratio of conditional logistic regression stratified by cases and healthy controls; [c]Odds ratio of conditional logistic regression stratified by cases and healthy controls and adjusted for gender and ethnicity; [c]*p*-value of 3x2 chi test for trend for genotype frequencies between tuberculosis patients and healthy controls adjusted for gender and ethnicity in a dominant model for rs:10735810; 731236; 7975232 and 1544410); *p*-value considered < 0.05 (confidence interval of 95%); OR, odds ratio.

Table 4. Adjustment for ethnicity on *BsmI VDR* frequencies in non-white tuberculosis patients and -healthy control subjects

Genotypes	Tuberculosis Patients Non-white N=70 (%)	Healthy Controls Non-white N=75 (%)	*P* Value[a]	OR (95% CI)
BB	17 (25.6)	11(14.7)	0.003	1.00
Bb	45 (64.3)	39 (52.0)		0.75 (0.31-1.78)
bb	8 (10.1)	25 (33.3)		0.21 (0.07-0.62)
B	79 (57.9)	61 (40.7)	0.007[*]	1.89 (1.15-3.10)
b	61 (42.1)	89 (59.3)		

[a]*p*-value of 3x2 chi test for trend in a dominant model for genotype frequencies between tuberculosis patients and healthy controls; [*]*p*-value of 2x2 chi test for overall allelic frequencies comparison between non-white tuberculosis patients and healthy controls; *p*-value considered < 0.05 (confidence interval of 95%); OR, odds ratio.

Table 5. Haplotype frequencies in polymorphisms of *VDR* gene in tuberculosis patients and healthy control subjects

Haplotypes	Tuberculosis Patients[a]	Healthy Controls[a]	*P* Value[b]	OR (95% CI)
FTab	0.1711	0.2034	-	1.00
FtAB	0.1241	0.1726	0.67	1.20 (0.52-2.80)
FTAb	0.1211	0.1192	0.63	0.83 (0.39 - 1.77)
FTAB	0.1404	0.088	0.07	0.49 (0.22 -1.07)
ftAB	0.1168	0.0883	0.06	0.47 (0.21 -1.05)
fTab	0.0762	0.0766	0.90	1.08 (0.32 – 3.66)
FTaB	0.0488	0.0633	0.85	0.90 (0.30 – 2.70
fTAB	0.0612	0.0336	0.11	0.39 (0.12 -1.23)
FtAb	0.0354	0.0444	0.51	0.66 (0.20 – 2.21)
FtaB	0.0255	0.0552	0.60	1.42 (0.38 – 5.27)

[a] *p*-value for comparison of haplotype frequencies between tuberculosis patients and healthy controls
[b] *p-value considere*d < 0.05 (confidence interval of 95%); OR, odds ratio. Haplotype analyzes were performed in statistical SNAP program

Table 6. Genotype and allele frequencies in *DBP* polymorphisms and Gc-globulin groups in tuberculosis patients and healthy control subjects

SNPs	Tuberculosis Patients n=148 (%)	Healthy Controls n=158 (%)	*P* Value[a]	OR[a]
HH	63 (42.6)	62 (39.2)	0.83	1.00
Hh	63 (42.6)	70 (44.3)		1.13 (0.69-1.84)
hh	22 (14.8)	26 (16.5)		1.20 (0.62-2.34)
H	189 (63.9)	194 (61.4)	0.52*	1.11(0.79-1.56)
h	107 (36.1)	122 (38.6)		
SS	93 (62.8)	85 (53.8)	0.28	1.00
Ss	45 (30.6)	60 (38.0)		1.46 (0.90-2.37)
ss	10 (6.8)	13 (8.2)		1.42 (0.59-3.41)
S	231 (78.1)	230 (72.8)	0.13*	1.33 (0.90-1.96)
s	65 (21.9)	86(27.2)		
Gc-globulin group				
1.1	95 (64.2)	85 (53.9)	0.12┼	1.00┼
1.2	42 (28.4)	60 (37.9)		0.63 (0.38-1.02)
2.2	11(7.4)	13 (8.2)		0.76 (0.32-1.78)

[a]*p*-value of 3x2 chi test for trend in a dominant model for genotype frequencies between tuberculosis patients and healthy controls; [*]*p*-value of 2x2 chi test for overall allelic frequencies comparison between tuberculosis patients and healthy controls; [a]Odds ratio of conditional logistic regression stratified by cases and healthy controls; ┼*p*-value of 3x2 chi test for trend in a dominant model for Gc-globulin group frequencies between tuberculosis patients and healthy controls;┼Odds ratio of conditional logistic regression stratified by cases and healthy controls; *p*-value considered < 0.05 (confidence interval of 95%); OR, odds ratio.

Table 7. Genotype frequencies in *VDR* polymorphisms and Gc-globulin groups in pulmonary tuberculosis patients and in healthy control subjects

	Gc-Globulin group 1-1				Gc-Globulin group 1-2				Gc-Globulin group 2-2			
SNPs *VDR*	Tuberculosis Patients n=(%)	Healthy Controls n=(%)	*P* value[a]	OR[a]	Tuberculosis Patients n=(%)	Healthy Controls n=(%)	*P* value[b]	OR[b]	Tuberculosis Patients n=(%)	Healthy Controls n=(%)	*P* value[c]	OR[c]
TT	47 (49.5)	36 (42.4)	0.27	1.00	18 (42.9)	22 (36.7)	0.47	1.00	2 (18.2)	5 (38.5)	0.51	1.00
Tt	42 (44.2)	38 (44.7)		1.18	20 (47.6)	35 (58.3)		1.43	6 (54.5)	6 (46.2)		0.40
tt	6 (6.3)	11 (12.9)		2.39	4 (9.5)	3 (5.0)		0.61	3 (27.3)	2 (15.3)		0.27
FF	41 (43.2)	43 (50.6)	0.52	1.00	21 (50.0)	30 (50.0)	0.86	1.00	3 (27.3)	9 (69.2)	0.10	1.00
Ff	46 (48.4)	34 (40.0)		0.70	17 (40.5)	26 (43.3)		1.07	5 (45.4)	3 (23.1)		0.20
ff	8 (8.4)	8 (9.4)		0.95	4 (9.5)	4 (6.7)		0.70	3 (27.3)	1 (7.7)		0.11
BB	20 (21.1)	21 (24.7)	0.03	1.00	11 (26.2)	9 (15.0)	0.26	1.00	4 (36.4)	2 (15.4)	0.09	1.00
Bb	64 (67.4)	43 (50.6)		0.64	24 (57.1)	35 (58.3)		0.64	7 (63.6)	8 (61.5)		2.29
b	11(11.6)	21 (24.7)		1.82	7 (16.7)	16 (26.7)		0.36	0 (0.0)	3 (23.1)		NA
AA	35 (36.8)	30 (35.3)	0.77	1.00	17 (40.5)	19 (31.7)	0.56	1.00	5 (45.5)	4 (30.8)	0.72	1.00
Aa	48 (50.5)	41 (48.2)		1.00	20 (47.6)	35 (58.3)		1.57	5 (45.5)	8 (61.5)		2.00
aa	12 (12.7)	14 (16.4)		1.36	5 (11.9)	6 (10.0)		1.07	1 (9.1)	1 (7.7)		1.25

[a,b,c]*p*-value of 3x2 chi test for trend in a dominant model for genotype frequencies between tuberculosis patients and healthy controls; [a,b,c] Odds ratio of conditional logistic regression stratified by cases and healthy controls; *p*-value considered < 0.05 (confidence interval of 95%); OR, odds ratio

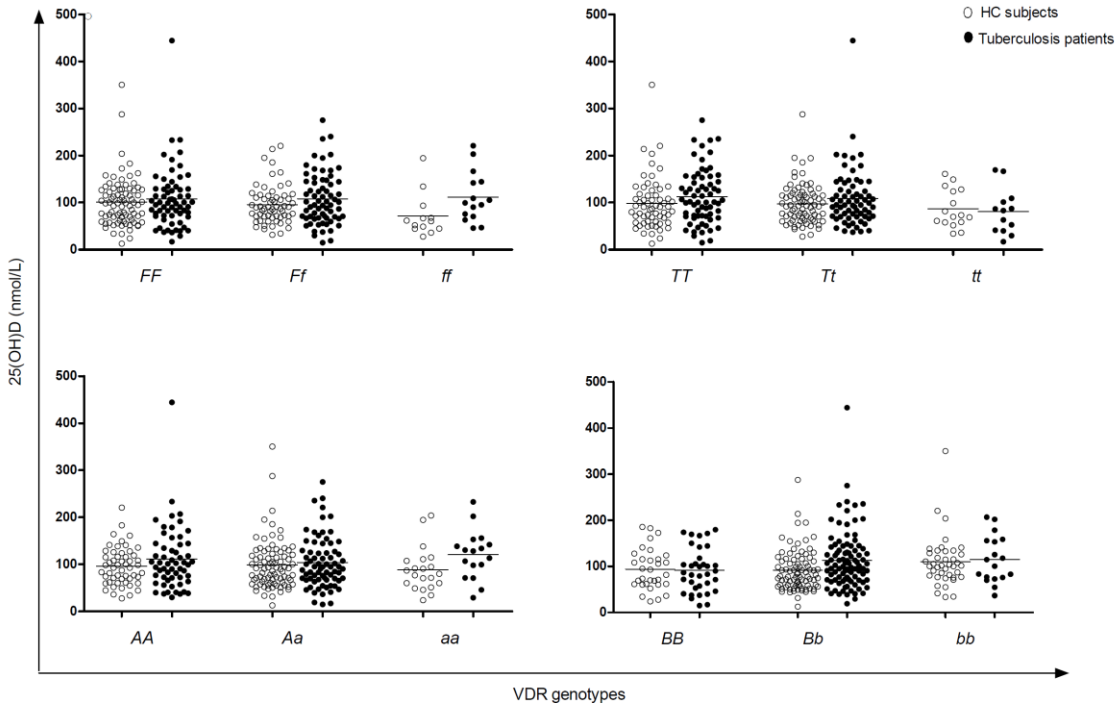

Figure 2: Comparative analysis of serum level of 25(OH)D and the genotypes of *VDR* in tuberculosis patients and healthy controls (p> 0.05)

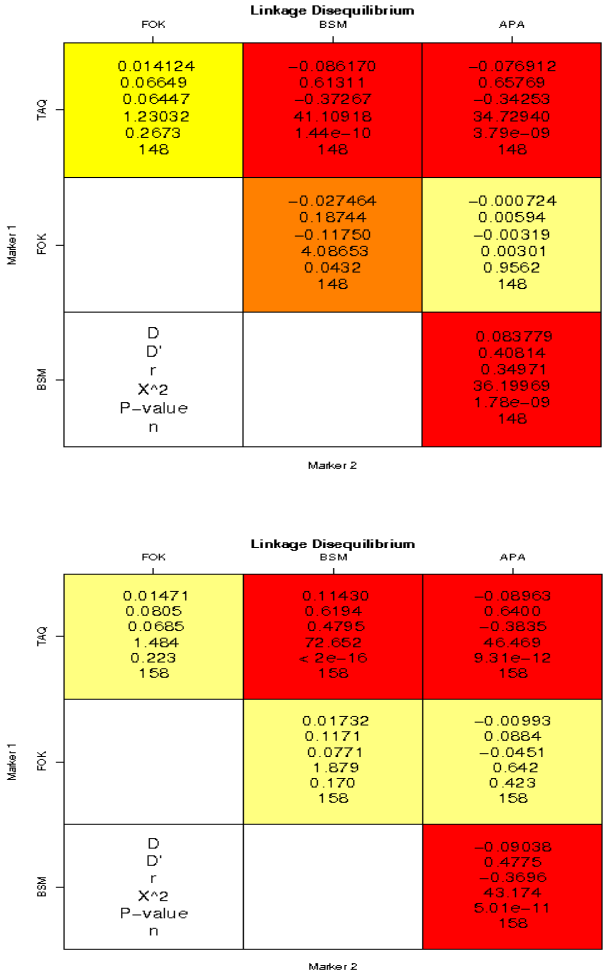

Figure 1. Linkage Desiquilibrium-D´values

4.3.2 Conclusões referentes ao artigo sibmetido *Tuberculosis Journal (2012)*

1- O alelo *F* de *FokI* foi associado a proteção contra a TB em negros, esse fato poderia estar relacionado ao aumento da capacidade de ligação ao fator de transcrição-TFIIB, acarretado em melhora na transcrição de genes alvos, que desempenham função moduladora do sistema imune, melhorando assim a respota contra o *Mtb*;

2- O genótipo *bb* e o alelo *b* de VDR mostraram estar associados com a resistência à TB em controles e controles não brancos com níveis de vitamina D dentro da faixa de normalidade, sugerindo o envolvimento deste polimorfismo na expressão do receptor de VD. Portadores deste genótipo expressam mais proteína, indicando um aumento da expressão do VDR e maior disponibilidade de VD para as células alvo e conseqüentemente em melhor acesso das células a esta vitamina, possibilitando um melhor desempenho do sistema imune na TB.

3- O haplótipo de VDR *ftBA* foi associado com susceptibilidade à TB, apenas em indivíduos com níveis séricos normais de 25(OH)D, sugerindo que em carreadore desta combinção alélica a VD não está influenciando os mecanismos da reposta imune contra o *Mtb* e que este haplótipo poderia estar relacionado com a diminuição da expressão ou funcionalidade deste receptor.

4- A combinação entre o grupo *Gc1-1* de *DBP* e *bb* de *VDR* foi associado com a proteção a TB. Porém outras combinações das variantes de *GC-globulin* e de *VDR* não demonstraram associação. Estes resultados sugerem que, apesar do grupo *Gc1-1* ser baixo produtor de catelicidina, esta produção pode ser suficiente. Além disso, o genótipo *bb* induz maior expressão da proteína VDR podendo ser um mecanismo de compensação, conferindo proteção, em indivíduos com 25(OH)D dentro da faixa de normalidade.

5- A falta de associação entre os polimorfismos de *DBP* e *VDR* com os níveis séricos de 25(OH)D sugerem que estes polimorfismos e a vitamina D sérica não representam fatores de risco no desenvolvimento

da TB, uma vez que no nosso estudo os pacientes apresentavam níveis séricos de VD dentro da faixa de normalidade.

6- Pacientes e controles apresentaram níveis séricos de VD dentro da faixa de normalidade independente da cor da pele, sugerindo que a ingesta da vitamina e a exposição solar destes indivíduos é o suficiente para produzir VD de forma adequada.

4.4 LACK OF ASSOCIATION OF *IFNG* AND *NOS2A* GENES POLYMORPHISM AND PRODUCTION OF NITRITE AND NITRATE IN TUBERCULOSIS SUSCEPTIBILITY AMONG BRAZILIAN POPULATION

Artigo a ser submetido *International Journal of Imunogenetic (2012)*

Entre os anos de 2004 e 2007, foram coletados amostras de sangue periférico de pacientes com tuberculose e controles saudáveis. Para este estudo, foram analisados os polimorfismos de *IFNG+874T/A* e *NOS2-954G/C* em indivíduos provenientes de área endêmica, através da técnica de PCR-RFLP. Além disso, foram availiadas a produção de nitrito e NOx total (Técnica de Griess). Os resultados das freqüências genotípicas e as dosagens de radicais de nitrogênio foram comparados entre pacientes e controles saudáveis e não foi possível detectar diferença entre os perfis genotípicos e a produção de nitrito e NOx total.

Os resultados obtidos respondem aos objetivos de nº 3 e 4 da tese de doutorado.

4.4.1 Artigo a ser submetido ao *International Journal of Imunogenetic (2012)*

LACK OF ASSOCIATION OF *IFNG* AND *NOS2A* GENES POLYMORPHISM AND PRODUCTION OF NITRITE AND NITRATE IN TUBERCULOSIS SUSCEPTIBILITY AMONG BRAZILIAN POPULATION

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Summary

Tuberculosis (TB) is one of the most common infectious diseases in the world. *Mycobacterium tuberculosis* infection leads to disease in approximately 5-10% of exposed individuals. Among other factors, the genetic predisposition to developing TB may involve multiple genes. Several genes, including *IFNG* and *NOS2A*, coding for different cytokines and proteins, may affect host susceptibility to TB. Thus, variations of these genes, including single-nucleotide polymorphisms may influence the susceptibility/resistance to TB development. Despite the possibility of multigenic predisposition, the individual demographic characteristics in different populations have been reported in TB susceptibility, where these polymorphisms have different impacts in different ethnic populations. Polymorphism of the *IFNG* gene at position *+874* and the *NOS2A* gene at position *-95*4 were determined by the amplification refractory mutation system (ARMS-PCR) and restriction fragment length polymorphism (RFLP), respectively. To evaluate the hypothesis that the functional *IFNG+T/A* and *NOS2A-954G/C* polymorphisms are associated with TB development in a multiracial and miscegenic population, we examined the genotype, allele and genotype combinations of these polymorphisms in 172 TB patients and 179 healthy controls (98 tuberculin skin test positive) and how these polymorphisms could be influence nitrite and nitrate secretion. No association with *IFNG+874T/A* and *NOS2A-954G/C* gene polymorphisms was observed between TB and healthy control groups or in the HC subgroup with TST-positive (possible latent TB). *NOS2A-954G/C* gene polymorphisms was not associated with serum levels of nitrite and nitrate. These results indicate that variants of *IFNG+874T/A* and *NOS2A-954G/C* are not influencing TB susceptibility nor the secretion of nitric oxide radicals.

KEYWORDS: tuberculosis, *IFNG+874T/A, NOS2A-954G/C*, polymorphism, IFN-γ, nitric oxide

Introduction

Tuberculosis (TB) has been declared as a major global healthy threat by the World Health Organization since 1993. In Brazil, pulmonary TB is highly prevalent with more than 111.000 new cases every year (WHO, 2010) and 9,789 reported in Rio de Janeiro (SNVS, 2006). The Brazilian population is one of the most heterogeneous in the world, descending from an admixture of Europeans, Amerindians and Africans during the last five centuries. The use of ancestry informative markers (AIMs) has revealed ample genetic heterogeneity in the Brazilian population (Callegari-Jacques et al. 2003; Parra et al. 2003; Marrero et al. 2005; Lins et al. 2010). The host genetic susceptibility to TB has been studied worldwide with or without association with single-nucleotide polymorphisms (SNP) in several genes, including *IFNG* (Lopez-Maderuelo et al. 2003; Mirsaeidi et al. 2006; Hwang et al. 2007; Cooke et al. 2006; Sallakci et al. 2007; Moran et al. 2007) and *NOS2A* genes (Gómez et al. 2007).

IFN-γ production is critical in the control of *M. tuberculosis* infection as demonstrated *in vitro* (Bonecini-Almeida et al. 1998), in experimental infection (Cooper et al. 1993) as well as in clinical observations. Polymorphisms in the first intron of human *IFNG* gene are associated with higher *in vitro* production of this cytokine and are correlated with a gene dosage effect in the presence of the *T* allele of *IFNG+874T/A* gene (Pravica et al 2000). While several reports have associated *IFNG+874T/A* gene with TB susceptibility (Cooke et al. 2006; Sallakci et al. 2007; Amim et al. 2007; Ben Selma et al. 2011) or with disease severity, others have not (Mirsaeidi et al. 2006; Moran et al. 2007; Hwang et al. 2007; Wu et al. 2008). Administration of IFN-γ to patients with tuberculosis speeds bacterial clearance (Dawson et al. 2009). In addition, IFN-γ induces apoptosis in mycobacteria-infected macrophages in a nitric oxide (NO) dependent manner (Kwon et al. 1997).

NO is a free radical and second messenger that has been shown to be important in the development of several diseases, including TB. NO plays a major role in the pulmonary host-defense mechanism in response to infections and is implicated in bacteriostatic and bactericidal processes. Nitric oxide (NO) is vital for macrophage function and granuloma formation in the immune

response and kills *M tuberculosis in vitro* (Gómez et al. 2007). NO production and NOS2 expression by alveolar macrophages is up regulated in response to heat-killed *M. tuberculosis* instilled into the lungs of rats (Greenberg et al. 1995) and plays a role in limiting the growth of *M. avium* complex (Bermudez et al. 1993). Contrary to TB murine models, the role of NO in killing or limiting the growth of the *M. tuberculosis* in humans is unclear. It has been proposed that NO produced by TB-infected human macrophages and by epithelial cells exhibits anti-mycobacterial behavior against *M. tuberculosis* (Rockett et al. 1998). Several reports, including some from our group, indicate that the alveolar macrophages from active TB patients express the inducible nitric oxide synthase (iNOS/NOS2) and may control mycobacteria growth *in vivo* (Nicholson et al. 1996) and *in vitro* (Nozaki et al. 1997). Thus, *NOS2A* gene may represent a pivotal protective locus against TB (MacMicking et al. 1997). Investigation is hampered by difficulty on estimating the production of NO *in vivo* meanly in lung tissues, but, genetic studies provide a potential means of examining the relation between *NOS2* expression and disease outcome. Given the biological and genetic plausibility of the role of NOS2 in the immune system, SNPs have been reported in many populations worldwide (Levesque et al. 1999; Kun et al. 2001; Jamieson et al. 2004). SNPs in the promoter region of the encoding *NOS2A* gene *(-954 G/C, -1173 C/T, -1659 A/T)* have been shown to increase NO synthesis (Hobbs et al. 2002; Burgner et al. 2003). The *NOS2A*-954*G/C* variant was originally reported in a malaria endemic area in Africa (Kun et al. 2001), suggesting that this mutation originated as a consequence of selective pressure of *Plasmodium* infection. Recent report described that peroxinitrite, superoxide and NO are able to induce antimycobacterial activities *in vitro* under stimulation of 1,25(OH)D3 (Molinari et al. 2011) or 1,25(OH)D3 plus IFN-γ (Rockett et al,. 1998). The triad IFN-γ, iNOS/NOS2 and vitamin D are important regulatory molecules in *M. tuberculosis* control (Leandro et al., 2009, Martineau et al., 2010). *NOS2A* gene variants may contribute to TB susceptibility, particularly in individuals of African descent, and may act synergistically with SNPs in *TLR4* and *IFNGR1* (Velez et al. 2009). The allele *G* of *NOS2A-954G/C* is not present in a Caucasian population (Kun et la., 1998; Coia et al. 2005) or in a Peruvian population (Martín et al. 1999). In Mexicans, in whom the allele *G* frequency of

NOS2A-954G/C was 5% this SNP was not associated with TB (Flores-Villanueva et al. 2005).

In this study, we analyzed TB patients and a healthy control population from Rio de Janeiro (Brazil) to determine whether SNPs in the *IFNG +874T/A* and *NOS2A-954G/C* genes, are associated with TB susceptibility in a highly admixed population located in a highly endemic TB geographic area. We also conducted functional studies to determine whether the modulation of nitric oxide radicals (nitrite and nitrate) secretion could vary according to genotypes or extended genotypes.

Materials and Methods

Study population

Patients and healthy controls (HC) were recruited from Evandro Chagas Clinical Research Institute at Fiocruz and from Municipal Health Centers, Rio de Janeiro, Brazil, between 2004 and 2009. All volunteers included in this study lived on the metropolitan area of Rio de Janeiro City (State of Rio de Janeiro, Brazil), were greater 18 years and provided written informed consent. Cases and HC were matched by age, socio-economic class and area of residence. HC were excluded if they had a history of prior anti-tuberculosis therapy, signs and symptoms of suggestive active tuberculosis. The diagnostic criteria for tuberculosis were defined as the presence of a positive smear for acid fast bacilli (WHO 2007) and/or culture positivity for *M. tuberculosis* in a sample from sputum and/or bronchial lavage and/or other clinical specimens according to Brazilian National Guidelines (2010). HIV-infected people and those taking immunosuppressant drugs were excluded from participation in the study. The protocol was approved by the Research Ethics Committees in Brazil (IPEC REC ref. 0008.0.009.000-04) and Rio de Janeiro Municipal Health Centre (REC ref. S/CRH/DRH/DIC3). Ethnic background was determined for each case and control volunteers by self-identification. All TB patients and HC were negative for HIV 1/2 infection (following standard diagnostic from Brazilian Minister of Health). Tuberculin skin test response to 5UT RT-23 (Statens Serum Institute, Denmark) was available in 98 (63%) individuals. The skin test response was measured at the diameter of induration 72h after the injection. These subjects were classified into who were naturally infected with *M. tuberculosis* (≥ 10 mm) and those who were uninfected (< 10 mm). BCG vaccination status was determined by the presence of the scars and/or oral vaccination when subjects were older than 29 years old. Blood samples were taken after informed consent was obtained from each subject. Patients and controls from the same family were not involved in the study.

Genotyping of +874 *IFNG* and -954G/C *NOS2A* genes polymorphisms

Genomic DNA was extracted from fresh or frozen EDTA blood using a DNA purification kit (QIamp DNA mini Kit, Qiagen, USA) according to the manufacturer's instructions. The SNP at *IFNG+874T/A* was detected by amplification refractory mutational system (ARMS-PCR) (Pravica et al. 2000). The SNP at *NOS2A-954G/C* gene was detected by restriction fragment length polymorphism (RFLP) (Kun et al. 2001). Amplifications were done in a 9700 Thermocycler (96-Well GeneAmp® PCR System 9700, Applied Biosystems, USA) using 2.5 UI and 1.5 UI for *IFNG* and *NOS2A* of Taq DNA polymerase, respectively (GoTaq flexi DNA polymerase, Promega, USA). Cycling PCR conditions for *IFNG+874T/A* were 3 minutes at 95oC followed by 10 cycles at 95^{o}C for 15 s, 65^{o}C for 50 s and 72^{o}C for 40 s; 20 cycles at 95^{o}C for 20 s, 55^{o}C for 50 s and 72oC for 50 s; 72^{o}C for 7 minutes and 4^{o}C until use. Cycling PCR conditions for -954 G/C *NOS2A* were 3 minutes at 95^{o}C followed by 30 cycles at 94^{o}C for 10 s, 60^{o}C for 30 s and 72^{o}C for 30 s; 72^{o}C for 7 minutes and 4^{o}C until use. The amplified products were evaluated by electrophoresis on a 1.5% (*IFNG*) and 2.5% (*NOS2A*) agarose gel containing ethidium bromide (0.5 µg/mL).

Detection of nitrite and nitrate on TB patients

Serum nitrite levels were quantified by a ready to use Griess reaction kit according to the manufactory's instruction (Promega). Since reduction of nitrate to nitrite occurs in acidic media, the detecting solution was present during reduction as described by Miranda *et al* (2001). Linear regression of the mean values of the absorbance at 420 or 540 nm for each standard set minus the blank values was utilized to determine the nitrite or nitrate after reduction with Vanadium III concentrations, respectively, in samples.

Statistical Analysis

Deviation from Hardy-Weinberg equilibrium for the genetic variants was assessed by the chi-square test (χ^2) in both case and control groups. We used the χ^2 test to compare the differences in each genotype, allele and extended genotypes of *IFNG* and *NOS2* polymorphism frequency. Additionally, we used unconditional univariate and multivariate logistic regression analyses to examine the associations between the selected SNPs and tuberculosis risk by estimating odds ratios (ORs) and 95% confidence intervals (CIs) with and without adjustment for gender, ethnicity, TST status, previous BCG vaccination between tuberculosis cases and HC. All statistical tests were two-sided, a *p* value of ≤ 0.05 was considered significant, and analyses were performed using Epi Info 6 (Version 6.04, July 1996, CDC, Atlanta GA, USA), SNPStats (http://bioinfo.iconcologia.net/SNPstats) and SPSS (Version 16, September 2007). Additionally, the distribution of *IFNG* and *NOS2* gene polymorphisms were compared among patients and in who TST were positive in HC group by the χ^2 or Fisher's exact test. Subgroup analyses for, genotype, allele and haplotype associations to tuberculosis were also conduct among individuals with TST-positive. The analysis of the skin test positive group was planned because it was thought that this would represent people with probable latent TB infection, whereas the skin test negative group would represent a mixture of people without tuberculous infection and people who were anergic, making interpretation of the findings more difficult and inconclusive. We used Anova to compare the nitrate/nitrite levels in association with *NOS2A-954G/C* genotypes and extended *IFNG/NOS2* genotype with the level of significance set at $p < 0.05$.

Results

The genotype distribution of *IFNG+874T/A* and *NOS2A-954G/C* were in Hardy-Weinberg equilibrium ($p>0.05$) in both cases and control groups.

Study population

There were 105 males (61.0%) and 67 females (39.0 %) in the TB group with a mean age of 36.9 ± 12.7 years (± standard deviation) and there were 63

males (35.2%) and 116 females (64.8%) with a mean age of 35.1 ± 11.5 years in the HC group. Age was not significantly different between the groups. However, we observed an inverse proportion of TB patients and HC regarding sex distribution inside the same groups. Brazilian ethnicity distribution is admixed and during the period of analysis a ratio of 2:1 (male: female) was observed in the outpatients Clinics, turning our cohort not matched by sex and ethnicity between groups. Enrolled volunteers were asked to define their own ethnic group as white (caucasian) and non-white (afro-descendents). No Indians or Asian backgrounds were identified in our cohort. Among TB patients, 136 (79.1%) individuals defined their ethnic group as white (52–38.2%) and non-white (84–61.8%), on the other hand, among HC subjects, 79 (49.4%) and 81of 160 (50.6%) were white and non-white, respectively. In the TB patients (n=172) group, the TST ranged from 0 to 35 (14.9±10.3) mm and from 0 to 57 (12.1±12.2) mm in the HC group. 60 (71%) of TB patients and 98 (63%) of HC were TST positive from total TST tests obtained (≥ 10 mm), confirming the high *M. tuberculosis* exposure in the endemic areas of Rio de Janeiro. The induration size after TST results was slight larger in males (n=34) (22.5±10.8 mm) than in females (n=51) (20.2±8.5 mm) in the HC group, even with elevated females number. The TST results considering ethnic characteristics from the same group was obtained in 130 individuals, 56 (45.4%) considered white and 74 (54.6 %) non-white, with mean indurations of 9.7±10.3 and 12.4±12.0 in white and non-white respectively. It was not found significance in TST reaction among ethnic groups and gender in either TB patients or HC groups (Table 1).

IFNG+874T/A distribution and association with tuberculosis

We evaluate the frequencies of *IFNG+874T/A* gene polymorphism (genotype and allele) in patients with active tuberculosis and in healthy volunteers. TB patients and HC group had very similar genotype distributions (p>0.05) (Table 2). The genotype frequencies was not significant in TB patients between the genotypes *A/A* and *A/T* and *T/T* (41.8, 45.3 and 12.8%, respectively), when compared with those in HC group (34.6, 50.8 and 14.5%, respectively). We next examined whether the genotype frequency could be a

relevant factor in determining *M. tuberculosis* latent infection. No statistical difference was observed between TB patients and HC whom were TST-positive (38.8, 49.0 and 12.2%, respectively). In addition, the genotypes *A/T* and *T/T* were 13.5 % more frequent in the HC than in TB patients group. The ability to respond to TST did not correlate with *IFNG+874T/A* genotypes in both TB and HC groups. There is no ethnicity interference in *IFNG+874T/A* genotypes between TB patients and HC groups ($p>0.05$).

No significant difference was observed in the allele frequency between TB and HC groups and in those HC whom TST-positive was identified. There was no association when univariate analysis was performed related to age, ethnicity and BCG vaccination status (Table 2).

NOS2-954G/C distribution and association with tuberculosis

We evaluate the frequencies of *NOS2-954G/C* gene polymorphism (genotype and allele) in patients with active tuberculosis and in HC group. The results of *NOS2A-954G/C* genotyping for the TB and HC groups were very similar and are presented in Table 2. The genotype frequencies was not significant ($p>0.05$) in TB patients between the genotypes *C/C* and *G/C* and *G/G* (0.6, 11.0 and 88.4%, respectively), when compared with those of HC group (0.6, 10.0 and 89.4%, respectively) and in those HC TST positive group (0, 13.3 and 85.7%, respectively) ($p>0.05$).

Allele *C* carries from *NOS2A-954C/G* polymorphism was very rare ($f=0.06$) and just one subject from each group was homozygote ($f=0.01$). When univariate analysis was performed age, gender, ethnicity and BCG vaccination no statistical difference was observed ($p>0.05$) (Table2).

Serum nitrite and nitrate levels and NOS2-954G/C polymorphism association

The Griess reaction was performed in 75 TB patients and 78 HC group, in according to calculations of proportionality, as described in the Table 3. The mean serum concentration of nitrite and total nitrate measurements were respectively in TB, (23.68 ± 15.74 μM and 32.61 ± 16.97 μM) and in HC group (23.99 ± 17.29 μM and 34.71 ± 19.39 μM), showing no statistical significance (*p*

= 0.90 and 0.47). We investigated whether serum levels of nitrite and nitrate could vary according to *NOS2A* genotypes in TB and HC groups. We were unable to demonstrate statistical association of nitrite and nitrate serum levels within *GG* and *GC* genotypes of *NOS2A*-954*G/C* between TB patients (*p* = 0.92 and 0.56) and HC (*p* = 0.60 and 0.67) (Figure 1), even in those TST-positive subgroup (p>0.05) (Table 3). These results demonstrated that the low *NOS-954G/G* or moderate *NOS-954G/C* nitric oxide producers were not associated with the modulation of nitrite and nitrate radical production in both TB and HC groups.

Association between extended genotype among IFNG+874T/A and NOS2A–954C/G polymorphism and tuberculosis

To determine whether the combination of these two genes was associated with susceptibility to TB, polymorphisms association was evaluated between pulmonary TB and HC groups. The rare *NOS2A-954C/C* genotype was not taken in account in the extended genotypes with *IFNG+874T/A* gene. No statistical significance was seen between *IFNG+874T/A* and *NOS2A-954G/C* genotypic combinations (Table 4) when TB cases and HC group was compared. In both groups, the most prevalent extended genotype was *ATGG* (44% and 44.9% in TB and HC groups, respectively), with no statistical difference (*p*=0.76). As, production of nitrogen radicals is directly dependent on IFN-γ, we analyzed the extended genotypes of *IFNG+874T/A* and *NOSA-954G/C*. The secretion of reactive nitrogen was not related with the extended genotypes (Table 5). These results demonstrated in our population, that the extended genotypes profiles of *IFNG+874T/A* (low *IFNG+874A/A*, moderate *A/T* or high *T/T* producers) and *NOS-954G/C* (low *NOS-954G/G* or moderate *G/C* producers) are not associated with the modulation of the production of nitrite and nitrate radicals in both TB and in HC or HC TST-positive groups.

Discussion

IFN-γ mediated immune activation has an important role in immunity to intracellular pathogens. IFN-γ is critical to macrophage activation and measurable levels are lower in patients with active TB than in controls subjects (Sánchez et al. 1994; Zhang et al. 1995), and are an unreliable correlation with protection (Lopez-Maderuelo et al. 2003). It has been more formally suggested that IFN-γ activity is a continuous, genetically controlled trait with genetic variability in both production, and responsiveness to IFN-γ (Flynn et al. 2001), although until recently there was little evidence to support a role for variability in IFN-γ production. For the *IFNG* gene, there are two intronic SNPs that contribute to its expression phenotypes*: +874T/A* and *+2109G/*A (Dupuis et a., 2000). The genotype *A/A* of *IFNG+874T/A* is thought to provide a low-secretor phenotype of IFN-γ*.* Conversely, the genotype *T/T* of *IFNG+874T/A* is thought to provide a high-secretor phenotype (Pravica et al. 1999; Henri et al. 2002). Some controversy exists concerning association of *IFNG* gene polymorphisms and susceptibility to pulmonary TB (Rossouw et al. 2003; Tso et al. 2005; Henao et al. 2006; Mirsaeidi et al. 2006; Hwang et al. 2007). Our results showed no evidence of an association between *IFNG* gene polymorphism and susceptibility to TB. The frequency genotype *A/A* of *IFNG+874T/A* in our control subjects (35.2%) was a little higher than in the Sicilian (26%) (Lio et al. 2002), Spanish (28%) (Lopez-Maderuelo et al. 2003) and Indian (11%) populations (Abhimanyu et al. 2011), but smaller than South African (47%) (Rossou et al. 2003), Hong Kong Chinese (46%) (Tso et al. 2005) and South Korea (74%) populations (Hwang et al. 2007). A search of provided reports on the *T* allele of *IFNG+874T/A* frequency revealed that there are ethnic differences in the allele distribution. It has been reported that the *T* allele frequency is significantly lower in a Japanese population (9%) (Sallakci et al. 2009) than in a South African population (32%) (Cooke et al. 2006) and an Italian Caucasian population (47%) (Poli et al. 2002). Rossouw et al (2002) reported a significantly lower allele *T of INFG+874T/A* frequency in patients with *M. tuberculosis* infection than in control subjects in a South Africa population with a high annual incidence of TB.

In Chinese population, Wang et al (2010) described that genotype *T/T* of *IFNG+874T/A* frequency (2%) was lower when compared with other genotypes in TB group. Similarly, Lio et al (2002) and Vallinoto et al (2010) found that T/T genotype was relatively rare in TB patients from Sicilian and Brazilian populations. However, a study conducted in a Brazilian population by Amim et al.(2007) showed different results from our study, where the genotype *A/A* of *IFNG+874T/A* was associated with TB susceptibility. Brazil was colonized by several ethnic groups that migrated from different countries. During the last 500 years these populations were mixed with native Indians and each Brazilian region has it own ethnical group descendents. Our patients were enrolled from Rio de Janeiro City that received migratory populations all over the country. Thus, the population in this city has a genetic background that represents a mix of all regions of the country, being unique and differentiated. This difference in genetic background inside the same continental country could explain the different results in gene frequency from our study.

There is convincing evidence that NO and related reactive nitrogen intermediate (RNI) can kill and/or inhibit intracellular pathogens such as mycobacteria (Kuo et al. 2000). IFN-γ knockout mice that are not capable of producing NO and RNI in response to the bacilli develop tuberculosis quickly, suggesting a role for NO and RNI in the defense mechanism against *M. tuberculosis* (Dalton et al. 1993). In contrast to murine models of TB, there is greater controversy about the role of NO in killing or limiting the growth of *M. tuberculosis* in humans. Nevertheless, several reports indicate that alveolar macrophages from TB patients produce increased amounts of NO compared to HC group (Nicholson et al. 1996; Kuo et al. 2000). Wang et al. (1998) demonstrated that increased NO exhalation in patients with TB was due to the up-regulation of NOS2 in alveolar macrophages. The expression of human *NOS2A* is inducible by cytokines, which has been documented in various types of cells, including respiratory epithelial cells, macrophages, hepatocytes, chondrocytes and retinocytes (Nathan et al. 1994a). The *NOS2A* gene is located in a region that has been linked to susceptibility to TB (Nathan et al. 1994b) and *NOS2* is encoded by this polymorphic gene at chromosome 17q11.2-q12. Several SNPs have been described in this gene (Jamieson et al. 2004), given the importance of this gene in the immune response to TB. Our

study verifies the influence of *NOS2A* polymorphisms on the risk of developing TB in a Brazilian population. We did not observe a significant difference between the *NOS2A-954G/C* genotype in our TB patients and HC subjects. The frequency of *NOS2A-954G/C p*olymorphisms may differ among populations. The C allele of *NOS2A-954G/C* has been shown to be absent from Caucasian populations (Kun et la., 1998) and in Peruvian population (Martín et al. 1999) and in low frequency in Asia (Kun et al., 2001). However the C allele has high frequency in African populations (Kun et al., 2001). A description of *NOS2A-954G/C* genotype study in Brazilian population, studying gastric cancer, showed different frequencies for *NOS2A-954G/C* polymorphism *G/G*, *G/C* and *C/C* (64.77, 28.69 and 6.54%, respectively, Jorge et al. 2010) compared with our study. In Mexicans, this SNP was not associated with TB. However, the frequency of C allele was 95% (Flores-Villanueva et al. 2005), contrary of our results and from others on Latin America (Martín et al. 1999, Jorge et al. 2010).

The involvement of nitric oxide with different populations has been studied in different pathologies and still has controversy results. In order to assess the nitric oxide radical levels and to compare with *NOS2A-954G/C* polymorphism and extended genotypes of *IFNG/NOS2A* in TB patients and HC group, the secretions of nitrite and nitrate were analyzed. No association was identified between cases and controls or between different genotype profiles or extended genotypes regarding nitrite and nitrate production. Our results indicated that even under iNOS/NOS2 and IFN-g pressure, *M tuberculosis* can avoid immune response surveillance and induce active TB, as showed by the absence of correlation with different SNP profile in our admixture population. The pulmonary milieu involving the immune response and bacilli interaction in different genetic background should be addressed to answer the question if *M tuberculosis* growth is controlled by several candidate genes.

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Table 1. Clinical data from Brazilian tuberculosis patients and healthy control subjects

		Tuberculosis Patients n=172 (%)	Healthy Controls n=179 (%)
Sex	M	105(61)	63 (35.2)
	F	67 (39)	116 (64.8)
Age (years)		36.9±12.7	35.1±11.5
Skin Color	white	136 (79.1)	79 (49.4)
	Non-white	84 (61.8)	81 (50.6)
BCG vaccine			
Yes		104(86)	149 (96)
No		16 (14)	6 (4)
TST	positive	60 (34.9)	98 (54.7)
	negative	24 (13.9)	57 (31.8)
	missing	88 (51.2)	24 (13.4)
	diameter (mm)	(14.9±10.3)	(12.1±12.2)

TST-tuberculin skin test

Table 2: Distribution of genotypes and allele frequencies of the +874T/A *IFNG* and *NOS2A* -954G/C SNPs among tuberculosis patients and healthy control subjects

	Tuberculosis Patients	Health Control			Health Control		
Genotype/Alleles	n=172(%)	n=179(%)	p^1	OR	TST positive n=98(%)	p^2	OR
AA	72 (41.8)	62 (34.6)	0.21	1.00	38 (38.8)	0.76	1.00
AT	78 (45.3)	91 (50.8)		0.74	48 (49.0)		0.86
TT	22 (12.8)	26 (14.5		0.73	12(12.2)		0.77
TT+ATvs AA	100 (58.1)	117 (65.3)	0.16	0.74 (0.47-1.16)	60 (61.2)	0.61	0.88 (0.51-1.51)
TTvsAA+AT	150 (87.2)	153 (85.4)	0.63	0.86 (0.45-1.66)	86 (87.8)	0.89	1.05 (0.47-2.38)
Alleles	n=344 (%)	n=358(%)			n=196(%)		
A	222 (64.5)	215 (60.1)	0.22	1.21	124 (63.3)	0.76	1.06
T	122 (35.5)	143 (39.9)		(0.88-1.66)	72 (36.7)		(0.72-1.55)
GG	152 (88.4)	160 (89.4)	0.77	1.00	84 (85.7)	0.78	1.00
GC	19 (11.0)	18 (10.0)		1.11	13 (13.3)		0.81
CC	1 (0.6)	1 (0.6)		1.05	0		NA
CCvsGC+GG	171 (99.4)	178 (99.4)	**0.97	1.04 (0.0-38.36)	97(100)	**0.45	-
GGvsCC+GC	20 (11.6)	19 (10.6)	0.76	0.90 (0.44-1.84)	13(13.3)	0.67	1.18 (0.52-2.63)
Alleles	n=344 (%)	n=358 (%)			n=196 (%)		
G	323 (93.9)	338 (94.4)	0.76	0.91	183 (93.4)	0.80	1.09
C	21 (6.1)	20 (5.6)		(0.46-1.66)	13(6.6)		(0.50-2.35)

p-value considered ≤ 0.05 (95% CI); OR, odds ratio

* **p*-value for *TT* and *AT* vesus *AA* or *TT* versus *AA* and *AT*. p^1-value from TB patients and whole HC group. p^2-value from Tb patients and HC TST positive group. TST positive, positive tuberculin skin test; **p*-value for GC and GG in respect to CC. p^1-value from TB patients and whole HC group. p^2-value from TB patients and HC TST positive group. TST positive, positive tuberculin skin test.

Table 3: Comparative analysis between polymorphisms *NOS2-954G/C* and serum nitrite and nitrate in patients with TB pulmonary and healthy controls

Genotypes	NO_2^+ (μM)	$NO_2^+ + NO_3^+$ (μM)
	p	*p*
Tuberculosis patients (intra group)		
GG vs GC	0,91	0,76
Healthy controls (intra group)		
GG vs GC	0,50	0,55
Tuberculosis patients vs Healthy controls		
GG vs GG	0,92	0,56
GC vs GC	0,60	0,67

p-value considered ≤ 0.05 (95% CI); NO_2^+-Nitrite; NO_3^+-Nitrate

Table 4: Genotype combination analysis of the *IFNG+874T/A* and *NOS2A-954 G/C* among TB patients and HC group (whole and TST positive).

Extended genotypes	Tuberculosis patients n =172 (%)	Healthy controls					
		n=179 (%)	ρ^1	OR	TST+ n=98(%)	ρ^2	OR
A-A/G-G	62 (36.0)	56 (31.3)	0.21	1.00	33 (33.7)	0.62	1.00
A-A/G-C	9 (5.2)	5 (2.8)		1.63	4 (4.1)		1.20
A-A/C-C	1(0.6)	1(0.6)		0.90	1(1.0)		0.53
A-T/G-G	70 (40.7)	80 (44.7)		0.79	39 (39.8)		0.96
A-T/G-C	8 (4.7)	11 (6.1)		0.66	9 (9.2)		0.47
T-T/G-G	20 (11.6)	24 (13.4)		0.75	12 (12.2)		0.89
T-T/G-C	2 (1.2)	2 (1.1)		0.90	0 (0)		N/A

p-value considered ≤ 0.05 (95% CI); p^1value comparing TB patients and Healthy controls. p^2value comparing TB patients and TST+ healthy controls; TST+, positive tuberculin skin test; OR, odds ratio; CI, confidence interval. The genotype combinations TT/GG and AT/GG were not present. AA/GG extended genotype was used as reference.

Table 5: Comparative analysis of the extended genotypes of IFN-γ +874T/A and NOS-954G/C and serum nitrite and NOx in patients with active pulmonary tuberculosis and healthy individuals

Extended genotypes *IFNG/NOS2*	Tuberculosis patients vs Healthy controls NO^-_2 (μM)	Tuberculosis patients vs Healthy controls $NO^-_2 + NO^-_3$ (μM)	*p*
A-A/G-G	0,37	0,97	>0.05
A-A/G-C	0,55	0,58	
A-T/G-G	0,84	0,60	
A-T/G-C	0,85	0,92	
T-T/G-G	0,22	0,31	
T-T/G-C	-	-	

p-value considered ≤ 0.05 (95% CI); NO^-_2 Nitrite; NO^-_3 Nitrate

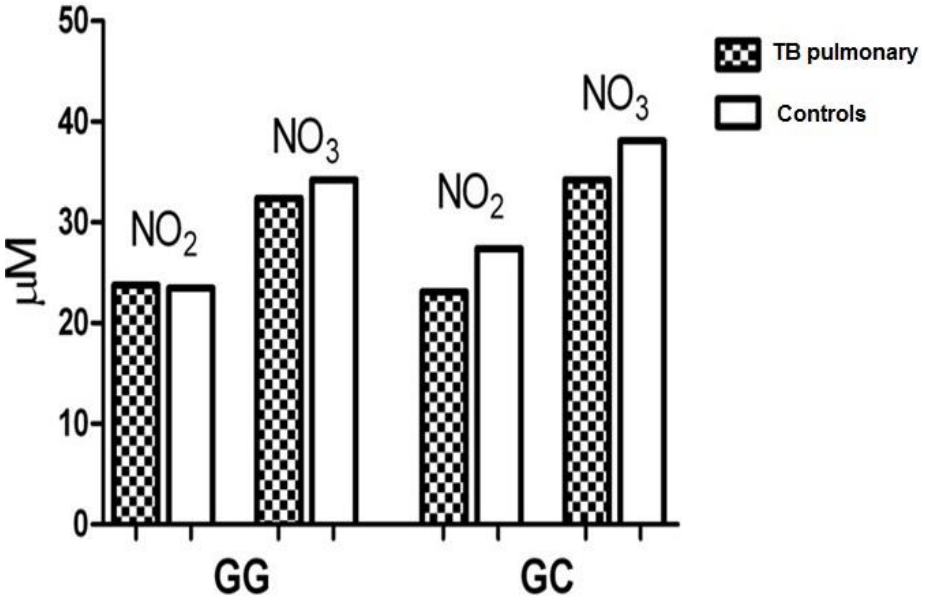

Figure 1: Comparative analysis of serum nitrite and nitrate (NO^-_2+ NO^-_3) concentration and *NOS2A*-954G/C genotypes association in tuberculosis patients and healthy control subjects (p> 0.05).

4.4.2 **Conclusões referentes ao artigo a ser submetido ao *International Journal of Imunogenetic* (2012)**

1- As variantes dos genes *IFNG+874T/A* e *NOS-954G/C* não foram associadas a susceptibilidade à infecção latente e a TB ativa. Apesar, destes polimorfismos serem descritos como alto, médio e baixo produtores desta citocina e proteína, as quantidades produzidas por nossos voluntários poderiam não ser o suficiente para influenciar na resistência à TB.

2- As análises funcionais demonstraram que os polimorfismos dos genes de *IFNG+874T/A* e *NOS-954G/C* e a resposta imune contra o *Mt,* avaliada através da produção e secreção de nitrito e NOx total, não foi influenciada pelos perfis genotípicos.

5 DISCUSSÃO

A VD desempenha um importante papel imunomodulatório na reposta imune contra o *Mtb*, assim pode-se concluir que níveis séricos adequados desta vitamina atuem como um fator de proteção contra o desenvolvimento da TB e contribui para um melhor prognóstico. Dessa forma, podemos assumir que SNPs no receptor de VD (*VDR)*, bem como, na proteína carreadora de VD (*DBP*), *NO2* e seu modulador *IFNG* podem ser importantes fatores para elucidar e compreender como estes diferentes perfis genotípicos podem afetar os mecanismos biológicos envolvidos no adoecimento ou na resistência na TB e de como a VD sérica é capaz de regular todos esses mecanismos.

Diferentes mecanismos de proteção contra TB têm sido descrito envolvendo a VD e TLR. O papel central dos receptores TLR na TB é o reconhecimento do *Mtb* e conseqüente ativação de sistemas de defesa contra este patógeno (Takeda et al. 2003). Depois do reconhecimento estes receptores podem iniciar vias de sinalização que ativam mecanismos da reposta imune inata, produção de citocinas e da imunidade adaptativa. A ativação de TLR estimula macrófagos a expressarem citocinas proinflamatórias (TNFα, IL-6, IL-12 e IFN-γ,) e genes antimicobacterianos como *NOS2* que leva a produção final de NO (Takeda et al. 2003). Além disso, estes receptores participam do aumento da expressão do VDR e da enzima 1-α hidroxilase que transforma a VD na sua forma inativa para ativa. A $1,25(OH)_2D$ é carreada pela proteína VDBP e polimorfismos nesta proteína estão relacionados com a disponibilidade e quantidades de VD presente no soro (Lauridsen et al. 2005) que em consequencia podem influenciar os eventos relacionados à resposta contra o *Mtb*. A forma ativa da VD ligada à proteína VDBP é reconhecida pelo VDR e internalizada pela célula, este mecanismo leva a indução do potente peptídeo antimicobacteriano catelicidina capaz de inibir o crescimento intracelular do *Mtb in vitro* (Liu et al. 2006). A $1,25(OH)_2D$, pode modular a reposta imune induzindo o aumento de resposta protetora pela indução da expressão do gene *NOS* (Rockett 1998) e da catelicidina (Martineau et al. 2007a), por outro lado, é capaz de induzir a diminuição da expressão do gene de *IFNG* (Cippitelli et al. 1998). Outro dado importante é que a $1,25(OH)_2D$ suprime a resposta tipo Th1, favorecendo o perfil Th2, modulando a resposta

do hospedeiro ao *Mtb*. De maneira diferente, a ativação de TLR em macrófagos e DCs levam a secreção de IL-12 resultando na resposta de células T do tipo Th1. As células Th1 secretam IFN-γ, que desempenha múltiplas funções como: ativar macrófagos, intensificar o poder de fagocitose e habilitadade microbicida (Adams et al. 2007). Esta citocina secretada por células T dentro do granuloma potencializa a expressão de enzima 1-α hidroxilase e subseqüente aumento dos níveis de VD ativa neste microambiente (Martineau et al. 2007a). Assim, é possível sugerir uma ligação entre níveis séricos de VD e de seu carreador (VDBP), a expressão de seu receptor (VDR), TLR e a produção de IFN-γ e NO. Dessa forma, podemos concluir que possíveis defeitos ou mutações nessas moléculas determinadas por diferentes polimorfismos poderiam estar associadas com susceptibilidade à TB (Ben-Ali et al. 2004 ; Ogus et al. 2004; Yim et al. 2006).

O estudo da genômica humana tem aumentado nosso entendimento nas diferenças interindividuais e interpopulacionais na susceptibilidade para a TB. Os genomas tanto do homem quanto do *Mtb* tem sido mapeados (Cole e cols., 1998) e que provavelmente devem auxiliar a identificar importantes redes imunológicas, moleculares e bioquímicas envolvidas na susceptibilidade e resistência para a TB e auxiliar no desenvolvimento de novas vacinas, métodos diagnósticos bem como terapias e agentes imunoterápicos.

Desde que somente 10% da população que se torna infectada pelo Mtb desenvolverá a doença clínica, acredita-se que fatores genéticos, bem como, fatores ambientais estejam intimamente envolvidos (Bellamy, 2003). Desta forma, determinar o perfil genotípico individual e conseqüentemente populacional seria de grande importância para o entendimento da TB e forneceria bases para compreender a relação parasita-hospedeiro quanto à imunopatogênese da TB. As interações do sistema imune e a alta complexidade da estrutura molecular do *Mtb* são multifuncionais. Assim, várias etapas da resposta imune contra o *Mtb* poderiam estar envolvidas na defesa contra o mesmo e que consequentemente, são controladas geneticamente (Davies & Grange, 2001). Dessa forma, quaisquer alterações na expressão dos fatores envolvidos no sistema imune contra o *Mtb* poderiam ocasionar um desequilíbrio levando ao hospedeiro a apresentar susceptibilidade ou resistência a esta doença.

O presente estudo teve por objetivo caracterizar diversos genes candidatos à resistência ou susceptibilidade para a TB, a partir do estudo de polimorfismos genéticos em genes intimamente ligados à imunopatogênese da TB, com vistas a buscar uma possível associação entre esses possíveis genes e a resistência ou susceptibilidade para TB, doença que vem acomentendo milhões de pessoas em todo mundo e que possui sua imunopatogenia pouco compreendida no hospedeiro, especificamente na população brasileira miscigenada. Dentre esses possíveis genes candidatos e que foram realizados neste estudo podemos descrever o *VDBP (HaeIII posição 416 e* StyI *posição 420, VDR (TaqI, FokI, BsmI* e *ApaI)*, *IFNG+874T/A,* e *NOS2A-954G/C*.

A clássica função de VDBP é estocar e prolongar a meia-vida dos metabólitos de vitamina D na circulação (White et al. 2000). Dessa forma, nosso grupo decidiu analisar a correlação entre as variantes do gene *DBP* ou *Gc-globulin* e TB. Não foi encontrada associação entre os carreadores dos genótipos e alelos de *DBP* entre pacientes com TB e indivíduos do grupo HC. Contudo, foi possível observar que o grupo *Gc1-1* foi o mais prevalente entre pacientes não brancos (73.9%), indicando que pacientes negros/pardos carreadores do grupo *Gc1-1* podem apresentar uma predisposição para o adoecimento. Em estudo recente, Chun et al. (2010), descreveram que o grupo *Gc1-1* induz de 2,4 a 2,5 vezes menos catelicidina do que os outros grupos (*Gc1-2* e *Gc2-2*, respectivamente), sugerindo que a resposta ao *Mtb* poderia ser menos eficiente. Martineau et al. (2010), demonstraram associação do genótipo *Gc2-2* com susceptibilidade à TB em população asiática, apenas quando apresentavam deficiência nos níveis séricos de VD, porém o mesmo não foi confirmando em população brasileira, indicando que a deficiencia de VD é um fator de risco mais importante do que o genótipo do indivíduo. Estes resultados divergem dos nossos e podem ser explicados pela diversidade do perfil genotípico dos voluntários incluídos no nosso estudo ou pela ingesta adequada de vitamina D e extrema exposição ao sol, especialmente no Rio de Janeiro, onde as pessoas praticam inúmeras atividades fora de casa em muitos dias da semana. De forma contrária, outro estudo em população de São Paulo, observou que no final do inverno, houve uma elevada prevalência de hipovitaminose D, em indivíduos saudáveis. Depois do verão, foi observada uma diminuição na prevalência de hipovitaminose D (Unger et al. 2010). A

diferença entre os nossos resultados pode estar relacionada com diferentes hábitos culturais, estilo de vida, poluição do ar e exposição ao sol.

Vários estudos têm descrito associação entre os níveis de vitamina D e os polimorfismos de VDR com doenças. O papel dos polimorfismos de *VDR* na susceptibilidade ou resistência a TB ainda permanece controverso. Por isso, consideramos apropriado analisar os polimorfismos de VDR com o objetivo de ter uma melhor compreensão destes marcadores genéticos como fatores de risco para o desenvolvimento da TB. No presente estudo não foi encontrada associação entre os genótipos, alelos e haplótipos de VDR com a TB. Contudo, o alelo *F* de *FokI* (OR= 3.2,) demonstrou ser um fator de proteção em indivíduos negros do grupo HC. Esse fato pode ser explicado, uma vez que, os polimorfismos de *FokI* têm sido associados com mudanças no número de aminoácidos presentes nas isoformas de VDR em humanos. O alelo *F* codifica uma proteína curta mais hábil em se associar ao fator de transcrição TFIIB (Jurutka et al. 2000), assim pode-se entender que portadores deste alelo apresentam maior expressão de VD e maior disponibilidade de VD celular. Essa maior expressão acarreta o aumento da produção de NO, catelicidina e maior expressão de VDR, que são importantes mecanismos de defesa contra o *Mtb*. Wilbur et al. (2007), descreveram um papel protetor dos genótipos *TT e FF* relacionado a tuberculose ativa e a infecção. Na população brasileira a distribuição das freqüências dos genótipos e alelos de 3 polimorfismos de *VDR*, não demonstraram diferença entre brancos e negros, incluindo os alelos *F* e *f* de *Fok* (Rezende et al. 2007). Interessantemente, populações distintas podem apresentar diferentes freqüências para os polimorfismos de *FokI,* como na população indiana, onde o genótipo *FF* foi associado com TB espinhal (Selvaraj et al. 2004), em chineses o genótipos *ff* e *FF* foram associados com TB espinhal (Zhang et al. 2010) e em negros norte americanos com TB extra pulmonar (Motsinger-Reif et al. 2010).

Ao comparar diferentes estudos realizados em população brasileira é possível identificar que o genótipo *BB* (10.2% a 20.9%) é menos freqüente que *bb* (38.5% a 41.5%) (Lazaretti-Castro., 1997; Hauache et al 1998; Mory et al. 2009), estas freqüências estão de acordo com os nossos resultados. Como descrito previamente em população da Índia (Selvaraj et al. 2004; Sharma et al. 2011) e da Turquia (Ates et al. 2011) o genótipo *bb* de *BsmI* também é pouco

representado em nossos pacientes, conferindo um papel protetor na TB ativa. Por outro lado, o alelo *b* confere risco para o desenvolvimento de TB em população iraniana (Merza et al. 2009). Nossos resultados confirmam que o genótipo *bb* está associado com a resistência à TB e que diferentemente, o genótipo *Bb* e o alelo *B* estão associados com suscetibilidade à TB. Em trabalho publicado por Selvaraj et al (2009), foi demonstrado em controles saudáveis, que os polimorfismos de *VDR in vitro* influenciam os níveis de expressão da proteína VDR, que é diminuída em carreadores do genótipo *BB*, sugerindo que este polimorfismo pode regular a expressão desta proteína. Assim, portadores de genótipo *bb* expressariam mais o VDR, o que possivelmente determinaria uma melhor resposta de células do sistema imune moduladas pela VD.

Em nosso estudo pacientes com TB apresentaram a média de 25(OH)D sérica levemente aumentada quando comparada aos controles, porém sem associação. Da mesma forma, em população indiana os níveis de 1,25(OH)2D3 foram aumentados em pacientes com TB, enquanto que *in vitro* os níveis da proteína VDR em cultura de PBMC foram significantemente diminuídos em pacientes com TB, sugerindo que o excesso de VD no microambiente podem induzir o *feedback* negativo diminuindo a expressão desses receptores (Selvaraj et al. 2009) e a disponibilidade de VD para célula. Outra explicação seria que níveis séricos de VD normais em pacientes com TB, não corresponderiam a uma menor produção de VD por células do sistema imune no sítio da infecção, justificando assim o adoecimento. Como pacientes e controles estão dentro da faixa de normalidade para VD, podemos concluir que níveis séricos desta vitamina não poderiam ser considerados sozinhos como fator de risco para o desenvolvimento da TB. Assim fica claro que existe interação entre proteínas e genes.

Estudos prévios descrevem a associação entre a cor da pele e a produção de vitamina D, mostrando que níveis de 25(OH)D no soro de afro americanos foram menores do que em caucasianos, devido ao aumento da produção de melanina na pele, diminuindo a produção de vitamina D e conseqüentemente de catelicidina ou LL-37, um potente peptídeo antimicrobicida que pode destruir o *Mtb in vitro* (Liu et al. 2006). Nossos pacientes e indivíduos do grupo HC não mostraram diferença nos níveis

séricos de 25(OH)D, em relação à cor da pele, assim define que em nossa casuística, pacientes negros e pardos estão recebendo quantidades adequadas de VD através da ingesta ou da luz UV. O Rio de Janeiro tem cerca de 200 dias de sol por ano e os voluntários do nosso estudo relataram constante exposição à luz solar. Estes podem ser fatores, além da qualidade da ingesta em nossa coorte, que podem estar relacionados às diferenças entre o nossos resultados e os relatados por Liu et al. (2006).

A citocina IFN-γ desempenha um importante papel na imunidade contra patógenos intracelulares. O IFN-γ atua na ativação de macrófagos e níveis desta citocina são menores em pacientes com TB ativa do que em indivíduos sadios (Sánchez et al. 1994; Zhang et al. 1995) e tem sido relacionado com a proteção (Lopez-Maderuelo et al. 2003). Nossos resultados não demonstraram evidências da associação entre os polimorfismos no gene *IFNG* e susceptibilidade à TB. A freqüência do genótipo *A/A* de *IFNG+874T/A* nos indivíduos controles (35.2%) foi maior do que em população siciliana (26%) (Lio et al. 2002), espanhola (28%) (Lopez-Maderuelo et al. 2003) e indiana (11%) (Abhimanyu et al. 2011) mas, menor do que nas populações sul africana (47%) (Rossou et al. 2003), chinesa (46%) (Tso et al. 2005) e sul coreana (74%) (Hwang et al. 2007). Trabalhos descrevem uma diminuição significante da freqüência do alelo *T* em população japonesa (9%) (Sallakci et al. 2009), do que em africana (17%) (Cooke et al. 2006) e em caucasianos da Itália (47%) (Poli et al. 2002)). Rossouw et al. (2002), descreveram uma diminuição significante da freqüência do alelo *T de IFNG+874T/A* em pacientes com TB na população sul africana com aumento anual da incidência de TB. Em chineses, He et al. (2010), descreveram que a freqüência do genótipo *T/T de IFNG+874T/A* foi de (2%) quando comparada com outros genótipos em pacientes com TB. Lio et al. (2002), descreveu que o genótipo *TT* foi relativamente raro em pacientes com TB na Sicilia. Vallinoto et al. (2010), descreveu uma baixa freqüência deste genótipo em pacientes com TB no sul do Brasil. Outro estudo realizado em população brasileira por Amim et al. (2007), mostrou diferentes resultados em relação ao nosso estudo, onde o genótipo *AA* de *IFNG+874T/A* foi associado com o desenvolvimento da TB. Nosso estudo foi realizado com voluntários provenientes do Rio de Janeiro, um dos maiores centros populacionais do Brasil, cuja população tem sido

miscigenada durante anos devido a imigração. A falta de associação observada em nosso estudo pode ser explicada pela heterogeneidade genética ou pela estratificação da população dentro de cada etnia em nosso estudo em comparação com diferentes populações avaliadas em outros estudos, que de fato conseguem refletir a diferença das freqüências entre os alelos. Assim, é possível que a prevalência de TB seja diferenciada por etnias que apresentam diferentes *back ground* genético que podem afetar a resposta imune na TB, uma vez que, a genômica funcional do indivíduo está diretamente relacionada a modulação do resposta imune efetora na TB. Desta forma, diferenças éticas podem ser consideradas como um fator determinante na distribuição das freqüências dos polimorfismos do gene *IFNG* na prevalência da TB. Assim, é importante entender a atividade funcional deste polimorfismo *in vitro* bem como entender porque os polimorfismos dessa citocina, que causariam diminuição na produção de IFN-γ, não estão associados a susceptibilidade à TB em nossa população, como em outras, uma vez que esta citocina é considerada chave em articular a resposta imune no combate ao *Mtb*.

Outro mecanismo importante na fagocitose da micobactéria e sua conseqüente eliminação é através dos radicais intermediários de nitrogênio ou mais precisamente através do NO. Esta substância é um radical livre que está intimamente ligado ao desenvolvimento da TB, uma vez que este possui ação bactericida no interior dos fagossomas dos macrófagos. Nicholson et al. (1996), observaram que macrófagos alveolares de pacientes com TB pulmonar expressam a NOS2 em maiores proporções que em indivíduos saudáveis (Nicholson e cols., 1996). Essas observações mostram o importante papel desta substância na resistência contra o *Mtb* e consequentemente para o desenvolvimento da TB (MacMicking e col., 1997).

Inúmeras evidências relacionam a produção de NO e radicais intermediários de nitrogênio (RNI) com a morte ou inibição de patógenos intracelulares (Kuo et al. 2000). Estudos indicam que macrófagos alveolares de pacientes com TB têm níveis de NOS2 aumentados em comparação com grupo controle (Nicholson et al. 1996; Kuo et al. 2000). A expressão de NOS2 humana é induzida por citocinas, principalmente o IFN-γ e tem sido descrita em diferentes grupos celulares, incluindo células epiteliais, macrófagos, hepatócitos, condrócitos e retinócitos (Nathan et al. 1994; Wang et al. 1998).

Devido à importância deste gene na resposta imune durante a TB, o objetivo do nosso estudo foi verificar a influencia do polimorfismo de *NOS2A* e o risco de desenvolvimento de TB em população brasileira. Não observamos associação entre os diferentes perfis genotípicos de *NOS2A-954G/C* nos grupos estudados, indicando que os diferentes perfis genotípicos encontrados em nosso estudo não estão relacionados á susceptibilidade à TB. Este fato pode ser explicado pela distribuição aproximada das freqüências destes polimorfismos entre pacientes e controles, não demonstrando significância. A freqüência do alelo *C* de *NOS2A-954G/C* não foi significantemente diferente entre pacientes com TB e indivíduos do grupo HC ou naqueles com TB latente. As freqüências dos polimorfismos de *NOS2A-954G/C* podem ser diferentes entre as diversas populações, como demonstrados nos polimorfismos de *IFNG+874T/A*. O alelo *C* do polimorfismo de *NOS2A-954G/C* é ausente em população caucasiana (Kun et la., 1998) e na população do Peru (Martín et al. 1999). Outro estudo em câncer gástrico na população Brasileira mostrou diferentes freqüências para os polimorfismos de *NOS2A-954G/C, GG* (64.77%), *GC* (28,69%) e *CC* (6,54%) (Jorge et al. 2010). Em população do México, este *SNP* não foi associado com TB, uma vez que o a freqüência do alelo *C* foi de aproximadamente 5% (Flores-Villanueva et al. 2005) . Estes resultados estão em acordo com os descritos neste trabalho, onde o alelo *C* de *NOS2A-954G/C*, estava presente em aproximadamente 0,6% do grupo de HC. Foi descrito por Liu et al. (2006), que em macrófagos e células epiteliais, o NO possui efeito microbicida contra o *Mtb* (Liu et al. 2006). Kwon et al. (1997), observaram *in vitro* a ocorrência de maior produção de NO em PBMC infectados com H37Ra quando comparado a infecção pelo H37Rv, indicando que a virulência da cepa poderá determinar a produção adequada de NO, contudo não observaram diferença entre pacientes com TB e controles.

Na TB níveis séricos de nitrito e nitrito total (NOx) foram estimados em casos e controles e relacionados às diferentes combinações de genótipos, alelos e genótipos estendidos de *IFNG/NOS2*, uma vez que a citocina IFN-γ, leva ao aumento da expressão do gene *NOS2A*, resultando no aumento da produção de NO (Bonecini-Almeida et al.1998 a e b). Em nosso estudo não observamos diferença estatística entre casos e controles em relação aos níveis séricos deste radical, sugerindo que pacientes com TB não foram capazes de

produzir quantidades elevadas de NO e assim serem adadequadas, a fim de montar uma resposta imune efetora contra o *Mtb.* E que apesar dos genótipos encontrados serem descritos como médio e baixo produtores desta proteína as quantidades produzidas por nossos voluntários poderiam não ser o suficiente para influenciar na resistência ou susceptibilidade à TB. Fiorenza et al. (2005) e Ratnikov et al. (2003), observaram que soro de pacientes com TB apresentavam menores níveis de NO quando comparados a controles saudáveis em população da Argentina e da Rússia, respectivamente.

Sabe-se que o controle genético da resposta imune na TB pode ser multigênico, assim, análises dos genótipos estendidos para *NOS2A-954G/C* e *IFNG+874T/A,* foram realizadas. Seis combinações genotípicas são possíveis e não foi encontrada significância estatística entre pacientes com TB e indivíduos dos grupos HC, bem como naqueles com PPD positivo. Da mesma forma, as análises dos níveis de nitrito e nitrito total no soro não apresentaram associação com os genótipos estendidos entre todos os grupos estudados. Possivelmente, o tamanho amostral não é adequado para demonstrar essas associações e um estudo com um número maior de voluntários incluídos seja mais informativo.

Assim, apesar de não haver associação no nosso estudo entre os genótipos e genótipos estendidos de *NOS2A-954G/C* e *IFNG+874T/A,* novos estudos deverão ser realizados com o objetivo de entender como as variantes destes genes sozinhos ou associados, podem estar envolvidos na patogênese da TB. Os resultados do nosso estudo sugerem a importância de considerar os níveis de vitamina D no soro de pacientes com TB e sua possível relação com os genótipos, alelos e haplótipos de *VDR* e de *DBP* em grandes estudos populacionais, incluindo não brancos, a fim de confirmar os efeitos combinados no sistema imune. Todos esses fatores estão envolvidos nos diferentes mecanismos de proteção e susceptibilidade na TB.

6 CONCLUSÕES

6.1CONCLUSÕES GERAIS

Na TB o maior desafio é entender como as variantes dos genes, podem influenciar na modulação da resposta imune do hospedeiro contra o *Mtb*. Como a TB é uma doença multifatorial, fatores epigenéticos devem também ser levados em consideração. Dessa forma, podemos concluir que em população miscigenada apenas estudos genéticos não são o suficiente para elucidar os mecanismos de desenvolvimento de TB.

6.2 CONCLUSÕES REFERENTES AO ESTUDO DO POLIMORFISMO DE *DBP* OU *GC-GLOBULIN*

O fato do genótipo *Gc2-2* estar ligado à susceptibilidade à TB apenas nos pacientes com deficiência de VD sugere a importância de concentração de VD sérica dentro da normalidade, uma vez que em indivíduos portadores deste genótipo com suficiência desta vitamina não foi encontrada associação com risco de adoecimento. Pode-se concluir que, apesar deste genótipo estar ligado ao risco de adoecimento a deficiência de VD nesta população representa maior fator de risco para a TB.

6.3 CONCLUSÕES REFERENTES AO ESTUDO DOS POLIMORFISMOS DE *VDR* E *DBP* OU *GC-GLOBULIN* E NÍVEIS DE VD

1- O alelo *F* de *FokI* foi associado a proteção contra a TB em negros, esse fato poderia estar relacionado ao aumento da capacidade de ligação ao fator de transcrição-TFIIB, acarretado em melhora na transcrição de genes alvos, que desempenham função moduladora do sistema imune, melhorando assim a respota contra o *Mtb*;
2- O genótipo *bb* e o alelo *b* de VDR mostraram estar associados com a resistência à TB em controles e controles não brancos com níveis de vitamina D dentro da faixa de normalidade, sugerindo o envolvimento deste polimorfismo na expressão do receptor de VD. Portadores deste

genótipo expressam mais proteína, indicando um aumento da expressão do VDR e maior disponibilidade de VD para as células alvo e conseqüentemente em melhor acesso das células a esta vitamina, possibilitando um melhor desempenho do sistema imune na TB.

3- O haplótipo de VDR *ftBA* foi associado com susceptibilidade à TB, apenas em indivíduos com níveis séricos normais de 25(OH)D, sugerindo que em carreadore desta combinção alélica a VD não está influenciando os mecanismos da reposta imune contra o *Mtb* e que este haplótipo poderia estar relacionado com a diminuição da expressão ou funcionalidade deste receptor.
4- A combinação entre o grupo *Gc1-1* de *DBP* e *bb* de *VDR* foi associado com a proteção a TB. Porém outras combinações das variantes de *GC-globulin* e de *VDR* não demonstraram associação. Estes resultados sugerem que, apesar do grupo *Gc1-1* ser baixo produtor de catelicidina, esta produção pode ser suficiente. Além disso, o genótipo *bb* induz maior expressão da proteína VDR podendo ser um mecanismo de compensação, conferindo proteção, em indivíduos com 25(OH)D dentro da faixa de normalidade.
5- A falta de associação entre os polimorfismos de *DBP* e *VDR* com os níveis séricos de 25(OH)D sugerem que estes polimorfismos e a vitamina D sérica não representam fatores de risco no desenvolvimento da TB, uma vez que no nosso estudo os pacientes apresentavam níveis séricos de VD dentro da faixa de normalidade.
6- Pacientes e controles apresentaram níveis séricos de VD dentro da faixa de normalidade independente da cor da pele, sugerindo que a ingesta da vitamina e a exposição solar destes indivíduos é o suficiente para produzir VD de forma adequada.

6.4 CONCLUSÕES REFERENTES AO ESTUDO DOS POLIMORFISMOS DE *IFNG* E *NOS2A*

Os polimorfismos dos genes de *IFNG +874T/A* e *NOS2A-954G/C* não foram capazes de influenciar na produção e secreção de nitrito e NOx total na resposta contra o *Mtb,* sugerindo que o perfil genotípico não confere proteção

ou representa um fator de risco. Apesar de, estes polimorfismos serem descritos como alto, médio e baixo produtores desta proteína e citocina, as quantidades produzidas por nossos voluntários poderiam não ser o suficiente para influenciar na resistência/susceptibilidade ou mesmo como proteção na progressão de infecção latente para TB ativa.

7 PERSPECTIVAS

- Realizar análises funcionais quanto à produção de *IFN-*γ e sua associação com os genótipos, com o objetivo de responder como esses SNPs estão associados à resistência/susceptibilidade à TB através da alta, média e baixa produção desta citocina;

- Evidenciar em estudos in vitro se a suplementação com VD é capaz de modular a resposta imunológica frente a infecção pelo Mtb, no intuito de identificar a dosagem fisiológica adequada desta vitamina que acarrete no controle ou destruição do bacilo;

-Quantificar as proteínas – VDR, VDBP e TLR *e* associar com as diferentes variantes genotípicas, a fim de compreender como os genótipos poderiam influenciar nesta expressão;

-Descrever as freqüências dos polimorfismos de TLR2 e 4 e associar com as análise funcionais de catelicidina e níveis séricos de VD, com o intuito de entender como os diferentes genótipos podem afetar a produção deste potente peptídeo e importante imunomodulador.

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