UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

ATIVIDADE DE ENZIMAS QUE DEGRADAM NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM PLAQUETAS DE PACIENTES COM TIREOIDITE DE HASHIMOTO EM TRATAMENTO COM LEVOTIROXINA SÓDICA

DISSERTAÇÃO DE MESTRADO

Cristiano Bicca Noal

Santa Maria, RS, Brasil

2012

ATIVIDADE DE ENZIMAS QUE DEGRADAM NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM PLAQUETAS DE PACIENTES COM TIREOIDITE DE HASHIMOTO EM TRATAMENTO COM LEVOTIROXINA SÓDICA

Cristiano Bicca Noal

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Ciências Farmacêuticas**.

Orientadora: Prof. (Dra) Daniela Bitencourt Rosa Leal

Santa Maria, RS, Brasil

2012

Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Ciências Farmacêuticas

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

ATIVIDADE DE ENZIMAS QUE DEGRADAM NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM PLAQUETAS DE PACIENTES COM TIREOIDITE DE HASHIMOTO EM TRATAMENTO COM LEVOTIROXINA SÓDICA

elaborada por
Cristiano Bicca Noal

como requisito parcial para obtenção do grau de
Mestre em Ciências Farmacêuticas

COMISSÃO EXAMINADORA:

Dra. Daniela Bitencourt Rosa Leal
(Presidente/Orientador)

Dr. Carlos Eduardo Blanco Linares (URI)

Dra. Maria Rosa Chitolina Schetinger (UFSM)

Santa Maria, 18 de Julho de 2012.

Dedico este trabalho...

... À minha amada esposa Kelly, por estar sempre comigo e por me fazer feliz todos os dias.

...Ao meu filho Carlos Eduardo, por me ensinar a superar os obstáculos que a vida nos impõe.

...Aos meus avós Ilo e Altair, que comemoraram comigo o início deste projeto, mas que infelizmente não puderam vê-lo concluído.

... Aos meus pais Rudi e Rosemary, ao meu irmão Luciano e à minha cunhada Juliana pelo apoio e incentivo.

...Sem eles nada disso seria possível.

"Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida, que o mais importante é o decidir."

(Cora Coralina)

AGRADECIMENTOS

Agradeço a Deus por tudo que conquistei até aqui.

Aos meus “anjos da guarda” Tias Vera e Rosângela pela presença constante em minha vida, pelo apoio e por tornarem essa conquista possível de ser realizada.

Aos meus sogros Plínio e Heloisa, que mesmo de longe sempre me apoiaram e incentivaram.

À minha orientadora Dra. Daniela Bittencourt, pela sua dedicação à docência e à pesquisa, por ter me acolhido em seu grupo e principalmente pelos ensinamentos durante este período de convivência.

Aos amigos e colegas do laboratório 4229, João, Jeandre, Jader, Josiane, Carine, Karine Silveira, Karine Schlemmer, Maria Luiza, Bruna, Francine e Pedro, pelo coleguismo, incentivo e pela colaboração para a realização deste trabalho.

Agradeço em especial às colegas Viviane, Lívia e Tatiana pelo apoio, paciência, parceria e incentivo.

Aos colegas do laboratório 2208, especialmente à Daniela e Andréia, pela contribuição a este trabalho.

À Dra. Sílvia Londero, pelos conselhos, ensinamentos e auxílio prestado no recrutamento dos pacientes.

A todos os professores e funcionários do Departamento de Microbiologia e Parasitologia e do Programa de Pós-Graduação em Ciências Farmacêuticas da UFSM, pelo suporte prestado.

A todos os pacientes que participaram deste estudo.

À Jocelaine, pela sua atenção especial e dedicação à farmácia e pelas palavras de incentivo, disposição e compreensão.

À banca examinadora, por aceitarem o convite e pelas contribuições que farão a este trabalho.

A todos que participaram deste trabalho de alguma forma, meu muito obrigado.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

ATIVIDADE DE ENZIMAS QUE DEGRADAM NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM PLAQUETAS DE PACIENTES COM TIREOIDITE DE HASHIMOTO EM TRATAMENTO COM LEVOTIROXINA SÓDICA

Autor: Cristiano Bicca Noal
Orientadora: Prof. (Dra) Daniela Bitencourt Rosa Leal
Data e local de Defesa: Santa Maria, 18 de Julho de 2012.

A Tireoidite de Hashimoto (TH) ou tireoidite linfocítica crônica é uma doença autoimune que causa a destruição da glândula tireóide por meio de infiltrados inflamatórios e consequente perda da função. Esta patologia encontra-se difundida mundialmente e acomete prevalentemente o sexo feminino. As consequências das alterações de sua atividade vão desde cretinismo à alterações vasculares. Sabe-se que as enzimas ectonucleosídeo trifosfato difosfo-hidrolase (E-NTPDase; E.C. 3.6.1.5; CD39), ecto-5'nucleotidase (E.C.3.1.3.5; CD73) e adenosina desaminase (ADA; E.C.3.5.4.4) participam tanto da regulação da resposta imune quanto de eventos trombóticos, uma vez que regulam os níveis extracelulares dos nucleotídeos e nucleosídeo da adenina. Visto que, pacientes portadores da TH possuem uma resposta autoimune e também apresentam alterações microvasculares, o objetivo deste estudo foi avaliar a influência da sinalização purinérgica na regulação das disfunções microvasculares desencadeadas pela doença, através da determinação da atividade de ectoenzimas envolvidas no metabolismo do ATP em plaquetas de pacientes com TH em tratamento com levotiroxina. Foram coletadas amostras de pacientes com TH em tratamento com levotiroxina e um grupo controle. Neste estudo determinamos a atividade das enzimas E-NTPDase, ecto-5'-nucleotidase e E-ADA, verificamos a expressão da enzima E-NTPDase em plaquetas e dosamos os níveis hormonais de TSH e fT_4 em pacientes com TH em tratamento com levotiroxina, bem como do grupo controle. Os resultados obtidos na atividade das enzimas e na concentração dos nucleotídeos e nucleosídeo da adenina, não demonstraram alterações significativas quando comparamos os pacientes com TH em reposição hormonal com levotiroxina, com o grupo controle. Em conclusão, sugere-se que a levotiroxina usada em pacientes com TH pode reverter os efeitos do hipotireoidismo quando usada regularmente por estes pacientes, mantendo as atividades das enzimas em níveis basais.

Palavras-chaves: Tireoidite de Hashimoto. Ectoenzimas. Levotiroxina. Plaquetas.

ABSTRACT

Dissertation
Graduate Program in Pharmaceutical Sciences
Federal University of Santa Maria

ACTIVITY OF ENZYMES DEGRATING ADENINE NUCLEOTIDES AND NUCLEOSIDE IN PLATELETS FROM PATIENTS WITH HASHIMOTO THYROIDITIS IN TREATMENT WITH LEVOTHYROXINE

Author: Cristiano Bicca Noal
Advisor: Prof Dr Daniela Leal Rosa Bitencourt
Date and place of Defense: Santa Maria, July 18, 2012.

Hashimoto Thyroiditis (HT) or chronic lymphocytic thyroiditis is an autoimmune disease that causes the destruction of the thyroid gland by inflammatory infiltrates and consequently loss of function. This disease is spread worldwide and predominantly affects females. The consequences of alterations in its activity range from cretinism to the vascular changes. It is known that the enzymes ectonucleoside diphosphohydrolase triphosphate (E-NTPDase, EC 3.6.1.5, CD39), ecto-5'nucleotidase (EC 3.1.3.5, CD73) and adenosine deaminase (ADA, EC 3.5.4.4) are involved in regulating the immune response and thrombotic events, since they regulate extracellular levels of adenine nucleotides and nucleoside. Since HT patients have an autoimmune response and present microvascular changes, the objective of this study was to evaluate the influence of purinergic signaling in the regulation of microvascular dysfunction triggered by the disease determining the activity of the ectoenzymes involved in the metabolism of ATP in platelets from patients with HT in treatment with levothyroxine. Samples were collected from patients with HT treated with levothyroxine and from a control group. In this study we determined the activity of the enzymes E-NTPDase, ecto-5'-nucleotidase and E-ADA; detected the expression of the enzyme in platelets and E-NTPDase; and measured hormone levels of TSH and fT_4 in patients with HT treated with levothyroxine and control group. Results obtained in the enzyme activity showed that patients with HT in hormone replacement with levothyroxine presented no significant changes when compared with the control group. In conclusion, levothyroxine used in patients with HT may reverse the effects of hypothyroidism when used regularly in these patients, while maintaining the enzyme activity in basal levels.

Keywords: Hashimoto’s thyroiditis. Ectoenzymes. Levothyroxine. Platelets

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

MANUSCRITO

LISTA DE ABREVIATURAS

ADP: Adenosina difosfato

AMP: Adenosina monofosfato

ATP: Adenosina trifosfato

ADA: Adenosina desaminase

APC: Célula apresentadora de antígeno

CTLA-4: Linfócito T antígeno 4

DAIT: Doenças autoimunes da tireóide

DG: Doença de Graves

E-NTPDase: ecto-nucleosídeo trifosfato difosfoidrolase

E-5'-NT: ecto-5'-nucleotidase

GT: Glândula tireóide

HLA: Antígeno leucocitário humano

IL-2: Interleucina-2

INF-γ: Interferon-gama

PTPN22: Proteína tirosina fosfatase 22

Treg: Células T reguladoras

T_3: Triiodotironina

T_4: Tiroxina

TH: Tireoidite de Hashimoto

TPO: Tireoperoxidase

TSH: Hormônio Tireoestimulante

TBG: Tireoglobulina

Th1: T *helper* 1

Th2: T *helper* 2

TNF-α: fator de necrose tumoral-alpha

TNF-β: fator de necrose tumoral-beta

Ca^{2+}: íon cálcio

Mg^{2+}: íon magnésio

LISTA DE ANEXOS

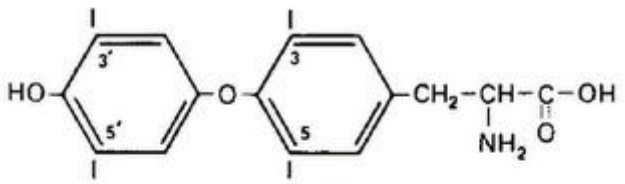
SUMÁRIO

APRESENTAÇÃO

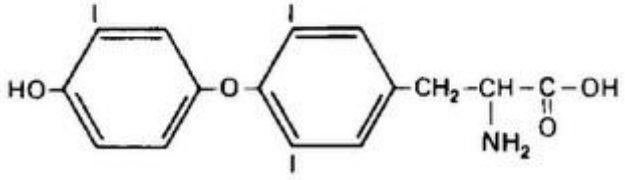
Esta dissertação está organizada na seguinte forma: primeiramente é apresentada a introdução. A seguir, os resultados, discussão e conclusões são apresentados na forma de manuscrito, o qual foi escrito, seguindo-se as normas do periódico ao qual o mesmo será submetido à publicação. As referências bibliográficas apresentadas no final da dissertação referem-se às citações que aparecem no item introdução. A formatação atende a MDT de 2012 da UFSM.

1 INTRODUÇÃO

A Glândula Tireóide (GT) é uma das maiores glândulas endócrinas do organismo e secreta dois hormônios importantes, a tiroxina (T_4) e a triiodotironina (T_3) (Figura 1), que têm efeito profundo de aumentar o metabolismo (GUYTON; HALL, 2002). Os hormônios da tiróide desempenham um papel vital na fisiologia humana normal (McLEOD, 2010), com efeitos pleiotrópicos, desempenhando papéis críticos no desenvolvimento inicial do cérebro, crescimento somático, maturação óssea, e síntese de RNAm de mais de 100 proteínas que regulam diferentes funções corporais (SARANAC et al., 2011).



3,5,5',5',- Tiroxina (T_4)



3,5,3',- Triiodotironina (T_3)

Figura 1- Estrutura química dos hormônios da tireóide (Adaptado de HARPER, 2003).

A GT é a única glândula endócrina que acumula o seu produto de secreção em quantidade apreciável. A síntese e o acúmulo de hormônios ocorrem em quatro etapas: síntese de tireoglobulina (TBG), captação de iodeto do sangue, ativação do iodeto e iodação dos resíduos de tirosina (catalisada pela peroxidase) (JUNQUEIRA; CARNEIRO, 2008). A TBG iodada é armazenada na luz do folículo tireoidiano como colóide e retirada através de endocitose (BERNE, 2004). A secreção destes hormônios é regulada por uma alça de retroalimentação que envolve o hipotálamo, a hipófise e a glândula tireóide. O hipotálamo produz o hormônio de liberação da tireotropina que estimula a produção do hormônio tireoestimulante (TSH) pela

hipófise. Por sua vez, o TSH estimula a secreção dos hormônios tireóideos, que exercem uma ação de retroalimentação sobre o hipotálamo e a hipófise, completando o círculo regulador (GOLDMAN; AUSIELO, 2005).

Uma vez liberados pela GT, os hormônios ligam-se às proteínas plasmáticas que servem como tampões, reservatórios e distribuidores para os tecidos, sendo que apenas 0,03% do T_3 e T_4 são encontrados em forma livre no sangue. As proteínas plasmáticas carreadoras são: TBG, a pré-albumina e a albumina. Cerca de 70% do T_3 e T_4 circulantes estão ligados à TBG; 10% a 15% estão ligados à outra proteína ligadora específica dos hormônios tireoidianos chamada transrretina. À albumina se ligam 15% a 20% e 3% se ligam a lipoproteínas (BERNE, 2004).

A tireóide é altamente vulnerável a doenças autoimunes e esses distúrbios começam a partir de uma complexa interação de fatores genéticos, ambientais e endógenos, e uma combinação desses fatores é necessária para iniciar a tireoidite autoimune (SARANAC et al., 2011).

As doenças tireoidianas autoimunes (DAIT), consideradas como arquétipo das doenças autoimunes órgão-específicas, afetam em especial mulheres adultas e idosas e são determinadas pela perda da autotolerância imunológica. Envolve espectro de fenótipos, cujos principais representantes são a doença de Graves (DG) e a tireoidite de Hashimoto (TH), ambas caracterizadas pela presença de infiltrado linfocitário de intensidade variável e produção de autoanticorpos tireoidianos dirigidos a antígenos específicos, determinantes da expressão clínica da enfermidade, que pode variar do hipertireoidismo ao hipotireoidismo (SGARBI; MACIEL, 2009).

O nome TH é em homenagem a Hakaru Hashimoto, médico japonês, nascido em 1881, que examinando amostras teciduais da tireóide, descobriu novas características patológicas da glândula. A doença, primeiramente, chamava-se struma linfomatosa e caracterizava-se por infiltrado linfocitário e formação de folículos linfóides com centro germinativo. A partir de 1931, a doença passou a ser conhecida na Europa e Estados Unidos como TH e foi um importante passo para a melhor compreendermos as autoimunidades e doenças endócrinas (AMINO et al., 2002).

A tireoidite é um termo genérico que comporta uma série de entidades clínicas que têm em comum o acometimento da glândula tireóide (YAMASHIRO, 2007). Entretanto, o termo TH deveria exclusivamente ser utilizado para descrever

pacientes com hipotireoidismo que demonstrem altos títulos de anticorpos antitireóide ou presença de citologia ou biópsia comprovando a enfermidade (ROBERTI et al., 2006).

A TH é a condição endócrina autoimune mais comum, afetando até 10% da população em geral (MICHELS; EISENBARTH, 2010). É cerca de 15 a 20 vezes mais comum em mulheres que em homens e frequentemente envolve pessoas com idades entre 30 e 50 anos de idade (STAII et al., 2010). Os principais sinais e sintomas clínicos são: ganho de peso, sensibilidade ao frio, cretinismo, pele ressecada, queda de cabelo, aumento dos níveis séricos de TSH, redução dos níveis de T_4 , perda gradual da função da tireóide, bócio e infiltração de células T em achados histológicos (MICHELS; EISENBARTH, 2010).

Estudos realizados sobre as associações genéticas de TH têm mostrado que o antígeno leucocitário humano (HLA), que desempenha um papel importante em outras desordens autoimunes, está associado com o desenvolvimento de TH (FISCHER, 2000), resultando em infiltrado na glândula por linfócitos T e expressão clínica do hipotireoidismo. Os linfócitos T desempenham um papel importante na patogênese da doença, reagindo com os antígenos da tireóide e secretando citocinas inflamatórias. São formados anticorpos contra a tireoperoxidase (TPO), a tireoglobulina e o receptor da tireotropina. Acredita-se que estes anticorpos aparecem após a destruição das células foliculares da tireóide, causadas por células T (SGARBI; MACIEL, 2009). Na determinação da TH, os anticorpos anti-TPO, estão intimamente associados com a atividade da doença (MICHELS; EISENBARTH, 2010).

Os genes imunomoduladores de suscetibilidade à TH são o complexo maior de histocompatibilidade classe II (MHC Classe II), o linfócito T citotóxico antígeno 4 (CTLA-4) e a proteína tirosina fosfatase-22 (PTPN22). Três regiões cromossômicas denominadas TH1 (cromossomo 13q33), TH2 (cromossomo 12q22) e a região 8q23-24, podem estar ligadas à TH (ERIKSSON et al., 2012; SGARBI; MACIEL, 2009).

O desenvolvimento da tolerância imunológica a autoantígenos envolve processo complexo de mecanismos centrais e periféricos. A tolerância central ocorre no timo pela deleção de células T que se liga com alta afinidade a peptídeos endógenos. Quando este processo falha, células T efetoras autorreativas podem escapar da seleção tímica e migrar para a periferia, onde são inibidas pelas células T (CD4+) regulatórias (Treg). As células Treg, geradas no timo, expressam as

moléculas CD25 e CTLA-4, consideradas essenciais para a supressão da resposta imune mediada por células T. Os polimorfismos do gene CTLA-4 ou a mutação do gene CD25 associam-se a doenças autoimunes nos seres humanos e a depleção de células Treg tem sido relacionada com o desenvolvimento de tireoidite autoimune (SGARBI; MACIEL, 2009).

A molécula CTLA-4 é o principal regulador negativo da ativação dos linfócitos (CHISTIAKOV; TURAKULOV, 2003), pela competição da ligação da proteína B7 (expressa na célula apresentadora de antígeno) à proteína co-estimuladora CD28. Portanto, mutações no gene CTLA-4, poderiam resultar em ativação exagerada de linfócitos T e desenvolvimento da autoimunidade. O bloqueio da molécula CTLA-4 com anticorpo monoclonal confere aumento da produção de células T e da produção de interleucina-2 (IL-2) (SGARBI; MACIEL, 2009).

A PTPN22, de modo semelhante ao CTLA-4, é inibidor potente da ativação dos linfócitos T (DUTZ et al., 2009) e polimorfismos deste gene (substituição do triptofano por arginina no códon R620W), causam hiperativação dos linfócitos e também tem sido associados como determinantes no desenvolvimento da TH, Diabetes tipo I e Artrite Reumatóide (FIORILLO et al., 2010).

Os fatores desencadeadores do processo autoimune na DAIT não são bem conhecidos, mas admite-se que o sinal inflamatório inicial seria emitido por lesão ou necrose celular desencadeada por múltiplos fatores, como anormalidades genéticas, infecção (viral ou bacteriana), estresse ou excesso de iodo, com liberação de autoantígenos, atração e infiltração glandular por células T. A lesão inicial atrairia quantidade expressiva de células apresentadoras de antígenos (APC) ”profissionais” para o meio intratireoidiano, que por sua vez, apresentariam os autoantígenos tireoidianos aos linfócitos T auxiliadores CD4+. As citocinas liberadas neste processo induziriam a liberação de moléculas MHC classe II na superfície da célula folicular transformando-as em APCs. A expressão aberrante de moléculas HLA de classe II na célula tireoidiana parece ter papel relevante no desenvolvimento das doenças autoimunes, entre elas a TH (SGARBI; MACIEL 2009; YOKOI et al., 2012), pois células T reconhecem e respondem a um antígeno pela interação com complexo composto de peptídeo antigênico apresentado por moléculas HLA, uma vez ligados, os peptídeos seriam apresentados e reconhecidos por receptores de células T (TCR) em células que teriam escapado da tolerância imunológica (SGARBI; MACIEL, 2009).

Havendo falha na tolerância imunológica, os autoantígenos não seriam reconhecidos, resultando ativação de células T autorreativas, com resposta inflamatória excessiva e inapropriada. O recrutamento de linfócitos na TH envolve um processo complexo com atuação de moléculas de adesão e, principalmente, de quimiocinas, uma família especializada de citocinas que controlam a migração e leucócitos (quimiotaxia) durante o processo inflamatório, essas quimiocinas recrutariam linfócitos T helper 1 (Th1), que secretam interferon gama (IFN-γ), portanto perpetuando o processo autoimune (Figura 2) (SGARBI; MACIEL, 2009).

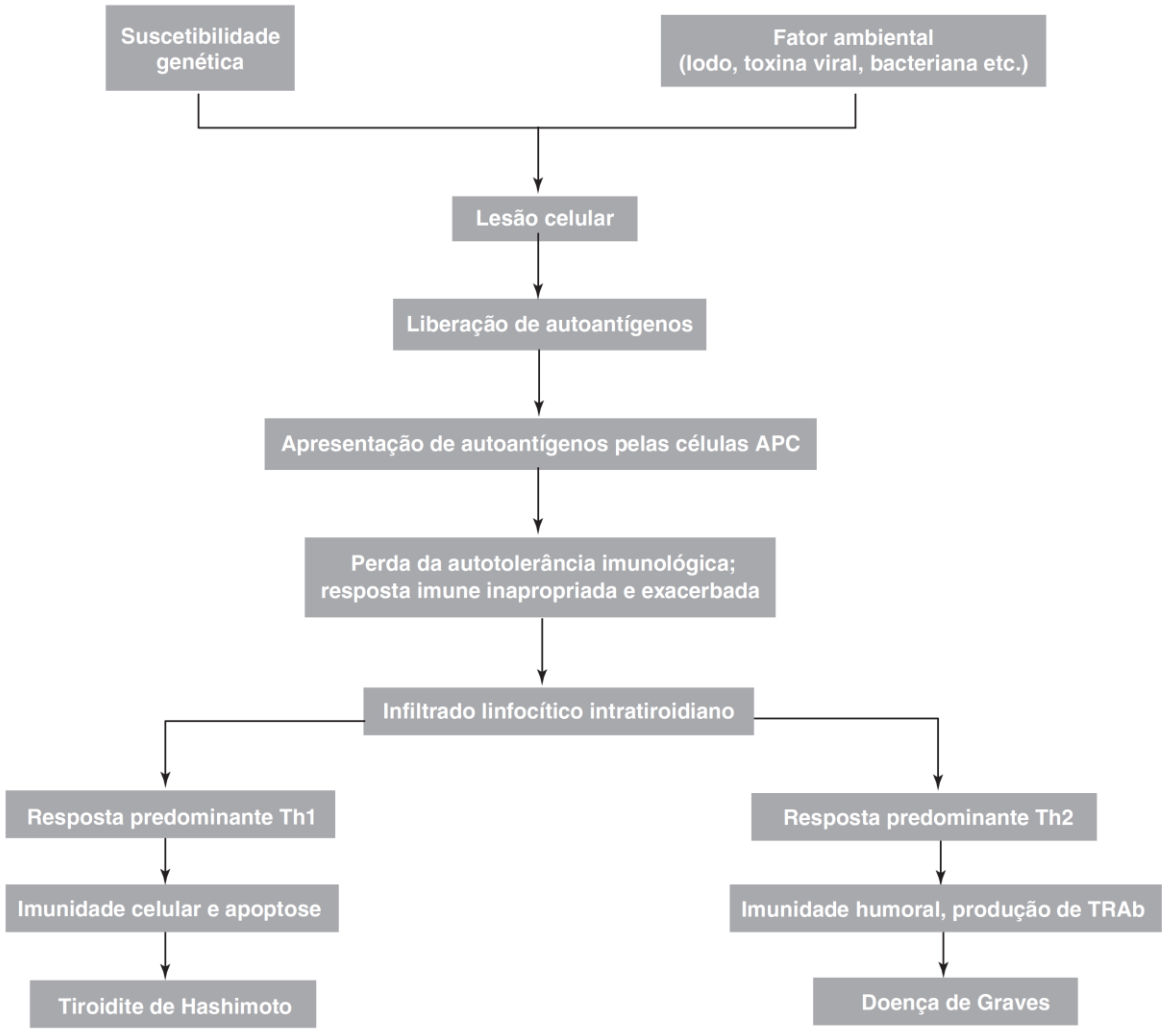


Figura 2 - Patogênese das doenças tireoidianas autoimunes (Adaptado de SGARBI; MACIEL, 2009).

Na TH, a maioria do infiltrado linfocítico age como Th1, que secretam IL-2, IFN-γ e TNF-α, com a ausência de interleucina-4 ou interleucina-10, favorecendo a imunidade celular e favorecendo a apoptose celular (BOSSOWSKI et al., 2011). Ligantes apoptóticos e receptores, como o TNF, Fas e o ligante indutor de apoptose ligado à necrose tumoral são expressos na célula tireoidiana, mas, em condições fisiológicas, permanecem inativados. No entanto, a expressão do FasL, induzidas por citocinas Th1 no infiltrado linfocítico tireoidiano, determina a apoptose. Defeitos nas células Treg resultam na hiperprodução das citocinas Th1 e poderiam estar envolvidos na patogênese da TH (SGARBI; MACIEL, 2009).

As patologias envolvendo hormônios da tireóide são muito comuns na prática clínica. Em pessoas adultas, a deficiência ou o excesso de hormônios da tireoide, possui uma ampla gama de manifestações clínicas, incluindo alterações no sistema vascular. Estes hormônios possuem um efeito inibitório na agregação plaquetária *in vivo e in vitro*, com isto, pacientes com hipotireoidismo apresentam aumento na agregação plaquetária (BRUNO, 2005).

O hipotireoidismo está associado a diversas anormalidades no sistema de coagulação que envolve hemostasia primária e secundária e que podem variar desde alterações subclínicas até importantes transtornos clínicos (YANGO et al., 2011).

A hemostasia é a resposta fisiológica normal ao dano vascular, sendo que as plaquetas possuem um importante papel neste processo (MARCUS; SAFIER, 1993). Quando um vaso ou um tecido é lesionado química ou fisicamente, a hemostasia é acionada através da vasoconstrição, da formação do tampão plaquetário, da formação do coágulo sanguíneo e da sua posterior dissolução. Nessas etapas, ocorre interação entre os componentes teciduais, as proteínas plasmáticas, e os receptores celulares, permitindo a formação rápida e eficiente de coágulos sólidos para bloquear pontos de ruptura ou lesão vascular e fazendo com que o sangue permaneça líquido e sem coágulos dentro do sistema vascular (REVEL; DOGHMI, 2004).

As plaquetas são células discóides e anucleadas (LORENZI, 1999). Sua membrana é lipoproteica composta por fosfolipídeos, contendo muitos receptores responsáveis pelo desencadeamento da ativação plaquetária e de reações de adesão/agregação. No citoplasma das plaquetas encontram-se os grânulos. Os grânulos alfa possuem diversos tipos de proteínas, como o fibrinogênio, fator de Von

willebrand (vWF), fibronectina, vitronectina e trombospondina que são proteínas adesivas. Já os grânulos densos contêm nucleotídeos como adenosina difosfato (ADP), adenosina trifosfato (ATP), serotonina e o cálcio (Ca^{2+}), substâncias que promovem agregação plaquetária (ZAGO et al., 2004).

O dano vascular inicia com a exposição do colágeno subendotelial, ativação das plaquetas e a iniciação da cascata de coagulação (KAHNER et al., 2006; RUSSEL, 1999). A ativação das plaquetas é caracterizada por: formação de pseudópodes (KAHNER et al., 2006; VORCHHEIMER; BECKER, 2006), liberação do conteúdo granular, geração dos mediadores lipídicos e agregação. A ativação plaquetária é alcançada através de vários receptores de superfície incluindo receptores acoplados a proteína G (GPCRs), integrinas e receptores de glicoproteínas. Os mediadores secundários, como ADP e tromboxano A2 (gerado no interior das plaquetas) ativam o restante das plaquetas, resultando na amplificação da resposta homeostática inicial (KAHNER et al., 2006).

As plaquetas tem um importante papel nas respostas imunes inatas e adaptativos estando presentes no início da inflamação, angiogênese, aterosclerose, desenvolvimento linfático e crescimento de tumores. Isso ocorre devido à sua grande quantidade na circulação e a capacidade de liberar mediadores inflamatórios. As plaquetas são posicionadas para agirem como sentinelas e para se comunicarem rapidamente com outras células do sistema imune. Recentes estudos indicam que as plaquetas podem armazenar substâncias imunomoduladoras de uma forma específica, a fim de responderem a diferentes tipos de danos aos tecidos (SEMPLE et al., 2011).

A manutenção da função vascular normal, também tem sido relacionada com o sistema purinérgico. Os nucleotídeos da adenina, ATP, ADP e AMP e o nucleosídeo adenosina estão envolvidos em muitos dos mecanismos homeostáticos no local da lesão vascular. Dependendo da concentração o ATP pode estimular ou inibir a agregação plaquetária (BRUNO, 2005), e o ADP caracteriza-se por ser o agente indutor da agregação plaquetária em presença de cálcio e fibrinogênio (VANNI et al., 2007), via interação com receptores $P2Y_{12}$. Com isto, antagonistas do receptor $P2Y_{12}$ são indicados como agentes antitrombóticos (BRUNO, 2005).

A hiperatividade plaquetária pode levar à formação patológica de trombos e oclusão vascular (DAHLBACK, 2005; KEATING et al., 2004; REMIJIN et al., 2002; WAGNER; BURGUER, 2003), além de desencadear a resposta inflamatória

(STRUKOVA, 2001). O sistema purinérgico é uma via de sinalização importante em diversos tecidos, desencadeando múltiplos efeitos celulares, incluindo resposta imune, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK; KNIGHT, 2004).

A sinalização purinérgica envolve três principais componentes que são os nucleotídeos e nucleosídeo da adenina; receptores purinérgicos específicos, através dos quais as purinas exercem seus efeitos; e as ectonucleotidases, que são as proteínas responsáveis pelo controle dos níveis dessas moléculas no meio extracelular (ATKINSON et al., 2006).

Os nucleotídeos são liberados para o meio extracelular por células sanguíneas e vasculares, como eritrócitos, plaquetas, e endotélio (DUBYAK et al., 1993; LUTHJE, 1989), mas também podem ser liberados frente ao dano celular, nos sítios inflamatórios ou de estresse oxidativo, onde há um aumento da liberação de nucleotídeos. Já a adenosina pode ser liberada no meio extracelular como resultado da degradação do ATP e ADP por enzimas específicas (HUNSUCKER et al., 2005; YEGUTKIN, 2008), ou através de transportadores na membrana das células, que transportam a adenosina de dentro das células para o meio extracelular (BOROWIEC et al., 2006).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (DI VIRGILIO et al., 2001). Nestas condições, o ATP extracelular, por exemplo, pode formar poros nas membranas celulares, resultando em mudanças osmóticas na célula (LEAL et al., 2005). Já em altas concentrações, pode atuar como uma molécula citotóxica e levar à morte celular (DI VIRGÍLIO et al., 2001). O ADP é um reconhecido promotor da agregação plaquetária (SOSLAU; YOUNGPRAPAKORN, 1997). Os nucleotídeos extracelulares como ATP e ADP não atravessam a membrana celular, mas podem realizar suas ações biológicas através de receptores específicos na superfície celular, denominados receptores purinérgicos (DI VIRGÍLIO et al., 2001).

A adenosina é um potente inibidor da agregação plaquetária e um importante modulador do tônus vascular, agindo como agente protetor em lesões e tendo um papel importante no sistema imune, promovendo a maturação de monócitos. Além disso, a adenosina têm propriedades anti-inflamatórias e imunossupressoras, inibindo a proliferação de células T através da ativação de receptores A_2A e da liberação de citocinas pró-inflamatórias (ZANINI et al., 2012). A ação de purinas e

pirimidinas é mediada através de uma extensa família de purinoreceptores, divididos entre receptores P1 para adenosina e P2 para o ATP e demais nucleotídeos (BURNSTOCK; VERKHRATSKY, 2010).

Existem quatro subtipos de receptores P1 (A_1, A_2A, A_2B e A_3), sete subtipos de receptores P2X ligados a canais iônicos ($P2X_1$ a $P2X_7$) e oito subtipos de receptores P2Y (Figura 3) acoplados a proteína G ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) (BURNSTOCK; VERKHRATSKY, 2010).

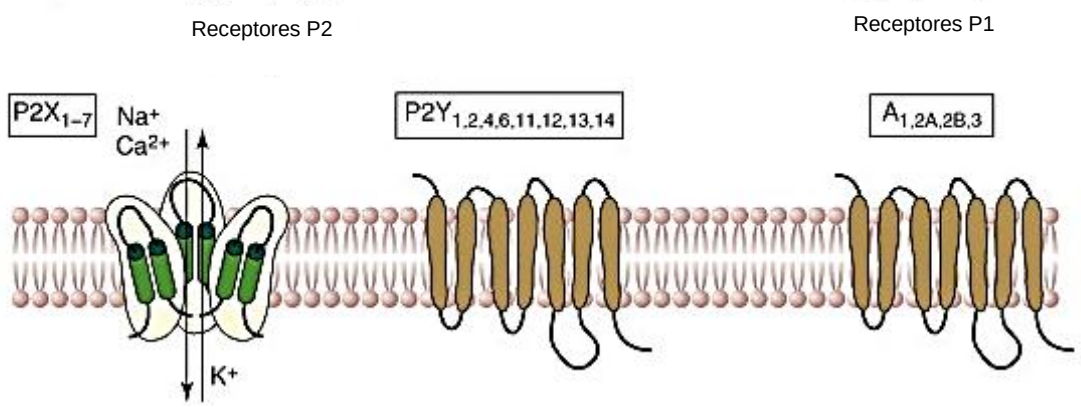


Figura 3 – Receptores purinérgicos (adaptado de ABBRACCHIO et al., 2009).

O ATP liberado no meio extracelular exerce seus efeitos ao se ligar a receptores P2X ou a P2Y. Os receptores P2X1 respondem somente ao ATP, enquanto que os receptores P2Y, respondem ao ATP e a outros nucleotídeos, incluindo o ADP, o UTP e o UDP, mas não ao AMP. Quando o ADP é liberado ao ambiente extracelular, interage com receptores metabotrópicos (P2Y) para desempenhar suas ações (DI VIRGILIO et al., 2001). Porém é necessário que haja um controle dos níveis extracelulares de ADP, para a regulação dos processos trombóticos e/ou hemorrágicos, o qual é modulado principalmente pela ação das ecto-nucleotidases (RALEVIC; BURNSTOCK, 2003). O AMP exerce a função de sinalizador em situações de desequilíbrio no metabolismo, servindo também como substrato para a formação da adenosina (CUNHA, 2001; LATINI; PEDATA, 2001). Quando a adenosina liga-se a receptores P1, mais especificamente ao A_2A ocorre

aumento da agregação plaquetária e da resposta inflamatória (BURNSTOCK; VERKHRATSKY, 2010).

Após exercerem seus efeitos no meio extracelular, os nucleotídeos da adenina são hidrolisados por enzimas conhecidas como ectonucleotidases. Dentre elas, destacam-se a E-NTPDase (apirase, CD39, ATP difosfo-hidrolase), a E-5'-nucleotidase e a E-NPP (ecto-nucleotideo pirofosfatase/fosfodiesterase), que são enzimas capazes de controlar a disponibilidade de ligantes como ATP, ADP e AMP a seus receptores (ZIMMERMANN et al., 2001; ZIMMERMANN et al., 2012)

A enzima adenosina desaminase (ADA) (E.C. 3.5.4.4.) é de suma importância no metabolismo das purinas, pois ela catalisa a desaminação irreversível da adenosina em inosina (Figura 4) (RESTA et al., 1998; ROBSON et al., 2006).

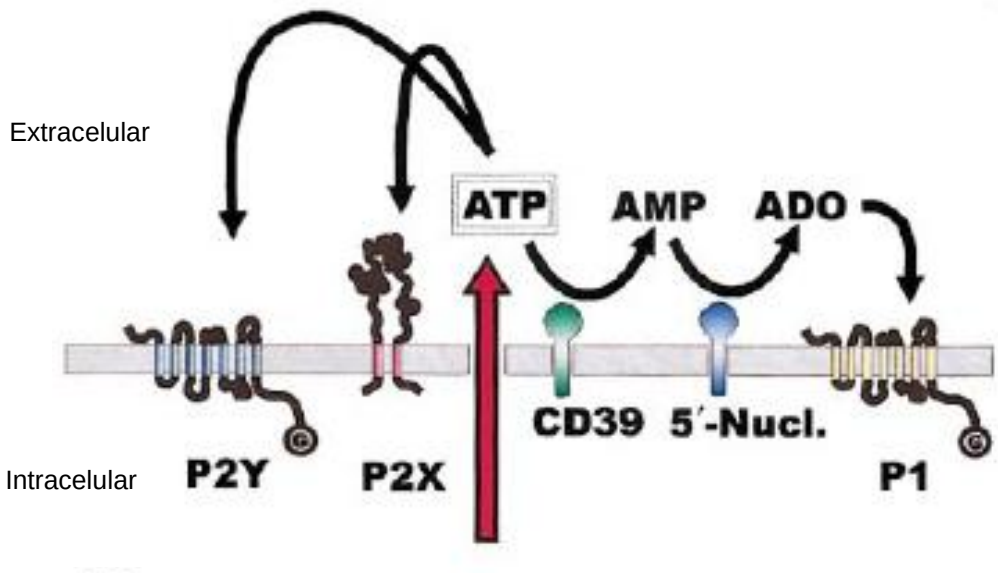


Figura 4 - Destino do ATP liberado: possível papel na quimiotaxia de leucócitos (Adaptado de DI VIRGILIO et al., 2001).

A cascata purinérgica é iniciada pela E-NTPDase, essa classe de enzimas inclui oito membros, que diferem quanto à especificidade de substratos, distribuição tecidual e localização celular (SHI et al., 2001; ZIMMERMANN, 2001; BIGOENNESE et al., 2004), na qual quatro estão localizados na membrana celular com o sítio catalítico voltado para o meio extracelular (NTPDase 1, 2, 3, 8); e quatro exibem uma localização intracelular (NTPDase 4,5,6,7) (ROBSON et al., 2006) (Figura 5).

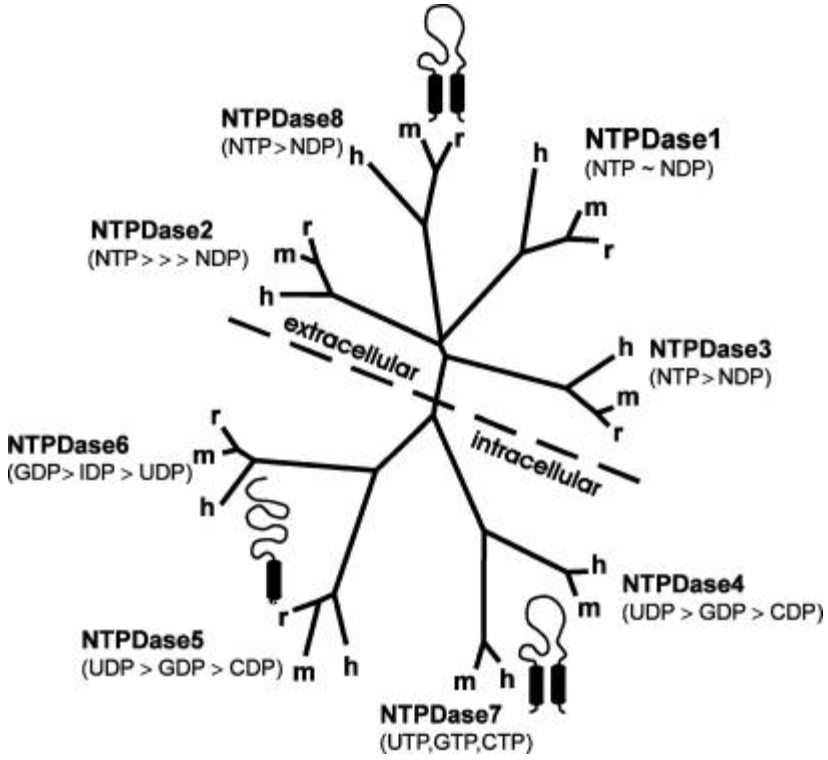


Figura 5 - Membros da família das NTPDases (Adaptado de ROBSON et al., 2006).

A função geral da NTPDase tem sido atribuída à hidrólise extracelular dos nucleotídeos ATP e ADP e, dependendo da localização tecidual, a atividade enzimática possui diferentes papéis fisiológicos (BONAN et al., 2001; SARKIS et al., 1995; ZIMMERMANN, 1999). A primeira NTPDase identificada foi a NTPDase-1, que está ancorada à membrana via dois domínios transmembrana e que hidrolisa os nucleotídeos ATP e ADP em proporções semelhantes, formando AMP e para isto requer a presença de íons Ca^{2+} e Mg^{2+}. Uma expressão abundante foi encontrada no endotélio vascular, nas células da musculatura lisa, no pâncreas, nas células dendríticas e em células sanguíneas como linfócitos, plaquetas e eritrócitos, bem como no plasma (FRASSETO et al., 1993; PILLA et al., 1996; SARKIS et al., 1995; YEGUTKIN, 2008; ZIGANSHIN et al.,1994; ZIMMERMANN, 2001). A NTPDase-2 é associada ao sistema nervoso central e periférico. A NTPDase-3 é associada com estruturas neuronais, agindo na regulação dos níveis de ATP pré-sinápticos (YEGUTKIN, 2008). Já as NTPDases 4, 5, 6 e 7 estão localizadas no meio intracelular (ZIMMERMANN, 2001).

Após a hidrólise de ATP e ADP pela E-NTPDase, a ecto-5’-nucleotidase (CD73, EC 3.1.3.5) será responsável pela hidrólise de AMP. Esta é uma enzima que está ancorada à membrana plasmática com seu sítio catalítico voltado para o meio extracelular (figura 6). Entretanto, formas solúveis e clivadas desta enzima também já foram descritas (HUNSUCKER et al., 2005; ZIMMERMANN, 2001), cinco membros estão localizados no citoplasma, um na matriz mitocondrial e um ancorado à membrana plasmática externa, sendo este uma ecto-5’-NT (E-5’-NT) (HUNSUCKER et al., 2005).

Esta enzima catalisa a desfosforilação de vários nucleotídeos 5’-monofosfatados como CMP, IMP, UMP, GMP e AMP a seus respectivos nucleosídeos (ZIMMERMANN, 1996). Porém, foi comprovado que a 5’-nucleotidase hidrolisa mais eficazmente o AMP, sendo por isto considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN, 1996, 2001; ZIMMERMANN et al., 1998).

As funções da ecto-5’-nucleotidase correlacionam-se diretamente no seu papel na produção de adenosina. Assim, de acordo com sua localização tecidual, ela desempenha importantes funções como, por exemplo, o controle da agregação plaquetária, a regulação do tônus vascular e atua também na neuromodulação e neuroproteção do sistema nervoso central (DUNWIDDIE; MASINO, 2001; KAWASHIMA et al., 2000; ZIMMERMANN et al., 1998).

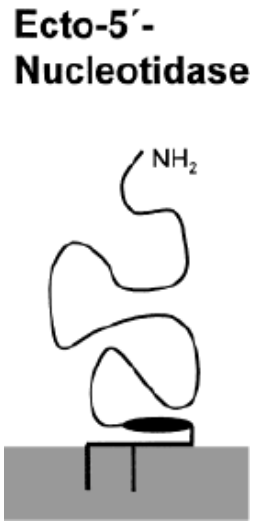


Figura 6 - Estrutura da ecto-5’-nucleotidase ancorada à membrana (ZIMMERMANN, 2001).

E na sequência da cascata purinérgica a enzima adenosina desaminase (ADA; E.C. 3.5.4.4) faz parte do conjunto de enzimas responsáveis pela degradação

sequencial dos nucleotídeos e nucleosídeo da adenina (YEGUTKIN, 2008). É responsável por catalisar a desaminação irreversível da adenosina e 2'-deoxiadenosina em inosina e 2'- deoxinosina, respectivamente (RESTA et al., 1998; ROBSON et al., 2006; YEGUTKIN, 2008). Em humanos existe na forma de duas isoenzimas classificadas como: ADA1 e ADA2, cada uma com suas particulares propriedades bioquímicas (SHAROYAN et al.,2006).

A ADA1 é encontrada em todas as células e tecidos humanos (Figura 7), apresentando alta atividade em linfócitos e monócitos (ZAVIALOV; ENGSTROM, 2005). A maioria das células humanas contém pequena quantidade de ADA2 e provavelmente sua maior fonte seja o sistema monócito-macrófago (GAKIS, 1996). Foi sugerido que a ADA2 no plasma humano pode ser secretada por monócitos ativados em processos inflamatórios, tendo a habilidade de regular a proliferação celular (IWAKI-EGAWA et al., 2006).

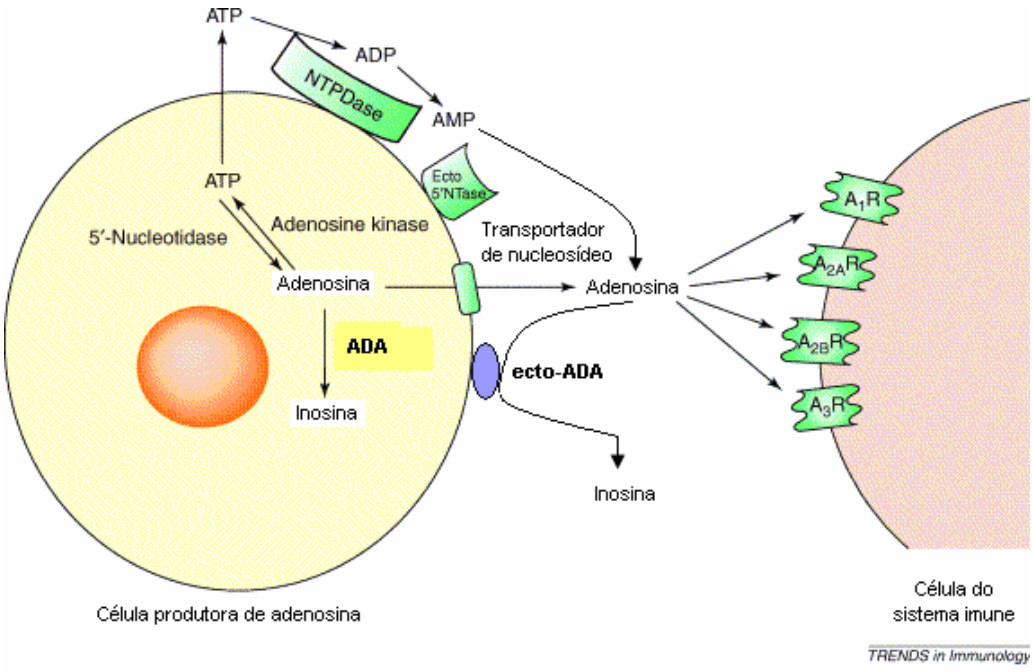


Figura 7 – Principais vias envolvidas no metabolismo da adenosina (Adaptado de HASKÓ & CRONSTEIN, 2004).

A deficiência da E-ADA, normalmente causa imunodeficiência combinada severa em crianças, que apresenta atraso no crescimento, infecções oportunistas, linfopenia e defeitos nas funções celular e humoral (OZSAHIN et al.; 1997).

Alterações na atividade da ecto-ADA podem ocorrer como consequência da variação em sua expressão em tecidos e células (ARAN et al., 1991; GORELL et al., 2001 FRANCO et al., 1997).

Na TH, ocorre a deficiência dos hormônios da tireóide. A reposição com hormônios tireoidianos é a mais simples das terapias hormonais. A droga de escolha é a levotiroxina sódica. Sua vida média é de 7 dias, e a resposta máxima é atingida na segunda semana de tratamento, tendo sido convertido em grande parte a T_3. É administrada uma vez ao dia, pela manhã. A principal vantagem desta terapia, é que o mecanismo de deiodinação periférica pode contribuir para produzir a quantidade de T_3 necessários para a manutenção dos níveis fisiológicos deste hormônio (SETIAN, 2007; RAJPUT; CHATTERJEE; RAJPUT, 2011).

Visto que pacientes portadores de TH possuem uma resposta autoimune e também apresentam alterações microvasculares que são moduladas pela sinalização purinérgica, é relevante a verificação da atividade das enzimas E-NTPDase, E-5´-nucleotidase e E-ADA, envolvidas no metabolismo do nucleosídeo e dos nucleotídeos da adenina em pacientes com TH que fazem uso da levotiroxina sódica para reposição hormonal.

2 OBJETIVOS

2.1 Objetivo geral

Determinar a atividade de enzimas que degradam nucleotídeos e nucleosídeo da adenina em plaquetas de pacientes com TH em tratamento com levotiroxina sódica.

2.2 Objetivos específicos

- Determinar a atividade das ectonucleotidases E-NTPDase e ecto-5'-nucleotidase em plaquetas de pacientes com TH em tratamento com levotiroxina sódica;
- Determinar a atividade enzimática da adenosina desaminase em plaquetas de pacientes com TH em tratamento com levotiroxina sódica;

- Avaliar a expressão de CD39 em plaquetas de pacientes com TH em tratamento com levotiroxina sódica.

3 MANUSCRITO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se compondo o próprio manuscrito e representam a íntegra deste estudo.

O manuscrito será submetido à revista Thrombosis Research.

Regular Article

ACTIVITIES OF ENZYMES DEGRADING ADENINE NUCLEOTIDES AND NUCLEOSIDE IN PLATELETS FROM PATIENTS WITH HASHIMOTO THYROIDITIS IN TREATMENT WITH LEVOTHYROXINE

Cristiano B. Noal[a], Viviane do C. G. de Souza[b], Jeandre A. dos S. Jaques[b], Lívia G. Castilhos[a], Tatiana D. Bertoldo[a], Jader B. Ruschel[b], Bruna C. Rocha[a], Karine B. Schlemmer[a], Cláudio A. M. Leal[c], Daniela Zanini[b], Sílvia K. Londero[c], Daniela B.R. Leal[a]

[a] Departamento de Microbiologia e Parasitologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

[b] Departamento de Química, Laboratório de Enzimologia Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

[c] Ambulatório de Endocrinologia, Hospital Universitário de Santa Maria, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

Corresponding author:

Prof Dr Daniela Bitencourt Rosa Leal

Departamento de Microbiologia e Parasitologia/CCS/UFSM – Universidade Federal de Santa Maria (UFSM)

Prédio 20 – Sala 4102

Phone:+55 55 3220 9581

ACTIVITIES OF ENZYMES DEGRADING ADENINE NUCLEOTIDES AND NUCLEOSIDE IN PLATELETS FROM PATIENTS WITH HASHIMOTO THYROIDITIS IN TREATMENT WITH LEVOTHYROXINE

Abstract

INTRODUCTION: Hashimoto Thyroiditis (HT) or chronic lymphocytic thyroiditis is an auto-immune disease, characterized by the infiltrated thyroidal parenchyma, which results from the incapacity of the suppressor T lymphocytes in destroying clones of lymphocytes sensitized by thyroidal antigens. As a consequence cytotoxicity mediated by “natural killers” cells is observed, and antibodies are produced against thyroidal components. Extracellular levels of the nucleotides ATP, ADP, and AMP and their derivative nucleoside adenosine are regulated by enzymes such as Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-5’-nucleotidase (E-5’-NT) and ecto-adenosine-deaminase (E-ADA). These nucleotides and nucleoside are secreted by immune cells, platelets and damaged endothelial cells, used as mediators to induce different patterns of immune response and modulate the inflammatory process.

OBJECTIVE: This study aimed to investigate the activities of the ectoenzymes E-NTPDase, E-5’-nucleotidase and E-ADA present on surface of the platelets from patients with HT in a current treatment with levothyroxine.

MATERIALS AND METHODS: Twenty patients diagnosed with HT in treatment with levothyroxine and 20 healthy subjects were selected. E-NTPDase, E-5’-NT and E-ADA activities and E-NTPDase expression were evaluated in platelets isolated from these individuals.

RESULTS: Results demonstrated no significant differences neither in the activities of E-NTPDase, E-5’-NT and E-ADA nor in E-NTPDase expressed in platelets from patients with HT treated with levothyroxine when compared with the control group.

CONCLUSION: Knowing that HT is an autoimmune disease and alters the basal metabolism, we conclude that the daily reposition of the thyroid hormone maintained the activity of the E-NTPDase, E-5’-NT and E-ADA unaltered at levels considered basal.

Keywords: adenosina triphoshate, Hashimoto’s thyroiditis, platelet, T3, T4, Levothyroxine.

Abbreviations

HT: Hashimoto's Thyroiditis

T_3: Triiodothyronine

T_4: Thyroxine

fT_4: Free thyroxine

TSH: Thyroid stimulating hormone

TPO: Thyroid peroxidase

Anti-TPO: Anti thyroid peroxidase

ADP: adenosine diphosphate

ATP: adenosine triphosphate

AMP: adenosine monophosphate

NTPDase: Nucleoside triphosphate diphosphohydrolase

5'-NT: 5'-nucleotidase

ADA: Adenosine deaminase

PRP: platelets-rich plasma

PPP: platelets-poor plasma

LDH: lactate dehydrogenase

TCA: trichloroacetic acid

Pi: inorganic phosphate

Introduction

Autoimmune thyroid diseases, comprising Grave´s disease (GD) and Hashimoto's disease (HT) are common, affecting up to 2% of the population. There are marked gender differences in prevalence with reported 5 to 10-fold excesses in females for both diseases. There is also a significant clustering of disease in family member with thyroid disorders [1].

Thyroid hormones play a vital role in normal human physiology with effects on almost all tissues to influence growth and development, maintain normal cognition, cardiovascular function, bone health, and energy balance [2].

Hypothyroidism is the most common disorder arising from hormone deficiency [3]. Hashimoto's thyroiditis is an autoimmune disorder in which Th1-mediated response contributes to the destruction of thyroid follicular cells (TFCs) [4], with presence of autoantibodies against thyroperoxidase (TPO) at high concentration. They are involved in thyroid cell destruction through cytotoxic mechanisms mediated by effector cells and/or complement activation and are the first to show thyroid antibody-dependent cell cytotoxicity (ADCC) in HT [5,6]. The degradation of the cells may be compensated by the increased thyroid-stimulating hormone (TSH) levels and the hyperplasia of epithelial cells [7].

The purinergic signaling system plays an important regulatory role in inflammation, cellular activation, blood flow and vascular thrombosis by extracellular biomolecules such as adenine nucleotides (ATP, ADP, and AMP) and their derivative nucleoside adenosine [8]. The ectonucleotidase chain or cascade, as initiated by E-NTPDase can be terminated by E-5′-nucleotidase (CD73; EC 3.1.3.5) with hydrolysis of nucleoside monophosphates (AMP). Together, E-5′-nucleotidase and E-adenosine deaminase (ADA; EC 3.5.4.4), another ectoenzyme converts adenosine to inosine, closely regulate local and pericellular extracellular and plasma concentrations of adenosine [9]. The ectoenzymes described above are capable of regulating the extracellular concentration of adenine nucleotides and nucleosides, which are important both in maintaining blood homeostasis through the regulation of platelet aggregation and in controlling inflammatory processes [10].

Therapy with levothyroxine seems to be associated with a quality of life of hypothyroid patients comparable to that enjoyed by healthy people. Replacement

therapy with levothyroxine is the “gold standard” for the treatment of hypothyroidism [11].

Taking into account that the activities of ectoenzymes are involved in the maintenance of hemostasis, we aimed to evaluate the activity of E-NTPDase, E-5’-nucleotidase and E-ADA enzymes in the thromboregulation of patients with HT in treatment with levothyroxine, since changes in the activity of this enzyme are found in other autoimmune diseases.

Material and Methods

Chemicals

The substrates adenosine 5’-triphosphate disodium salt (ATP), adenosine 5’-diphosphate sodium salt (ADP), 5’-monophosphate sodium salt (AMP), adenosine, as well as bovine serum albumin, Trizma base, HEPES and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals used in this experiment were of analytical grade and of the highest purity.

Patients and samples

Twenty patients with a diagnosis of HT from the Federal University of Santa Maria Hospital were included in this study. The group of patients showed an average age of **55.0 ± 1.5** years old and positive thyroid peroxidase (TPO) antibody test. The control group was composed of 20 healthy subjects with an average age of **45.9 ± 2.3** years old and negative serology for HT. All the patients with HT had been in a current treatment with levothyroxine for at least two years. Fifteen milliliters of peripheral blood was obtained from each patient and used for platelet-rich plasm (PRP) preparation. The same procedure was carried out for the control group. The protocol was approved by the Human Ethics Committee from Federal University of Santa Maria, protocol number 23081.004278/2011-60.

Hormone measurements

Serum TSH (reference range 0.1-4.0 mU/L) and fT_4 levels (reference range 0.89-1.76 ng/dl) were measured by a chemiluminescent immunoassay method.

Quantitative determination of platelets

Total blood was collected in tubes containing 7.2 mg dipotassium EDTA as anticoagulant and the quantitative determination of platelets was performed by an automated haematology analyzer (SYSMEX XT-1800i, Roche Diagnostic, USA).

Platelet preparation

PRP was prepared by the method of Pilla et al. [12] modified by Lunkes et al. [13]. Briefly, peripheral blood was collected in 129 mM sodium citrate as anticoagulant and centrifuged at 160 x g for 15 min. Afterwards, the PRP was centrifuged at 1400 x g for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The washed platelets were resuspended in HEPES isosmolar buffer.

Cellular integrity

The integrity of the platelet preparation was confirmed by determining the lactate dehydrogenase (LDH) activity in intact and disrupted platelets using the kinetic method of Labquest apparatus (Diagnostics Gold Analyzer). The procedure was repeated before and after the incubation period. The protocol was carried out according to the manufacturer instructions. Triton X-100 (0,1 %, final concentration) was used to disrupt the platelet preparation. The enzymatic activity is expressed as units per liter; one unit (1U) corresponds to 1 μM of NADH formed per minute.

Protein determination

Protein was determined by the Coomassie blue method using bovine serum albumin as standard [14].

E-NTPDase and E-5'-NT activity determination

The E-NTPDase enzymatic assay in platelets was carried out in a reaction medium containing 5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose and 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 200 µL as described by Lunkes et al. [13]. For AMP hydrolysis, the E-5'-nucleotidase activity was carried out as previously described, except that the 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$. Twenty microliters of the isolated platelets (8-12 µg of protein) was added to the reaction mixture and pre-incubation proceeded for 10 min at 37ºC. The reaction was started by the addition of ATP or ADP at a final concentration of 1 mM, and AMP at a final concentration of 2 mM, and the time of incubation was 60 min. Both enzyme assays were stopped by the addition of 200 µL of 10% TCA to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. The inorganic phosphate (Pi) released was measured by the method of Chan et al. [15] using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for nonenzymatic hydrolyses of nucleotides by adding enzyme preparation after 10% TCA addition. All samples were run in triplicate. Enzyme-specific activities are reported as nmol of Pi released/min/mg of protein.

E-ADA activity determination

E-ADA activity from platelets was determined according to Giusti and Galanti [16] based on the direct measurement of the formation of ammonia produced when adenosine deaminase acts in excess of adenosine. Briefly, 50 µL of platelets reacted with 21 mmol/L of adenosine and was incubated at 37 ºC for 60 min, pH 6.5.
Afterwards, the reaction was stopped by adding a solution of 106.2 mM phenol and 167.8 µM sodium nitroprussiate and a 0.082% hypochlorite solution. The amount of

ammonia produced was measured at 620 nm and results were expressed in units per milligrams of proteins (U/mg of protein). One unit (1U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Western blot of protein ecto-nucleoside triphosphate diphosphohydrolase1 (ENTPD1) – CD39

Electrophoresis was performed using 12% polyacrylamide in a Bio-Rad Mini-Protean III apparatus. For Western blotting assays, peripheral blood platelets were lysated inside microtubes containing an extraction buffer (50 mM Tris HCl, 1 mM EDTA, 1 mM PMSF, pH 7,5) in the presence of Triton® X-100 (0.5%, v/v), glass pearls and vortexed for a minute, twice, on ice. Samples were centrifuged at 10,000 x g for 20 min at 4^{o}C. The protein present in the supernatant, determined by colorimetric assay [14], was diluted (1:1, v:v) in the Bio-Rad Laemmli sample buffer (62.5 mM Tris HCl, pH 6,8; 25% glycerol, 2% SDS, 0,01% Bromophenol Blue) added 5% of mercaptoethanol and then loaded (10 ug) and size-separated in 15% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE, 100V). The running buffer used contained 25 mM Tris, 192 mM glycine, and 0.5% SDS. The proteins were blotted onto a polyvinylidine difluoride (PVDF) membrane for 2 h (Bio-Rad) in blotting buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Subsequently, the membrane was incubated with anti-human ENTPD1 polyclonal antibody (primary antibody used at a dilution of 1:2000; eBiosciences) at room temperature overnight. The sensitivity and specificity of this antibody for rat antigen have been previously validated. The amount of protein was corrected in order to load a fixed concentration of protein (25 ug) in 12% SDS-PAGE, and it was determined based on preliminary experiments by using different concentrations of proteins. To ensure equal protein loading, we used the Ponceau method to Western blot [17]. Membranes were developed using the substrate of alkaline phosphatase, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

In vitro tests

The *in vitro* effects of levothyroxine on E-NTPDase, E-5’-nucleotidase and E-ADA activities were evaluated. Isolated platelets from healthy subjects were incubated with different concentrations of levothyroxine in the medium reaction as previously described. The concentration of drug used *in vitro* represents approximately the mean plasma values of the medication. [18]

Statistical analysis

Variables were expressed as mean ± standard error of the mean (SEM). The results were analyzed by the Student’s t test for independent samples. Differences were considered significant when the probability was $p<0.05$.

Results

Patient characteristics

The characteristics of patients with HT and controls in this study regarding age, quantitative determination of platelets, fT4 and TSH measurements are shown in Table1, and show no significant changes between groups ($p>0.05$).

Cellular integrity

Table 2 shows the quantification of intact platelets on the preparation and effect of platelet lyses on LDH activity in both groups. The measurement of LDH activity showed that approximately 7% of the platelets from both groups was disrupted indicating that the preparation was predominantly intact after the isolation procedure.

E-NTPDase and E-5’-nucleotidase activities

Figure 1A shows the E-NTPDase activity using ATP as substrate. No significant differences between the control and patients were observed. HT group (13.24 ± 1.10

nmol of Pi released/min/mg of protein), control group (12.10 ± 1.04 nmol of Pi released/min/mg of protein) (results are expressed as means±SEM, $p>0.05$, n=20). Figure 1B shows the E-NTPDase activity using ADP as substrate. No significant differences between the control and patients were observed. HT group (7.59 ± 0.60 nmol of Pi released/min/mg of protein), control group (7.52 ± 0.67 nmol of Pi released/min/mg of protein) (results are expressed as means±SEM, $p>0.05$, n=20). Figure 2 shows the E-5'-NT activity by AMP hydrolysis. No significant differences between the control and patients were observed. HT group (6.38 ± 0.64 nmol of Pi released/min/mg of protein), control group (6.38 ± 0.44 nmol of Pi released/min/mg of protein) (results are expressed as means±SEM, $p>0.05$, n=20).

E-ADA activity

Figure 3 shows the results obtained for E-ADA enzymatic activity in platelets. As can be observed, there were no significant differences between groups. HT group (5.67 ± 0.73 U ADA/mg of protein), control group (5.29 ± 0.74 U ADA/mg of protein) (results are expressed as means±SEM, $p>0.05$, n=20).

Western blot of protein E-NTPDase1 (CD39)

The expression of E-NTPDase1 in peripheral blood platelets was evaluated by Western blot analysis (Figure 4). The Western blot quantification of E-NTPDase1 protein content did not show any difference between the groups.

Effects of the drug used in the treatment of HT in E-NTPDase, E-5'-nucleotidase and E-ADA activities in platelets

Concentration of levothyroxine (70, 7.0, 0.7, 0.07, and 0.007 µg/mL) were tested *in vitro*. The results obtained, shown in Table 3, demonstrate that E-NTPDase, E-5'-nucleotidase and E-ADA activities were not affected by the presence of the pharmacological preparation at the concentrations cited above ($p>0.05$).

Discussion

Hypothyroidism is a frequent endocrine disorder in the general population, especially in women. HT is the most common cause of hypothyroidism: it is characterized by gradual thyroid failure due to autoimmune-mediated destruction of the thyroid gland. It was demonstrated that hypothyroid patients have an increased risk of atherosclerosis and myocardial infarction and several mechanisms have been proposed in hypothyroid patients. High levels of cholesterol and triglycerides, hyperhomocysteinemia, immune-complex-mediated vascular damage, hemostatic profile changes, weight gain, sensitivity to cold, motor slowness, dry or brittle hair, increased TSH levels, reduced T_4 levels, T-cell infiltrate in thyroid tissues and recently, endothelial dysfunctions were detected in hypothyroid patients without treatment [19,20].

The immune process is modulated by purinergic signaling, which involves extracellular purines, ectoenzymes and purinergic receptors [21]. Adenine nucleotides (ATP and ADP) and nucleoside adenosine are released from such cells as platelets into extracellular medium during infection and inflammatory process and act modeling vascular response, as agonist and antagonist in the platelet aggregation, respectively [22,23]. The ectoenzymes E-NTPDase, E-5'-nucleotidase and E-ADA are capable of regulate the concentration of extracellular nucleotides and nucleoside of adenine, controlling the inflammatory process [10].

Platelets are known to play a major role in the maintenance of endothelial integrity and homeostasis. They also participate in inflammatory responses as well as events leading to thrombus formation [24]. Alterations in platelet aggregation are demonstrated in patients with HT. Gullu et al. [25] showed that these patients have a hypocoagulable state. It could be suggested that generalized diminution in protein synthesis in HT may also cause decreases in the coagulation factors, however levothyroxine treatment improved the bleeding tendency in hypothyroidism.

Alterations in the activities of ectoenzymes have been observed in various diseases, suggesting that these could be an important physiological and pathological parameters [10,26,27,28,29]. In our study, we verified the activity of enzymes that degrade adenine nucleotides and nucleosides in patients with thyroiditis that make hormone replacement with levothyroxine. Our results showed no significant differences in E-NTPDase, E-5'-nucleotidase and E-ADA activities in patients with HT

in treatment with levothyroxine when compared with the control group. Since the enzymes, E-NTPDase, E-5’-nucleotidase and E-ADA are a multiple system for extracellular nucleotide hydrolysis, we suggest that their activities suffered no changes, because the treatment with levothyroxine reversed the possible effects of hypothyroidism.

Corroborating our results, Bruno et al. [30] found that results obtained with ATP and ADP hydrolysis were not altered in platelets of hypothyroid rats with or without treatment with levothyroxine, suggesting that the pathological conditions studied do not affect platelet ATP-diphosphohydrolase activity. We also evaluated the expression of E-NTPDase and results showed that there was no change in the expression of E-NTPDase in patients with Hashimoto's thyroiditis treated with levothyroxine compared with the control group corroborating the activity which was unchanged.

In our study, we demonstrated that there no significant changes in the enzyme E-5’-nucleotidase in patients treated with levothyroxine when compared with controls. Bruno et al. [30] demonstrated alterations in the AMP hydrolysis in rat platelets after the induction of hypothyroidism. This result indicates that the hypothyroidism altered platelet E-5’-nucleotidase activity. AMP hydrolysis was decreased by 50% in response to thyroid hormone deficiency. Furthermore, the T_4 replacement treatment was effective in decreasing the inhibition found in the E-5’-nucleotidase activity in platelets from adult hypothyroid rats as demonstrated in our study. Also, according to Bruno et al 2005, platelet aggregation was not altered in patients treated with levothyroxine.

Regarding the activity of E-ADA, no changes were observed between the groups, may indicate that this nucleoside is present in physiological levels in the extracellular medium.

In order to exclude a direct effect of levothyroxine used by patients with HT, in this study, we also tested E-NTPDase, E-5’-nucleotidase and E-ADA activities *in vitro* in the presence of this hormone in different concentrations. Levothyroxine did not affect the activity of these enzymes.

In this study we measured the levels of fT_4 and TSH in both groups. Other studies have showed that alterations in these hormones may lead to an extensive array of clinical manifestations [25,31,32,33,34]. However, we demonstrated that

thyroid hormones in patients with HT in treatment were in normal range, and the abnormalities being reversible with HT substitutive treatment.

In conclusion, the treatment with levothyroxine in patients with HT avoids changes in the activity of ectoenzymes, suggesting that this treatment reversed possible alterations caused by hypothyroidism.

Table 1. General characteristics and hormonal parameters from patients with HT and control group.

Variable	HT men	HT women	HT Total n=20	Control men	Control women	Control Total n=20
Gender	40%	60%	-	35%	65%	-
Age (years)	58.3 ± 1.9	51.2 ± 2.1	55.0 ± 1.5	47.5 ± 2.5	44.0 ± 2.2	45.9 ± 2.3
Platelets ($x10^3/\mu L$)[a]	244.6± 12.9	265.1 ± 16.4	253.8±14.5	210.6 ± 13.6	262.9 ± 139.2	235.0 ± 10.5
TSH (mU/l)[a]	1.468 ±0,3	2.008 ± 0.4	1.738 ± 0.2	2.057 ± 0.4	2.283 ± 0.3	2.170 ± 0.2
fT4 (ng/dl)[a]	1.24 ± 0.1	1.18 ± 0.1	1.21 ± 0.08	1.41 ± 0.1	1.35 ± 0.1	1.38 ± 0.07

Continuous variables are present as means±SEM and the other variables are shown as percentage of individuals

TSH: thyroid stimulating hormone, fT4: free thyroxin

[(a)]No statistical significances between groups (One-way ANOVA, P>0.05)

Table 2. Evaluation of cellular integrity on platelet preparation

Variable	HT group	Control group
Platelets ($x10^7$/mL)	12.3 ± 1.6 (5)	16.6 ± 2.2 (5)
LDH (intact platelets)	152.6 ± 24.0 (5)	116.46 ± 26.5 (5)
LDH (lysed platelets)	2242 ± 389.6 (5)	1640 ± 161.4 (5)

Results are present as means ± SEM with the number of experiments given in parenthesis.

Lactate dehydrogenase (LDH) activity is expressed as U/L.

No statistical significances between groups (One-way ANOVA, P>0.05)

Table 3. Effect of Levothyroxine on E-NTPDase, E-5'-nucleotidase and E-ADA activities in platelets from control individuals.

Drug (µg/mL)	ATP	ADP	AMP	ADA
0	17.70±0.45	11.05±2.58	5.062±0.94	6.08±0.72
0.0070	15.55±4.19	9.479±2.96	4.534±0.91	6.01±1.21
0.070	15.90±2.96	9.429±1.52	3.175±1.14	5.97±1.20
0.70	13.46±1.96	9.092±3.38	3.015±0.68	6.31±0.70
7.0	15.74±3.41	11.19±3.47	5.045±1.37	5.00±0.87
70	15.15±2.66	10.95±2.48	5.817±0.68	7.07±0.95

Valves represent mean ± Standard error from six control individuals (SEM)

No statistical significances between groups (One-way ANOVA, P>0.05)

Acknowledgements.

The financial support by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), PRONEX and Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

Disclosure of Conflicts of Interests

Author declares there are no actual or potential conflicts of interest.

References

1. Manji N, Carr-Smith Jd, Boelaert K, Alahabadia A, Chatterjee VK, Lazarus JH, Pearce SHS, Vaidya B, Gough SC, Franklyn JA. Influences of Age, Gender, Smoking, and Family History on Autoimmune Thyroid Disease Phenotype. The Journal of Clinical Endocrinology & Metabolism 2006; 4873-4880.

2. Panicker V. Genetics of Thyroid Function ad Disease. Clinical Biochemistry Reviews. 2011;32: 165-175.

3. Kostaglou-Athanassiou I, Ntalles K. Hypothyroidism – new aspects of an old disease. Hippokratia 2010; 4,2: 8: 82-87.

4. Liu Z, Wang H, Xiao W, Wang Chen, Liu G, Hong T. Thyrocyte interleukin-18 expression is up-regulated by interferon- γ and may contribute to thyroid destruction in Hashimoto's thyroiditis. International Journal of Experimental Pathology 2010; 91:420-425.

5. Rebuffat SA, Nguyen B, Robert B, Castex F, Peraldi-Roux S. Antithyoperoxidase Antibody-Dependent Cytotoxicity in Autoimmune Thyroid Disease. Journal of Clinical Endocrinology & Metabolism 2008; 93:929-934.

6. Maclachlan SM, Rapoport B. Genetic and epitopic analysis of thyroid peroxidase (TPO) autoantibodies: markers of the human thyroid autoimmune response. Clinical & Experimental Immunology 1995; 101:200-206.

7. Özen S, Berk O, Simsek DG, Darcan S. Clinical Course of Hashimoto's Thyroiditis and Effects of Levothyroxine Therapy on the Clinical Course of the Disease in Children and Adolescents. Journal of Clinical Research in Pediatric Endocrinology 2011; 3(4):192-197.

8. Ralevic V, Burnstock G. Involvement of purinergic signaling in cardiovascular diseases. Drug News & Perspectives 2010; 16:133-140.

9. Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signalling 2006; 89:83-89.

10. Zanini D, Schmatz R, Pimentel VC, Gutierres JM, Maldonado PA, Thomé GR, Cardoso AM, Stefanello N, Oliveira L, Chiesa J, Leal DB, Morsch VM, Schetinger MR. Lung cancer alters the hydrolysis of nucleotides and nucleosides in platelets. Biomedicine & Pharmacotherapy 2012; 66:40-45.

11. Fadeyev VV, Morgunova TB, Sytch JP, Melnichenko GA. TSH and thyroid hormones concentrations in patients with hypothyroidism receiving replacement therapy with L-thyroxine alone or in combination with L-triiodothyronine**.** Hormones 2005; 4:101-107.

12. Pilla C, Emanuelli T, Frassetto SS, Battastini AMO, Dias RD, Sarkis JJF. ATP diphosphohydrolase activity (Apyrase, EC 3.6.1.5.) in human blood platelets. Platelets 1996; 7: 225-230.

13. Lunkes IG, Lunkes D, Stefanello F, Morch A, Morch MV, Mazzantti MC, Schetinger MCR. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thrombosis Research 2003; 109:189-194.

14. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of proteins-dye binding. Analytical Biochemistry 1976; 72:248-54.

15. Chan KM, Delfert D, Junger KD. A direct colorimetric assay for Ca2+-stimulated ATPase activity. Analytical Biochemistry 1986; 157:375-80.

16. Giusti G, Galanti B. Colorimetric Method. In: Bergmeyer HU, editor. Methods of enzymatic analysis. Verlag Chemie: Weinheim 1984; 315-323.

17. Abercrombie ED, Keefe KA, Difrischia DS, Zigmond MJ. Differential effects of stress on in vivo dopamine release in striatum, nucleus acumbens and medial frontal cortex. Journal of Neurochemistry 1989; 52: 1655-1658.

18. Silva CMS, Souza MVL. Autoimmune hypothyroidism nonresponsive to high doses of levothyroxine and severe hypocalcemia.. Arquivos Brasileiros de Endocrinologia & Metabologia 2005, vol.49, n.4, pp. 599-603.

19. Centikalp S, Tobu M, Karadeniz M, Buyukkeçeci f, Yilmaz C. The effect of hormone replacement treatment on thrombin-activatable fibrinolysis inhibitor activity levels in patients with Hashimoto thyroiditis. Internal Medicine 2009; 48:281-285.

20. Quarantino S. Models of autoimmune thyroiditis. Drug Discovery today: Disease Models 2004; 1:417-423.

21. Ralevic V; Burnstock G. Receptors os Purines and Pyrimidines. Pharmacological Reviews 1998; 50:413-492.

22. Yegutkin, GG. Nucleotide and nucleoside converting ectoenzymes: important modulators of purinergic signalling cascade. Biochimica et Biophysica Acta 2008; 1783: 673-694.

23. Gordon EL, Pearson JD, Dickinson ES, Moreau D, Slakey LL, The hydrolysis of extracellular adenine nucleotides by arterial smooth muscle cells. Regulation of adenosine production at the cell surface. The Journal of Biological Chemistry 1989; 264:18986-95.

24. Marcus AJ, Broekman MJ, Drosopoulos JH, et al. The endotelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. Journal of Clinical Investigation 1997;996:1351-1360.

25. Gullu S, Sav H, Kamel N. Effects of levothyroxine treatment on biochemical and hemostasis parameters in patients with hypothyroidism. European journal of endocrinology 2005; 152:355:361.

26. Maldonado PA, Corrêa MC, Becker LV, et al. Ectonucleotide pyrophosphatase/ phosphodiesterase (E-NPP) and adenosine deaminase (ADA) activities in patients with uterine cervix neoplasia. Clinical Biochemistry 2008;41:400-406.

27. Bagatini MD, Martins CC, Battisti V, et al. Hydrolysis of adenine nucleotides in platelets from patients with acute myocardial infarction. Clinical Biochemistry 2008;41:1181-1185.

28. Schmatz R, Schetinger MRC, Spavanello RM, et al. Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats. Life Sciences 2009; 84:345-350.

29. Lara V. Becker LV, Rosa CS, Souza VCG, Bagatini MD, Casali EA, Leal CAM, Silva JCN, Moretto MB, Pinheiro FV, Morsch VM, Schetinger MRC, Leal DBR. Activities of enzymes that hydrolyze adenine nucleotides in platelets from patients with rheumatoid arthritis. Clinical Biochemistry 2010;43:1096-1100.

30. Bruno AN, Pochmann D, Ricachenevsky FK, Bonan CD, Barreto-Chaves MLM, Sarkis JJF. 5'nucleotidase activity is altered by hypo – and hyperthyroidism in platelets from adult rats. Platelets 2005; 16:25-30.

31. Mayer Jr O, Simon J, Filipovsky J, Plásková M, Pikner R. Hypothyroidism in coronary heart disease and its relation to selected risk factors. Vascular Health and Risk Management 2006; 2:499-506.

32. Okosieme OE, Belludi G, Spittle K, kadiyala R, Richards J. Adequacy of thyroid hormone replacement in a general population. Q. J. Med 2011;104:395-401.

33. Gerdes AM, Lervasi G. Thyroid replacement therapy and heart failure. Circulation 2010;122:385-393.

34. Massunaga R, Nagasaka A, Nakai A, Kotake M, Sawai Y, Oda N, et al. Alterations of platelet agregation in patients with thyroid disorders. Metabolism 1997;46:1128-1131.

Figure legends

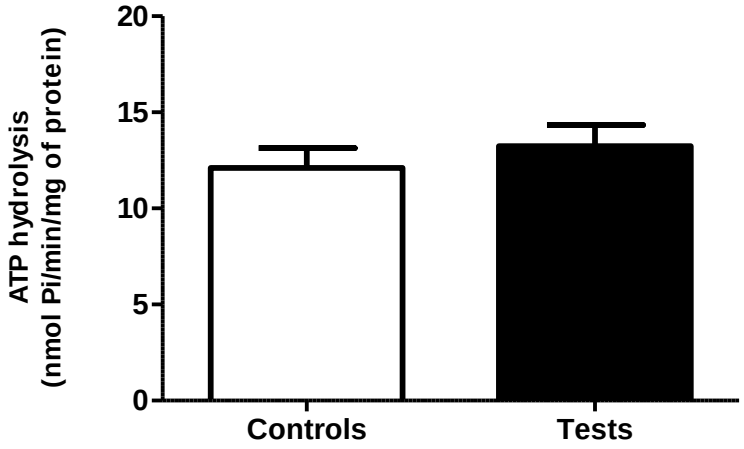
Figure 1. ATP(A) and ADP(B) hydrolysis in platelets of patients with HT in treatment with levothyroxine. Results are expressed as means±SEM. (Student's T test, *P*>0.05, n=20). Enzymes specific activities are reported as nmol Pi released/min/mg of protein.

Figure 2. AMP hydrolysis in platelets of patients with HT in treatment with levothyroxine. Results are expressed as means±SEM (Student's T test, *P*>0.05, n=20). Enzymes specific activities are reported as nmol Pi released/min/mg of protein.

Figure 3. E-ADA activity in platelets of patients with HT in treatment with levothyroxine. Results are expressed as means±SEM (Student's T test, *P>0.05, n=20).* Enzyme activities are reported as U/mg of protein.

Figure 4. Expression of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase1), in platelets of patients with HT in treatment with levothyroxine. Densitometric analysis (arbitrary units A.U.) of the protein NTPDase1. Data are represented as means ± SEM (Student's T test, p>0.05, n=6).

(A)


ATP hydrolysis
(nmol Pi/min/mg of protein)
20
15
10
5
0
Controls
Tests

(B)

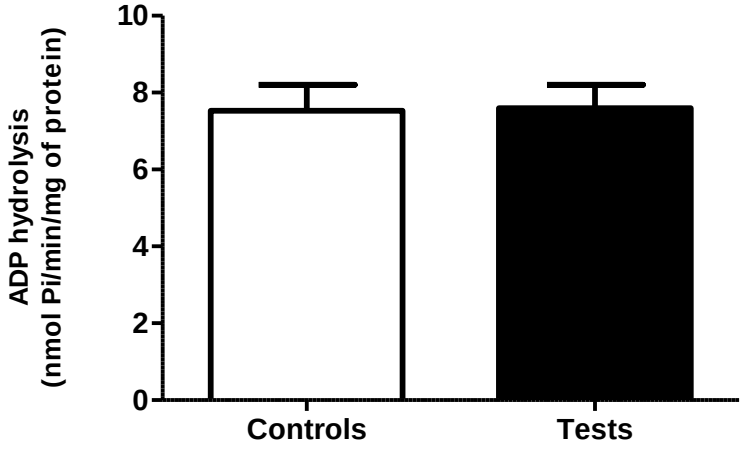

ADP hydrolysis
(nmol Pi/min/mg of protein)
10
8
6
4
2
0
Controls
Tests

Figure 1

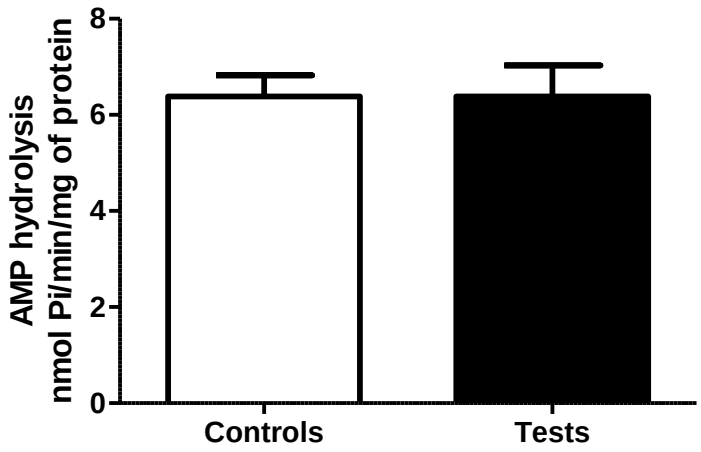

AMP hydrolysis
nmol Pi/min/mg of protein
8
6
4
2
0
Controls
Tests

Figure 2

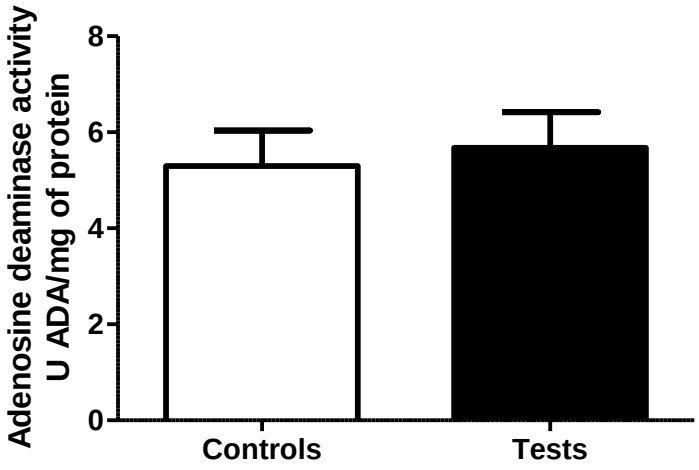

Adenosine deaminase activity
U ADA/mg of protein
8
6
4
2
0
Controls
Tests

Figure 3

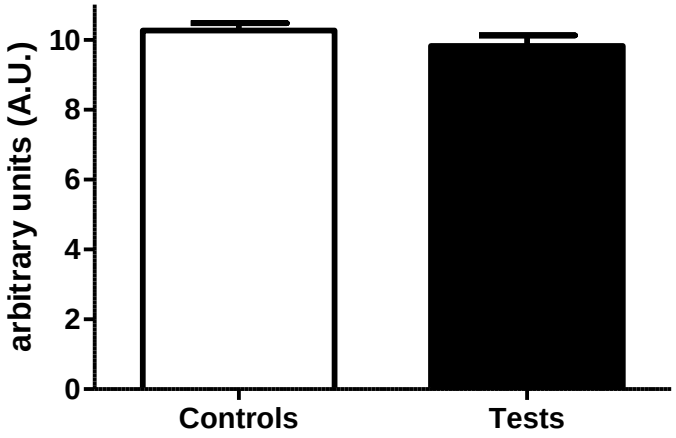


Figure 4

4 CONCLUSÕES

. A atividade das enzimas E-NTPDase, E-5’-nucleotidase e E-ADA, assim como a expressão da E-NTPDase1, não apresentou alterações significativas nos pacientes com TH em tratamento com levotiroxina em comparação com o grupo controle, sugerindo-se que a reposição hormonal nesses pacientes pode reverter os efeitos causados pelo hipotireoidismo não tratado.

. A levotiroxina, por si só, não foi capaz de alterar a atividade das enzimas E-NTPDase, E-5’-nucleotidase e E-ADA *in vitro*.

5 REFERÊNCIAS BIBLIOGRÁFICAS

ABBRACCHIO, M.P. et al. Purinergic signalling in the nervous system: an overview. **Trends in Neurosciences**, v.32, p. 19-29, 2009.

AMINO, N. et al. Hashimoto's Disease and Dr. Hakaru Hashimoto. **Endocrine Journal**, v.49, p. 393-397,2002.

ARAN, J.M. et al. Presence of adenosine deaminase on the surface of mononuclear blood cells: immunochemical localization using light and electron microscopy. **The Journal of Histochemistry and Cytochemistry**, v. 39, p. 1001-1008, 1991.

ATKINSON, B. et al. Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: potential as therapeutic targets. **Blood Cells, Molecules & Diseases**, v.36, p.217-222, 2006.

BERNE, R.M. et al. **Fisiologia**. 5. ed. Rio de Janeiro: Elsevier, p.915-940, 2004.

BIOGONESE, F. et al. Cloning and characterization of mouse triphosphate diphosphohydrolase 8. **Biochemistry**, v.43, p.5511-5519, 2004.

BONAN, C.D. et al. Ectonucleotidases and Synaptic Plasticity: implications in physiological and pathological conditions. **Drug Development Research**, v.52, p. 57-65, 2001.

BOROWIEC, A. et al. Adenosine as a metabolic regulator of tissue function:production of adenosine by cytoplasmic 5'-nucleotidase. **Acta Biochimica Polonica**, v.53, p. 269-278, 2006.

BOSSOWSKI, A. et al. Cytometric evaluation of intracellular IFN-γ and IL-4 levels in thyroid follicular cells from patients with autoimmune thyroid diseases. **Thyroid Research**. v. 4, p.5-13, 2011.

BRUNO, A.N. et al. 5'nucleotidase activity is altered by hypo –and hyperthyroidism in platelets from adult rats. **Platelets**. v. 16, p. 25-30, 2005.

BURNSTOCK, G.; KNIGHT, G.E. Cellular distribution and functions of P2 receptors subtypes in different systems. **International Reviews of Cytology**, v.240, p.31-304, 2004.

BURNSTOCK, G.; VERKHRATSKY, A. Long-term (trophic) purinergic signalling: purinoceptors control cell proliferation, differentiation and death. **Cell Death and Disease**, v. 9, 1-10, 2010.

CHISTIAKOV, D. A.; TURAKULOV, R. I. CTLA-4 and its role in autoimmune thyroid disease. **Journal of Molecular Endocrinology**. v. 31, p. 21-36, 2003.

CUNHA, R.A. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. **Neurochemistry International**, v. 38, p. 107-25, 2001.

DAHLBÄCK B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. **Journal Internal Medicine**, v. 257, p. 209-223, 2005.

DI VIRGILIO, F. et al. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. **Blood**, v.97, n.3, p.587-600, 2001.

DUBYAK, G.R.; ELMOATASSIM, C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. **The American Journal of Physiology**, v.265, p.577-606, 1993.

DULTZ, G. et al. The Protein Tyrosine Phosphatase Non-Receptor Type 22 C1857T Polymorphism Is a Joint Susceptibility Locus for Immunthyroiditis and Autoimmune Diabetes. **Thyroid**, v.19, p.143-148, 2009.

DUNWIDDIE, T.; MASINO, S. The role and regulation of adenosine in the central nervous system. **Annual Review of Neuroscience**, v.24, p.31-55, 2001.

ERIKSSON, N. et al. Novel Associations for Hypothiroidism Include Know Autoimmune Risk Loci. **PLoS ONE**, v.7,e34442, 2012.

FIORILLO, E. et al. Autoimmune-associated PTPN22 R620W Variation Reduces Phosphorylation of Lymphoid Phosphatase on an Inhibitory Tyrosine residue. **Journal of Biological Chemistry**,v.285, p.26506-26518, 2010.

FISCHER, G. F. Molecular Genetics of HLA. **Vox Sanguinis**, V. 78, p.261-264, 2000.

FRANCO, R. et al. Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. **Immunological Reviews**, v. 161, p. 27-42, 1998.

FRASSETTO, S.S.; DIAS, R.D.; SARKIS, J.J.F. Characterization of an ATP diphosphohydrolase activity (EC 3615) in rat blood platelets. **Molecular and Cellular Biochemistry**, v. 1, p. 47-55, 1993.

GAKIS, C. Adenosine deaminase (ADA) isoenzymes ADA1 and ADA2: diagnostic and biological role. **The European Respiratory Journal**, v. 9, p. 623-624, 1996.

GOLDMAN, L.; AUSIELO, D. **Cecil-Tratado de Medicina Interna**. 22. ed. 2v. Rio de Janeiro: Elsevier, p. 1617-1636, 2005.

GORELL, M.D.; GYSBERS, V.; McCAUGHAN, G.W. CD26: a multifunctional integralmembrane and secreted protein of activated lymphocytes. **Scandinavian Journal of Immunology**, v. 54, p. 249-264, 2001.

GUYTON, A.C.; HALL, J.E. **Tratado de Fisiologia Médica**. 2v. 10. ed. Rio de Janeiro: Guanabara e Koogan, p. 802-812, 2002.

HARPER'S ILLUSTRATED BIOCHEMISTRY, 26ª Ed. International edition, The McGraw-Hill Companies,434, 2003.

HASKÓ, G.; CRONSTEIN, B.N. Adenosine: an endogenous regulator of innate immunity. **Trends in Immunology**, v. 25, p. 33-39, 2004.

HUNSUCKER, S.A.; MITCHELL, B.S.; SPYCHALA, J. The 5'-nucleotidases as regulators of nucleotide and drug metabolism. **Pharmacology & Therapeutics**, v.107, p. 1-30, 2005.

IWAKI-EGAWA, S.; YAMAMOTO, T.; WATANABE, Y. Human plasma adenosine deaminase 2 is secreted by activated monocytes. **Biological Chemistry**, v. 387, p. 319-321, 2006.

JUNQUEIRA, L.C.; CARNEIRO, J. **Histologia Básica**. 11. ed. Rio de Janeiro: Guanabara e Koogan, p. 406-411, 2008.

KAHNER, B.N. et al. Nucleotide receptor signaling in platelets. **Journal of Thrombosis and Haemostasis**. v.4, p. 2317-2326, 2006.

KAWASHIMA, Y.; NAGASAWA, T.; NINOMIYA, H. Contribuition of ecto-5'-nucleotidase to the inhibition of platelet aggregation by human endothelial cells. **Blood**, v. 96, p. 2157-2162, 2000.

KEATING, F. et al. Relation of augment platelet reactivity the magnitude of distribuition of atherosclerosis. **The American Journal of Cardiology**, v.94, p. 725-728, 2004.

LATINI, S.; PEDATA, F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. **Journal of Neurochemistry**, v. 79, p. 463-484, 2001.

LEAL, D.B.R. et al. Characterization of NTPDase (NTPDase1; ecto-apyrase; ecto-diphosphohydrolase; CD39; E.C. 3.6.1.5) activity in humans lymphocytes. **Biochimica et Biophysica Acta**, v. 1721, p.9-11, 2005.

LORENZI. **Manual de Hematologia**. 2º ed., Editora MEDSI, Rio de Janeiro,1999.

LUTHJE, J. Origin, metabolism and function of extracellular adenine nucleotides in the blood. **Klinische Wochenschrift**, v.67, p.317-327, 1989.

MARCUS, A.J.; SAFIER, L.B. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. **The FASEB Journal**, v.7, p.516-22, 1993.

McLEOD, D. S. Current concepts and future directions in differentiated thyroid cancer. **The Clinical Biochemist Reviews**. v. 31, p. 9-19, 2010.

MICHELS, A.W.; EISENBARTH, G.S. Immunologic endocrine disorder. **Journal of Allergy and Clinical Immunology**, v.125, p.226-237, 2010.

OZSAHIN, H. et al. Adenosine Deaminase Deficiency in adults. **Blood**, v.89, p.2849-2855, 1997.

PILLA, C. et al. ATP diphosphohydrolase activity (apyrase EC 3.6.1.5.) in human blood platelets. **Platelets**, v.7, p.225-230, 1996.

RAJPUT, R.; CHATTERJEE, S.; RAJPUT, M. Can levothyroxine be taken as evening dose? Comparative evaluation of morning versus evening dose of levothyroxine in treatment of hypothyroidism. **Journal of Thyroid Research**, v.2011, p. 1-5, 2011.

RALEVIC, V.; BURNSTOCK, G. Involvement of purinergic signaling in cardiovascular disease. **Drug News & Perspectives**, v.16, p.133-140, 2003.

REMIJIN, J.A. et al. Role of ADP receptor P2y12 in platelet adhesion and thrombus formation in flowing blood**. Aterioscleros**is**, Thrombosis and Vascular Biology**, v.22, p.686-691, 2002.

RESTA, R.; YAMASHITA, Y.; THOMPSON, L.F. Ecto-enzyme and signaling functions of lymphocyte CD73. **Immunological Reviews**, v.161, p.95-109, 1998.

REVEL, T.; DOGHMI, K. Physiologie de l'hémostase. **EMC-Odontologie**, v. 1, p. 71-81, 2004.

ROBERTI. A. et al. Concomitância da tireoidite de Hashimoto e o carcinoma diferenciado da tireóide. **Revista do Colégio Brasileiro de Cirurgiões**, v.33, p.345-349, 2006.

ROBSON, S.C.; SÉVIGNY, J.; ZIMMERMANN, H. The NTPDase family of ectonucleotidases: structure function relationship and pathophysiological significance. **Purinergic Signalling**, v.2, p.409-430, 2006.

RUSSELL, R. Mechanisms of disease: Atherosclerosis – an inflammatory disease. **The New England Journal of Medicine**, v.320, n.2, p.115-126, 1999.

SARANAC L. et al. Why is the thyroid so prone to autoimmune disease? **Hormone research in Paediatrics**, v.75, p.157-165, 2011.

SARKIS, J. et al. ATP diphosphohydrolases: and overview. **Journal of the Brazilian Association for the Advancement of Science**, v.43, p.131-136, 1995.

SEMPLE J.W.; ITALIANO JR. J.E.; and FREEDMAN J. Platelets and the immune continuum. **Nature Reviews Immunology**, v.11, p. 264-274, 2011.

SETIAN N. Hypothyroidism in children: diagnosis and treatment. **Jornal de Pediatria**, v.83, p.209-216, 2007.

SGARBI J.A.; MACIEL R.M.B. Patogênese das doenças tiroidianas autoimunes. **Arquivo Brasileiro de Endocrinologia & Metabologia**, v,53. P.5-13, 2009.

SHAROYAN, S. et al. Influence of dipeptidyl peptidase IV on enzymatic properties of adenosine deaminase. **Acta Biochimica Polonica**, v.53, p. 539- 546, 2006.

SHI, J. et al. Molecular cloning and characterization of a novel mammalian endo apyrase. **The Journal of Biological Chemistry**, v. 276, p.17471-17478, 2001.

SOSLAU, G.; YOUNGPRAPAKORN, D. A possible dual physiological role of extracellular ATP in the modulation of platelet aggregation. **Biochimica et Biophysica Acta**, v. 1355, p. 131-140, 1997.

STAII, A. et al. Hashimoto thyroiditis is more frequent than expected when diagnosed by cytology which uncovers a pre-clinical state. **Thyroid Research,** v.3, p. 1-7, 2010.

STRUKOVA, S.M. Thrombin as a regulator of inflammation and reparative processes in tissues. **Biochemistry**, v.66, p.8-18, 2001.

VANNI, D.S. et al. Óxido nítrico: inibição das plaquetas e participação na formação do trombo. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v.43, n.3, p.181-189, 2007.

VORCHHEIMER, D.A.; BECKER R. Platelets in atherothrombosis. **Mayo Clinic Proceedings**, v.81, p.59-68, 2006.

WAGNER, D.D.; BURGER, P. Platelets in inflammation and thrombosis. Ateriosclerosis. **Thrombosis and Vascular Biology**, v. 23, p.2131-2137, 2003.

YAMASHIRO I. et al. Achados ultra-sonográficos na tireoidite. **Radiologia Brasileira**, v.40, p.75-79, 2007.

YANGO, J. et al. Evaluation of the respective influence of thyroid hormones and TSH on blood oagulation parameters after total thyroidectomy. **European Journal of Endocrinology**, v.164, p.599-603, 2011.

YEGUTKIN, G.G. Nucleotide and nucleoside converting ectoenzymes: important modulators of purinergic signalling cascade. **Biochimica et Biophysica Acta**, v.1783, p.673-694, 2008.

YOKOI, N. et al. Role of major histocompatibility complex class II in the development of autoimune type 1 diabetes and thyroiditis in rats. **Genes in Immunity**. v. 13, p. 139-145, 2012.

ZAGO, M. A.; FALCÃO, R. P.; PASQUINI, R. **Hematologia: fundamentos e prática**. 1º ed. São Paulo: Atheneu, 2004.

ZANINI, D. et al. Lung cancer alters the hydrolysis of nucleotides and nucleosides in platelets. **Biomedicine & Pharmacoterapy**, v.66, p.40-45, 2012.

ZAVIALOV, A.V; ENGSTROM, A. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. **The Biochemical Journal**, v. 391, p. 51-57, 2005.

ZIGANSHIN, A. U.; HOYLE, C.; BURNSTOCK, G. Ecto-enzimes and metabolism of extracellular ATP. **Drug Developmental Research**. v. 32, p. 134-146, 1994.

ZIMMERMANN, H. Biochemistry, localization and functional roles of ecto nucleotidases in the nervous system. **Progress in Neurobiology**, v. 49, n°6, p. 589-618, 1996.

ZIMMERMANN, H. et al. New insights into molecular structure and function of ectonucleotidases in the nervous system. **Neurochemistry International**, v.32, p.421-425, 1998.

ZIMMERMANN, H. Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. **Trends in Pharmacological Sciences**, v.20, p.231-236, 1999.

ZIMMERMANN, H. Ectonucleotidases: some recent developments and a note on nomenclature. **Drug Developmental Research**, v. 52, p.44-56, 2001.

ZIMMERMANN, H.; ZEBISCH M,; STRATER, N. Cellular function and molecular structure of ecto-nucleotidases. **Purinergic Signalling**, v.8, p.437-502, 2012.

6 Anexos

6.1 Anexo A - Carta de Aprovação do Comitê de Ética

MINISTÉRIO DA SAÚDE Conselho Nacional de Saúde Comissão Nacional de Ética em Pesquisa (CONEP)	UNIVERSIDADE FEDERAL DE SANTA MARIA Pró-Reitoria de Pós-Graduação e Pesquisa Comitê de Ética em Pesquisa - CEP- UFSM REGISTRO CONEP: 243

CARTA DE APROVAÇÃO

O Comitê de Ética em Pesquisa – UFSM, reconhecido pela Comissão Nacional de Ética em Pesquisa – (CONEP/MS) analisou o protocolo de pesquisa:

Título: Atividade de enzimas que degradam nucleosídeos e nucleotídeos da adenina em linfócitos e plaquetas em pacientes com tireóide de hashimoto.
Número do processo: 23081.004278/2011-60
CAAE (Certificado de Apresentação para Apreciação Ética): 0043.0.243.000-11
Pesquisador Responsável: Daniela Rosa Leal

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes estabelecidas na Resolução 196/96 e complementares do Conselho Nacional de Saúde. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.
O pesquisador deve apresentar ao CEP:

Agosto/ 2012- Relatório final

Os membros do CEP-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 15/06/2011

Santa Maria, 15 de Junho de 2011

Félix A. Antunes Soares
Coordenador do Comitê de Ética em Pesquisa-UFSM
Registro CONEP N. 243.

6.2 Anexo B – Questionário utilizado com os pacientes

COLETA DE DADOS

Título do projeto:

“A avaliação enzimática em linfócitos e plaquetas de pacientes com Tireoidite de Hashimoto”

Pesquisadora responsável: Profª. Drª. Daniela Bitencourt Rosa Leal – UFSM

Instituição/Departamento: Departamento de Microbiologia e Parasitologia – UFSM

Telefone para contato: (55) 3220-9581 e/ou (55) 9144-5635

Identificação/número:_____

Data da coleta:_____

Responsável pela coleta:_____

1. Paciente com Tireoidite de Hashimoto está em tratamento?

( ) SIM ( ) NÂO Se sim, com qual medicamento e dose:_____

2. Porque você procurou auxílio médico?_____

3. Quais sintomas você tem:

( ) Sonolência

( ) Cansaço

( ) Falta de memória

( ) Ganho de peso

( ) Cabelo fino, fraco

( ) Bócio

( ) Infertilidade

( ) Outros. Quais?_____

4. Paciente é fumante? ( ) Sim ( ) Não

5. Possui alguma das seguintes doenças?

( ) Hipertensão ( ) Diabetes tipo I ( ) Lúpus ( ) Artrite Reumatóide ( ) Vitiligo

Outras? Quais_____

6. Possui familiares com Tireoidite de Hashimoto? ( ) Sim ( ) Não

Se sim, qual o grau de parentesco?_____

Observações:_____
_____

6.3 Anexo C – Normas do periódico Thrombosis Research

Guide for Authors

I. SUBMISSION OF ARTICLES

A. All Articles and Material should be submitted on-line via EES http://ees.elsevier.com/tr. Please refer to the 'Tutorial for Authors' located on the EES site for guidance on the electronic submission process.

If you are unable to submit online or have any general queries, please contact the Editorial Offices regarding alternatives.

Editor in Chief for the Americas (USA, Canada, Mid - and South America): Dr Charles Francis, Hematology/Oncology, University of Rochester Medical Center, PO Box 610, Rochester NY 14642, USA;

Editor-in-Chief for the Rest of the world: P.M.Sandset, Ulleval University Hospital, Department of Hematology, Hematological Research Lab., N-00407 Oslo, Norway; Email: thrombosis-research@ioks.uio.no

Senior Associate Editor for Review Articles:: Dr Nigel Key, University of North Carolina, 932 Mary Ellen Jones Building, CB #7035, Chapel Hill, NC 27599, USA; Email: nigel_key@med.unc.edu

B. Letters to the Editors-in-Chief should be submitted online via EES http://ees.elsevier.com/tr/

Communication Categories: Several categories of manuscripts will be considered for publication. (1) Original Articles are full length reports of original work. (2) Review Articles Review Articles constitute a literature review of a particular area and can be clinical or concentrate on a basic science topic. Review Articles are often commissioned but if you would like to submit a proposal, please contact Dr Nigel Key at the above address. Proposals for a series of review articles under one main heading will also be considered. Mini reviews, providing a concise overview of the current research and/or clinical status of the topics under discussion, will also be considered. (3)Letters to the Editors-in-Chief. These contributions should be

concerned with matters of opinion and criticism on contributions published in the journal and other matters of interest to researchers in our field. (4) Editorials provide comments on matters significant to the readers of Thrombosis Research. (5) Reports of Scientific Meetings are published from time-to-time. Please contact the Editors-in-Chief regarding these. (6) Supplement issues may cover various topics in the field of thrombosis and hemostasis. They are approved by the Editors-in-Chief and edited preferably by one of the Editors of Thrombosis Research. In addition to the above categories various News Items and Announcements are printed.

Length of Communications: Authors are asked to limit their (a) Original Articles and Reports of Scientific Meetings to 5-6,000 words, (b) Letters to the Editors-in-Chief to 1,500 words, and (c) News Items and Announcements to 500 words. The length of review articles and mini reviews is flexible and should be discussed with the Senior Associate Editor for Review Articles on a case-by-case basis.

Authorship: All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. Please list up to six authors before using "et al".

Acknowledgements: All contributors who do not meet the criteria for authorship as defined above should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Authors should disclose whether they had any writing assistance and identify the entity that paid for this assistance.

Conflict of interest: At the end of the text, under a subheading "Conflict of interest statement" all authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Role of the funding source: All sources of funding should be declared as an acknowledgement at the end of the text. Authors should declare the role of study sponsors, if any, in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. If the study sponsors had no such involvement, the authors should so state.

Randomized controlled trials

All randomized controlled trials submitted for publication in Thrombosis Research should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart. Please refer to the CONSORT statement website at http://www.consort-statement.org for more information. Thrombosis Research has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) which require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Trials must register at or before the onset of patient enrolment. The clinical trial registration number should be included at the end of the abstract of the article. For this purpose, a clinical trial is defined as any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects of health outcomes. Health-related interventions include any intervention used to modify a biomedical or health-related outcome (for example drugs, surgical procedures, devices, behavioral treatments, dietary interventions, and process-of-care changes). Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) will not require registration. Further information can be found at http://www.icmje.org. If a CONSORT flow chart is not included with the submitted manuscript, authors should provide a statement explaining the omission.

Disclosure of clinical trial results

In line with the position of the International Committee of Medical Journal Editors, Thrombosis Research is willing to consider manuscripts which include results posted in the same clinical trials registry in which primary registration resides. It will not consider such postings to be prior publication, providing the results are presented in

the form of a brief structured (500 words) abstract or table. However, divulging results in other circumstances (eg, investors' meetings) is strongly discouraged and may jeopardize consideration of the manuscript. Authors should fully disclose all postings in registries of the same or closely related work.

Ethics:

Identifying information, including patients' images, names, initials, or hospital numbers, should not be included in videos, recordings, written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and you have obtained written informed consent for publication in print and electronic form from the patient (or parent, guardian or next of kin where applicable). If such consent is made subject to any conditions, Elsevier must be made aware of all such conditions. Written consents must be provided to Elsevier on request. Even where consent has been given, identifying details should be omitted if they are not essential. If identifying characteristics are altered to protect anonymity, such as in genetic pedigrees, authors should provide assurance that alterations do not distort scientific meaning and editors should so note. If such consent has not been obtained, personal details of patients included in any part of the paper and in any supplementary materials (including all illustrations and videos) must be removed before submission.

Supplementary material

Thrombosis Research accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please ensure that data is provided in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions

Online-only Publication

Thrombosis Research offers authors the opportunity to select online-only publication as their preferred option for publishing their paper in the journal, rather than print publication. If authors wish to do this, their paper will be published online on Science Direct as a paginated and fully citable electronic article. It will be listed in the contents page of a printed issue and the full citation and abstract will be published in print. The citation and abstract of the paper will also still appear in the usual abstracting and indexing databases.

Authors will be asked to select which publication option they would prefer when submitting their paper to the Editorial Office. Please note that e-publication is selected at the Editors discretion. Authors will be notified if their manuscript has been selected for e-publication upon acceptance of the manuscript.

II. FORMATTING INSTRUCTIONS

General Information:

It is the responsibility of the authors to write in standard, grammatical English. Spelling may be British or American, but must be consistent throughout the text, tables and legends to tables and figures. A word count should be provided in the 'Enter Comments' section of EES and on the title page of the manuscript file. Original articles and Review articles should be organized as follows: (a) Title page; (b) Abstract, Keywords, and Abbreviations; (c) Text with the following sections; Introduction, Materials and Methods, Results, Discussion, Acknowledgments; (d) References; (e) Tables; (f) Table Legends and Figure Legends. The pages should be numbered consecutively; the Title page is page 1, the Abstract page 2, etc. throughout the manuscript (including References, Tables, and Legends to Tables and Figures). Brief Communications have no abstract but a summary at the end of the discussion.

(a) Title Page: Identify the category of the communication on top of the page. Include a brief and descriptive Title of the article, the full Name(s) of the Author(s)(in the format First Name, Initials, and Surname) and the Name and Location of the institution where the research was carried out. A word count of the text should include Tables and Legends. Exclude the Abstract and Reference list. The name,

postal and email addresses, telephone and fax numbers of the corresponding author should be included at the bottom of the title page as well as, if necessary, additional addresses of other Authors. If the manuscript was presented at a meeting, the name of the organization, the place and the date on which it was read must be indicated.

(b) Abstract Page: The abstract is essential and the most read part of the paper. It should be informative, not descriptive, and should avoid abbreviations except for units of measure. An abstract for a regular article should not exceed 250 words and should end with the principal conclusions of the study. Structured abstracts are encouraged and should use the following headings: Introduction, Materials and Methods, Results and Conclusions.

Keywords and Abbreviations should follow the abstract and be on the same page (or on a separate page if no abstract). List up to 6 key words for subject indexing, preferably to be taken from Index Medicus. List all abbreviations used.

(c) Text of Articles: The text should be arranged as follows: Introduction, without heading, should state the purpose of the investigation and give a short review of pertinent literature.

Materials and methods should be described in detail with appropriate information about patients or experimental animals. Authors should stipulate that informed consent was obtained when applicable to research on humans, with the comment that the study was approved by the institutional Ethics Committee on human research. Manuscripts reporting animal experiments must include the statement that all animals received care in compliance with the American, European, or any other Convention on Animal Care, with the comment that the study was approved by the institutional Ethics Committee. Generic names of drugs and equipment should be used throughout the manuscript with brand names (proprietary name) and the name and location (city, state, country) of the manufacturer in parentheses when first mentioned in the text.

Results should be reported concisely. Results presented either in tables or figures should be commented on in the text.

Discussion is an interpretation of the results and their significance with reference to pertinent work by other authors. It should be clear and concise. The importance of the study and its limitations should be discussed.

Acknowledgments of financial or personal assistance should be placed at the end of the text.

(d) References: Consecutive numbers in square brackets should be used to indicate references in the text, e.g., [1,2], as part of the text and not raised above it.

The full reference should be cited in a numbered list essentially according to the Vancouver Uniform Requirements (5th ed., Ann Intern Med 1997;126(1):36-47).

References should contain names of all authors in small letters (surnames first followed by initials), Title of communication in lower case lettering, Title of Journal [abbreviated according to International Serials Data System-List of Serial title Word Abbreviations, 1985 (ISDS-ISO International Centre, 20 rue Bachaumont, 75002 Paris, France)], year of publication; volume number: first and last page number (see Ref.1).

Reference to Journal Supplement, cf. example (Ref. 2).

References to books should contain Author Name(s) in the same format as above: Title. Publisher's location: Name; Year of publication. page range (see Ref. 3).

References to multi-author books with editor(s) should contain Author Name(s) in the same format as above: Title of contribution. In: Name(s) of editor(s). Title of book. Publisher's location: Name; Year of publication. If necessary page range (see Ref. 4)

For communications which have been accepted for publication, but not yet printed, the reference must contain the journal name and year (see Ref. 5). Material referred to by the phrase "personal communication" or "submitted for publication" are not considered full references and should only be placed in parentheses at the appropriate place in the text, e.g., (Hessel 1997 personal communication).

Examples of references are shown below.

1. Ordljin TM, Shainoff JR, Lawrence SO, and Simpson-Haidaris PJ. Thrombin cleavage enhances exposure of heparin binding domain in the N-terminus of the fibrin beta chain. Blood 1996;88:2050-61.

2. Copley AL. The endoendothelial fibrin lining. Thromb Res 1983;(SV):1-154.

3. Davies JT, Rideal EK. Interfacial Phenomena. New York-London: Academic Press; 1961. p. 110-30.

4. Blomback B. Fibrinogen to fibrin transformation. In: Seegers WH, editor.

Blood Clotting Enzymology. New York-London: Academic Press; 1967. p. 143-215.

5. Leshner AI. Molecular mechanisms of cocaine addiction. N Engl J Med. In Press 1996.

(e) Tables and Figures: Tables and figures are submitted via the online submission system EES with the accompanying article. Each Table should be included on a separate page. Tables should supplement but not duplicate the text. A brief title should be provided for each. Abbreviations used in Tables should be defined. Legends to Tables should be included at the end of the manuscript file. Figures should be in black and white, all details clear enough to permit reproduction, and legible in the actual size in which they should be published. If the number of tables and/or figures is excessive, the author(s) could be asked to eliminate some of them. Authors requesting color figures in the print version of the Journal will be required to share in their production expenses. Thrombosis Research is part of the programme "Colourful e-Products". This allows illustrations that appear in black and white in the print version of the Journal to be included in colour online, in ScienceDirect. Authors wishing to make use of this facility should ensure that the artwork is in an acceptable format (TIFF, EPS, or MS Office files) and at the correct resolution. In addition, for colour online and black and white in print, both colour and black and white artwork (file and/or hard copy as specified above) must be provided. There is no extra charge for authors who participate in this facility. For more detailed specification on submitting electronic illustrations, please see the following webpage: http://www.elsevier.com/locate/authorartwork

Copyright: Upon acceptance of an article, authors will be asked to sign a "Journal Publishing Agreement" (for more information on this and copyright see http://www.elsevier.com/wps/find/authorshome.authors/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An email (or letter) will be sent to the corresponding author confirming receipt of the manuscript together with a "Journal Publishing Agreement" form.

If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: contact Elsevier's Rights Department, Philadelphia, PA, USA: Tel. (+1) 215 238 7869; Fax (+1) 215 238 2239; email healthpermissions@elsevier.com. Reqiests may also be completed online via the Elsevier homepage (http://www.elsevier.com/locate/permissions).

Peer Review: Regular Articles, Review Articles, and Mini-Reviews are subjected to review by two referees. Acceptance is based upon the significance, originality and validity of the material presented. If you are interested in reviewing for the Journal, please contact the Editors-in-Chief.

Revised Manuscripts: When returning a revised manuscript to the Editor, provide a covering letter in the 'Enter Comments' section of Editorial Manager, replying to the Editor's and referees' comments, describing the changes which have been made in the revised version. Highlight the changes in the revised manuscript to facilitate editorial reassessment. Time for revision must never exceed 60 days.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors who publish in Elsevier journals to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies

III. GALLEY PROOFS.

One set of page proofs in PDF format will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post). Elsevier now sends PDF proofs which can be annotated; for this you will need to

download Adobe Reader version 7 available free from http://www.adobe.com/products/acrobat/readstep2.html. Instructions on how to annotate PDF files will accompany the proofs. The exact system requirements are given at the Adobe site: http://www.adobe.com/products/acrobat/acrrsystemreqs.html#70win

If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post.

Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately. Therefore, it is important to ensure that all of your corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Author Names

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts: Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the

Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed. After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Offprints:

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use.