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MANUELLA MARIA SILVA SANTOS

Saccharomyces cerevisiae como modelo genético para estudo da deficiência da mevalonato quinase (MKD) em humanos

Recife
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Tese apresentada ao Programa de Pós-Graduação em Genética, Área de Concentração Genética, da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Genética.

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Orientador: Profº. Dr. Sergio Crovella

Coorientadores: Profº. Dr. Marcos Antônio de Morais Junior

Profª. Drª.Jaqueline de Azevêdo Silva

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BANCA EXAMINADORA:

Prof° Dr. Marcos Antônio de Morais Júnior (Co-orientador)
Universidade Federal de Pernambuco

Profª Drª Paula Sandrin Garcia (Examinador interno)
Universidade Federal de Pernambuco

Profª Drª Maíra Galdino da Rocha Pitta (Examinador externo)
Universidade Federal de Pernambuco

Profª Drª Cláudia Jacques Lagranha (examinador externo)
Universidade Federal de Pernambuco

Prof° Dr. Mário Ribeiro de Melo Júnior (Examinador externo)
Universidade Federal de Pernambuco

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RESUMO

A deficiência da mevalonato quinase (MKD) é uma doença genética humana rara e a patogênese ainda não elucidada é promovida por mutações no gene que codifica a enzima mevalonato quinase (MVK). Células da levedura *Saccharomyces cerevisiae* que apresentam mutação no gene *ERG12* (ortólogo de MVK em humanos) apresentam fenótipos semelhantes às células humanas, o que indica o possível uso desta levedura como modelo biológico para estudo da MKD. Assim, o presente trabalho utilizou o mutante *erg12*-d que reduz em 90% a atividade MVK. Dados fisiológicos mostraram a deficiência do mutante na utilização de glicerol como fonte de carbono, e a incapacidade de se restaurar o fenótipo com a suplementação com isoprenóides. Em seguida, análise de expressão gênica mostrou a repressão de genes do metabolismo respiratório, indução de genes envolvidos nas vias de biossíntese de aminoácidos sulfurados e ubiquinona e dos genes de autofagia (ATG). Esses dados indicaram que a escassez de ubiquinona, e não a falta de isoprenóides, deve ser a principal causa da deficiência respiratória, do mau funcionamento mitocondrial e da possível mitofagia incompleta no fenótipo MKD. Por fim, consolidamos as evidências com a análise de expressão gênica de genes *ATG* e localização da proteína mitocondrial Idhp1 nas células mutantes. Isso confirma a hipótese de indução mitofágica e podem ser a base da resposta inflamatória inespecífica observada em pacientes com MKD humano.

Palavras-chave: Mitofagia. Expressão gênica. Microarranjo. Metabolismo respiratório. Leveduras.

ABSTRACT

Mevalonate kinase deficiency (MKD) is a rare human genetic disorder and the pathogenesis yet to be elucidated is promoted by mutations in the gene encoding the enzyme mevalonate kinase (MVK). Saccharomyces cerevisiae yeast cells that have a mutation in the ERG12 gene (MVK ortholog in humans) present phenotypes similar to human cells, indicating the possible use of this yeast as a biological model for the study of MKD. Thus, the present work used the erg12-d mutant that reduces MVK activity by 90%. Physiological data showed the deficiency of the mutant in the use of glycerol as a carbon source, and the inability to restore the phenotype with isoprenoid supplementation. Next, gene expression analysis showed the repression of respiratory metabolism genes, induction of genes involved in sulfur amino acid and ubiquinone biosynthesis pathways and autophagy (ATG) genes. These data indicate that the shortage of ubiquinone, not the lack of isoprenoids, should be the main cause of respiratory deficiency, mitochondrial malfunction and possible incomplete mitofagia in the MKD phenotype. Finally, we consolidated the evidence with the analysis of gene expression of ATG genes and localization of mitochondrial protein Idhp1 in mutant cells. This confirms the hypothesis of mitophagic induction and may be the basis of the nonspecific inflammatory response observed in patients with human MKD.

Key words: Mitophagy. Gene expression. Microarray. Respiratory metabolism. Yeast.

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LISTA DE ABREVIATURAS E SIGLAS

3' UTR	3' região não traduzida ou *3' Untranslated region*
ATG	Proteína relacionada com a autofagia
Atg1	Gene 1 relacionados com a autofagia
Atg7	Gene 7 relacionados com a autofagia
Atg8	Gene 8 relacionados com a autofagia
Atg9	Gene 9 relacionados com a autofagia
Atg29	Gene 29 relacionados com a autofagia
Atg32	Gene 32 relacionados com a autofagia
Atg41	Gene 41 relacionados com a autofagia
Atg49	Gene 49 relacionados com a autofagia
ATP sintase	Trifosfato de adenosina sintase
COQ	Ubiquinona ou Coenzima Q
COX4	Subunidade 4 citocromo c oxidase
Cvt	Via de transporte do citoplasma para o vacúolo
ERO	Espécie reativas de oxigênio
FOH	Farnesol
FPP	Farnesil pirofosfato
FPPS	Farnesil difosfato sintase
GFP	*Green Fluorencence Protein*
GGOH	Geranilgerniol
GGPP	Geranilgeranil pirofosfato
GOH	Geraniol
GTPases	Guanosina trifosfato
Gut1p	Glicerol quinase

HIDS	Síndrome da hiperimunoglobulina D e febre periódica
HMG-CoA	Hidroximetilglutaril coenzima A
HMGR	Hidroximetilglutaril coenzima A redutase
Idh1	Isocitrato desidrogenase 1
IgA	Imunoglobulina A
IgD	Imunoglobulina D
IL-1b	Interleucina 1 beta
IL-6	Interleucina 6
IPP	Isopentenil pirofosfato
LPS	Lipopolissacarídeo bacteriano
MA	Acidúria Mevalônica
MET1	Metionina sintase 1
MET6	Metionina sintase 6
MET10	Metionina sintase 10
MET14	Metionina sintase 14
MK	Mevalonato quinase (proteína)
MKD	Deficiência da mevalonato quinase
MVA	Ácido mevalônico
MVAP	Mevalonato 5 fosfato
MVK	Mevalonato quinase (gene)
NAC	N-acetilcisteína
NADH	Nicotinamida adenina dinucleotídeo
OLI1	subunidade da ATP sintase
PAS	Estrutura pré-autofágica
ROS	Reactive Oxygen Species

RNAt	Ácido ribonucléico transportador
SAM2	S-adenosilmetionina
SH	Grupo sulfifrila
SNP	*Single Nucleotide Polymorphisms*
SREBP	Proteínas de ligação aos elementos reguladores do esterol
SREs	Elementos reguladores do esterol
RNAr	Ácido ribonucléico ribossomal
RhoA	mMembro da família Ras de pequenas GTPases
TNF-a	Fator de necrose tumoral alfa

SUMÁRIO

1 INTRODUÇÃO

A deficiência de Mevalonato Quinase (MVK) é uma doença genética autossômica recessiva humana, causada por mutações no gene da enzima mevalonato quinase (MVK). A base da deficiência da mevalonato quinase (MKD) está no comprometimento da via mevalonato, um processo metabólico de suma importância na síntese do colesterol e isoprenóides não-esteróis. A MKD é uma doença com mecanismos bioquímicos ainda não completamente elucidados, de diferentes apresentações clínicas e que ainda não tem medicamento específico para o seu tratamento. A terapia atualmente adotada é referente aos efeitos inflamatórios decorrentes da acidúria mevalônica causada pela deficiência desta enzima.

A mevalonato quinase (MVK) atua como a segunda enzima da via mevalonato, que converte ácido mevalônico (MVA) em mevalonato 5-fosfato (MVAP). A via é comum à maioria das células eucariotas e é essencial nos mecanismos celulares como prenilação de proteínas e regulação do ciclo celular. Apesar de muitos estudos reportarem modelos biológicos para a doença, até o momento nenhum modelo mimetiza o defeito genético, no qual é a base da deficiência da mevalonato quinase.

Dadas as semelhanças fenotípicas das células heterozigóticas MVK entre seres humanos e a levedura *S. cerevisiae*, objetivamos neste trabalho identificar o aspecto chave da deficiência de MVK nesta levedura usando um mutante que expressa apenas atividade residual desta enzima. Desta forma, visamos contribuir para a compreensão, pelo menos em parte, da genética ~~e~~ bioquímica desta síndrome.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Identificar aspectos fisiológicos e genéticos relacionados com deficiência da enzima mevalonato quinase, usando como modelo biológico a levedura *Saccharomyces cerevisiae* com atividade residual da enzima.

1.1.2 Objetivos específicos

1. Investigar o papel de isoprenóides exógenos no modelo biológico;

2. Determinar o papel da deficiência de MVK na função mitocondrial;

3. Investigar do perfil de alteração transcricional global na linhagem de *S. cerevisiae*;

4. Avaliar genes que participam da ativação da autofagia e formação do autofagossomo;

5. Monitorar a mitofagia no modelo Erg12-d em Saccharomyces cerevisiae

2 REFERENCIAL TEÓRICO

2.1 DEFICIÊNCIA DA MEVALONATO QUINASE

Com a natureza genética conhecida, a MKD é uma doença rara, autossômica recessiva causada por mutações no gene *MVK* (12q24.11), que codifica a mevalonato quinase (MVK), segunda enzima da via da mevalonato quinase responsável pela síntese do colesterol (Figura 1) (VAN DER BURGH *et al*., 2013). Apesar de ser uma doença metabólica, indivíduos portadores desecandeiam febres episódicas e inflamação generalizada na primeira infância (VAN DER BURGH *et al*., 2013; BADER-MEUNIER *et al*., 2011).

Descrita inicialmente como uma variação da doença de Still, na qual os pacientes apresentavam elevadas taxas de IgA e IgD, a doença foi nomeada de síndrome de hiper-IgD (VAN DER MEER *et al*.,1984). Quanto a denominação de MKD, na última década surgiram uma série de discussões com relação a mudança de nomenclatura (STOFFELS; SIMON, 2011; CELSI *et al*., 2014; STOFFELS *et al*., 2014). Contudo, os mais recentes artigos adotaram a deficiência da mevalonato quinase como nomenclatura padrão, na qual possui duas variantes fenótipicas, a síndrome da hiperimunoglobulina D e febre periódica (HIDS, MIM# 260920), menos severa e mais comum, e a acidúria mevalônica (MA, MIM# 610377), abordada como uma variante fenotípica de um quadro inflamatório mais severo e raro (VAN DER MEER *et al*.,1984; BERGER *et al*., 1985; VAN DER BURGH *et al*., 2013). Os fenótipos HIDS e MA são desordens claramente definidas, mas a MKD engloba pacientes com características sobrepostas e com o curso da doença clinicamente silencioso (SIMON *et al*., 2004).

As duas variantes possuem ocorrências inflamatórias similares, com febres e rápida resposta de fase aguda, acompanhados de linfadenopatias, hepatoesplenomegalia, dor abdominal, diarreia, vômitos, mialgias e úlceras nas mucosas. As elevações dos níveis séricos de IgD é uma característica da forma mais branda da doença. Além desta manifestação, ocorre no fenótipo da acidúria mevalônica, retardo no crescimento pré e pós-natal, problemas oculares e neurológicos (PRASAD *et al*., 2012).

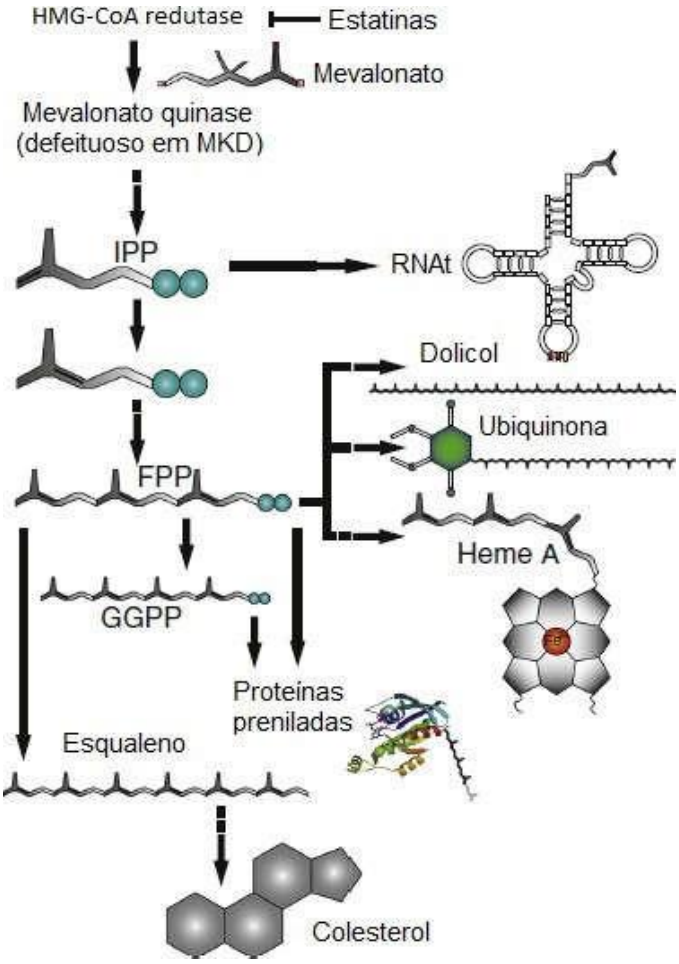

Figura 1. Representação esquemática da via mevalonato. FONTE: Adaptado de van der Burgh et al., 2013.

2.2 CARACTERÍSTICAS E VARIAÇÕES CLÍNICAS DA MKD

Descobertos em meados da década de 80, a acidúria mevalônica e a síndrome da hiperimunoglobulinemia D e febre periódica eram diagnosticados como doenças distintas (VAN DER MEER *et al*., 1984; BERGER *et al*., 1985). Apenas em 1999 com a descoberta da mutação compartilhada no gene *MVK*, os dois fenótipos clínicos foram relacionados (DRENTH *et al*., 1999). A atividade residual da mevalonato quinase varia de acordo com o fenótipo associado. Em pacientes HIDS é de 1,8% a 28%, enquanto que pacientes MA apresentam pouca ou nenhuma atividade residual (VAN DER BURGH *et al*., 2013; HOFFMANN *et al*., 1993). Reciprocamente, as concentrações de ácido mevalônico, no plasma e na urina, variam de médio a moderadamente elevado em pacientes com HIDS, e muito

elevadas em pacientes com MA (HOFFMANN *et al*., 1993; BADER-MEUNIER *et al*., 2011; VAN DER BURGH *et al*., 2013). Na maioria dos pacientes HIDS elevadas concentrações de IgD podem sugerir um possível diagnóstico, mas não pode ser utilizado como tal, pois alguns pacientes não apresentam aumento na concentração da imunoglobulina (HOFFMANN *et al*., 1993; VAN DER HILST *et al*., 2008; BADER-MEUNIER *et al*., 2011). Apesar dos dois fenótipos serem relacionados ao MKD, a severidade da doença é bem distinta.

A acidúria mevalônica apresenta uma heterogeneidade clínica e afeta severamente os pacientes desde o nascimento com malformações congênitas, como microcefalia, fissuras nas pálpebras e catarata (PRASAD *et al*., 2014; SCHULERT *et al*., 2015). A interrupção do fluxo nos canais biliares (colestase) está presente em alguns pacientes e pode ocasionar a morte por septicemia (HINSON ET AL., 1998; RAUP *et al*., 2004; PRASAD *et al*., 2014; SCHULERT *et al*., 2015). As manifestações cardinais tardias incluem, atraso psicomotor leve e grave, crises recorrentes (febres, vômitos e diarreia) e perda do tônus muscular (hipotonia), nos quais não parecem ser de origem infecciosa (PRASAD *et al*., 2012). Os episódios ocorrem com a frequência de 25 vezes por ano, com duração média de 4 a 5 dias. Ocasionalmente, anormalidades hematológicas como, leucocitose, trombocitopenia e formas anormais de células sanguíneas podem direcionar à diagnósticos incorretos de infecção congênitas ou síndrome mielodisplásica (PRASAD *et al*., 2012). Após a idade pré-escolar, as manifestações dos casos menos graves, caracterizam-se pela baixa estatura e ataxia devido a uma progressiva atrofia cerebelar (SIMON *et al*., 2004; PRASAD *et al*., 2012; TER HAAR *et al*., 2013).

Em HIDS, o quadro clínico é dominado por recorrentes ataques febris, que surgem antes do final do primeiro ano de vida (STOFFELS; SIMON, 2011). Esses ataques febris duram em média de 4 a 6 dias e podem ser provocados por vacinação, cirurgia ou estresse, geralmente associado com dores abdominais, vômitos, diarreia e aumento dos linfonodos (linfadenopatia cervical) (DRENTH *et al*., 1994; FAVIER; SCHULERT, 2016). Em 63% dos casos, as vacinas foram atribuídas como primeiro ativador da doença, o que poderia sugerir que a doença é iniciada por uma pequena estimulação imune (DRENTH *et al*., 1994). A hepatoesplenomegalia, dor de cabeça e erupções cutâneas são outros sintomas comuns. Após o período de febres recorrentes, os pacientes não apresentam outros sintomas, embora os sintomas na pele desaparecerem lentamente (VAN DER HILST *et al*., 2008; BADER-

MEUNIER *et al*., 2011). A maioria dos pacientes não apresentam malformações, nem anormalidades neurológicas, contudo um subgrupo de pacientes adultos também desenvolve anormalidade neurológicas de grau variável, tais como atraso mental, ataxia, sintomas oculares e epilepsia (HOFFMAN *et al*., 1993; SIMON *et al*., 2004; HAAS *et al*., 2006). A existência deste subgrupo reflete no possível espectro contínuo entre MA e HIDS.

2.3 EPIDEMIOLOGIA DA MKD

Por ser uma doença autossômica recessiva, a MKD acomete ambos os sexos, e embora a epidemiologia da doença apresente grandes variações de acordo com a região, a maior prevalência documentada está na Holanda (SIMON *et al*., 2003). Baseados nos dados do registro internacional de HIDS (www.hids.net) e Eurofever, patrocinada pela união europeia (http://www.printo.it/eurofever), o número estimado de pacientes com MKD é de cerca de 300 pessoas em todo o mundo, dessas apenas 30 são pacientes com acidúria mevalônica (HAAS *et al*., 2006; MEVALONATE KINASE DEFICIENCY/HIDS, 2016). No Brasil, apenas nove pacientes foram diagnosticados com a doença, segundo os dados da Eurofever. Certamente, o valor real da prevalência está subestimado, visto que muitos pacientes não são diagnosticados corretamente, havendo um atraso no diagnóstico inicial de pelo menos 10 anos (VAN DER HILST *et al*., 2008; TOPLAK *et al*., 2012).

Um estudo, durou em média 9,9 anos para chegar ao diagnóstico de pacientes sintomáticos, devido à raridade e variabilidade na apresentação (VAN DER HILST *et al*., 2008). A frequência da doença na população holandesa é bem estimada, com cerca de 1: 200 mil afetados em todo o país (VAN DER HILST *et al*., 2008). Isto ocorre devido a frequência relativamente alta de portadores de mutação MVK na população holandesa, que é estimado em 1:65, embora, isso implicaria um número significativamente maior de casos MKD do que foi descrito (HOUTEN *et al*., 2003).

Baseados nos dados acima, a MKD se encaixa entre os casos de doenças raras. Doenças raras (também conhecidas como doenças órfãs) são oficialmente definidas como doenças ou condições que atingem menos de 200 000 pessoas no mundo, e todos subgrupos demográficos (THE GENOME OF THE NETHERLANDS CONSORTIUM, 2014). Esse tipo de condição tem um papel de liderança na inovação e no avanço da ciência médica, em grande parte porque eles revelam

mecanismos que não são evidentes quando sistemas e vias metabólicas estão funcionando normalmente (por exemplo, ausência ou deficiência de uma enzima endógena).

2.4 VIA DA MEVALONATO

A mevalonato quinase (MK) é a enzima chave da via da mevalonato, uma rota biossintética responsável pela produção de colesterol (humanos), ergosterol (microrganismos; e.g. *Saccharomyces cerevisiae*) e cadeias lipídicas ramificadas insaturadas, chamadas de isoprenóides não-esteróis (BULUA *et al*., 2011). A via da mevalonato é regulada por muitos mecanismos de *feedback,* no qual o mais importante é o nível de colesterol livre disponível (BULUA *et al*., 2011).

Esta via inicia com a enzima HMG-CoA redutase (HMGR), a primeira enzima da via, na qual sua taxa limitante é bem regulada. A regulação inicia no nível da transcrição; se colesterol ou outro isoprenóide esterol estão em falta, as proteínas de ligação aos elementos reguladores do esterol (SREBP) são ativadas. Estes vinculam-se aos elementos reguladores do esterol (SREs) presentes no promotor HMGR, aumentando sua transcrição. A HMG-CoA redutase converte a HMG-CoA, catalisando a fosforilação dependente de ATP de ácido mevalônico (MVA) em mevalonato 5-fosfato (MVAP) (VAN DER BURGH *et al*., 2012). A HMG-CoA redutase é um alvo farmacológico para redução do colesterol através da ação das estatinas, que agem como inibidores desta enzima. Através da mevalonato quinase o ácido mevalônico é fosforilado em 5-fosfomevalonato e, subsequentemente, é fosforilado para 5-pirofosfomevalonato (figura 1). A adição desses grupos fosfatos polares suporta a solubilidade dos metabólitos em água (MIZIORKO, 2011). Na etapa seguinte da via, o 5-pirofosfomevalonato é convertido a isopentenilpirofosfato (IPP), sendo este um importante substrato para processos biológicos essenciais (MIZIORKO, 2011).

Neste contexto, quando a atividade da mevalonato quinase é reduzida, leva a acumulação de ácido mevalônico e deficiência desses componentes *downstream* da via (MIZIORKO, 2011). A deficiência na síntese de IPP tem importantes consequências nos metabólitos posteriores, incluindo o requerimento do próprio IPP na modificação do RNA transportador (RNAt) (VAN DER BURGH *et al*., 2013).

Entretanto, seu papel chave é na síntese de isoprenóides como o farnesil pirofosfato (FPP) e geranilgeranil pirofosfato (GGPP), os quais são mediadores da prenilação proteica.

O processo de prenilação de proteínas é um tipo de modificação pós-traducional de proteínas pela adição de isoprenóides. Esse é um processo fisiológico chave para facilitar a interação proteína-proteína e o tráfego de proteínas associadas a membrana (MCTAGGART, 2006). O FPP e GGPP são covalentemente transferidos para resíduos de cisteína ou para o terminal carboxi, servindo tanto para localizar proteínas como âncoras de membrana, como para regular a atividade proteica, como as das proteínas da superfamília Ras de pequenas GTPases (ANDO *et al*., 1992; MCTAGGART, 2006; FAVIER; SCHULERT, 2016). Essas proteínas G acopladas participam de eventos de transdução de sinais como, controle do ciclo celular e do crescimento, diferenciação e tráfego de vesículas (ANDO *et al*., 1992; MCTAGGART, 2006; VAN DER BURGH *et al*., 2013).

Por fim, mais adiante na via, encontra-se a farnesil difosfato sintase (FPPS) que tem papel na síntese de colesterol, heme, esqualeno e ubiquinona (VAN DER BURGH *et al*., 2013). Assim como HMG-CoA, FPPS é um outro alvo farmacológico, na via mevalonato, inibido pela utilização dos aminobisfosfonatos, como o caso do alendronato, droga empregada no tratamento da osteoporose (RESZKA *et al*., 2008). Estudos com aminobisfosfonatos vêm acumulando evidências que muito das perturbações mecanismos celulares básicos e do fenótipo de MKD é devido as alterações no fluxo da via mevalonato (RUSSELL *et al*., 2008; KUIJK *et al*., 2008).

2.5 ASPECTOS GENÉTICOS DA MKD

A deficiência da mevalonato quinase é uma doença com herança autossômica recessiva e sua compreensão genética tem origem paralela com a descoberta de que pacientes com HIDS eram portadores de mutações no *MVK*, previamente apenas vinculadas à MA (STOFFELS; SIMON, 2011). Observando o aumento de ácido mevalônico na excreção urinária e a atividade diminuída da mevalonato quinase, Houten e col. (1999) direcionaram a pesquisa para o sequenciamento do gene *MVK.* No mesmo período, um grupo de pesquisa, através da análise de ligação genética, identificou o *MVK* como gene candidato em famílias com HIDS (DRENTH *et al*., 1999). O gene MVK está localizado no braço longo do

cromossomo 12 (12q24), possui 11 exons e codifica a enzima mevalonato quinase com 396 aminoácidos (Figura 2).

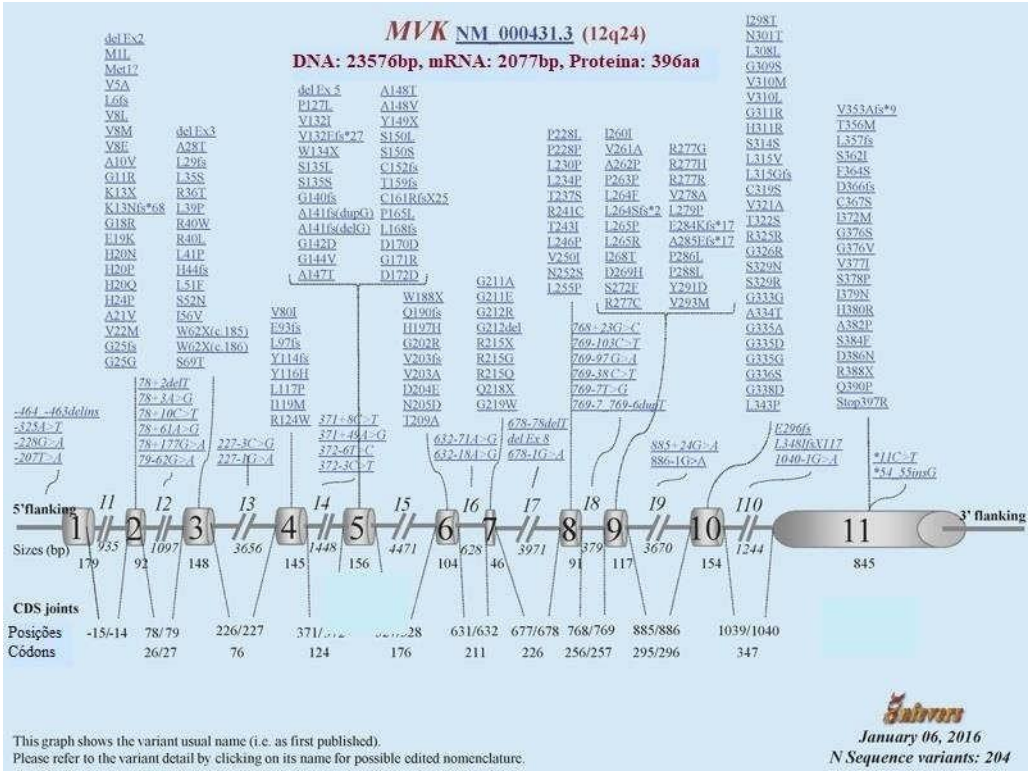

Figura 2. Representação do gene MVK. Fonte: Infevers (https://infevers.umai-montpellier.fr/upload/schema/MVKGeneInfevers06JANUARY2016.png)

Atualmente, existem 215 sequências de variantes neste gene, relatadas em associação com MKD (INFEVERS, 2016). As variantes mais relatadas são polimorfismos de base única (*SNPs: single-nucleotide polymorphisms*) sentido trocado, nos quais os indivíduos heterozigotos e apresentam uma significativa diminuição da atividade enzimática (D'OSUALDO et al., 2005; MANDEY *et al*., 2006; BADER-MEUNIER *et al*., 2011). Outras mutações incluem alterações sem sentido, deleções, inserções, defeitos de *splicing* e uma combinação de uma deleção e uma inserção (indel) (Figura 3) (MANDEY *et al*., 2006; BADER-MEUNIER *et al*., 2011). Embora a maioria dos pacientes possuam mutações bialélicas em *MVK*, foi identificado que alguns apresentam características clínicas com uma única mutação, possibilitando a correlação genótipo-fenótipo em MKD (MANDEY *et al*., 2006; TER HAAR *et al*., 2016).

Figura 3. Figura 3. Representação do número de sequências variantes de acordo com as alterações genéticas. Fonte: Infevers (Https://infevers.umaimontpellier.fr/web/stats.php?n=3&graph=alteration_pheno).

Variantes do gene MVK são associadas com o fenótipo mais severo da acidúria mevalônica e resulta em baixa atividade enzimática (HOUTEN *et al*., 2011; CUISSET *et al*., 2001). De fato, variantes associadas ao fenótipo MA podem ter efeitos mais profundos na estabilidade da MVK (CUISSET *et al*., 2001). Em contraste, outras variantes tais como a substituição C-terminal V377I, manifestam o fenótipo da HIDS, raramente associados com a MA. Curiosamente, esta variante é a mais comumente relatada em pacientes com MKD, com uma taxa de frequência alélica de cerca de 50% e presente somente no fenótipo para HIDS (HOUTEN *et al*., 2003; D'OSUALDO *et al*., 2005; VAN DER HILST *et al*., 2008; BADER-MEUNIER *et al*., 2011; TER HAAR *et al.,* 2013). Entretanto, um estudo mostrou que seis pacientes homozigotos para V377I possuem severidade distintas de MKD, variando entre assintomático, leve e severo (BADER-MEUNIER *et al*., 2011). A mutação I268T é a segunda mutação mais frequente no gene *MVK* com cerca de 8 a 15% de frequência alélica entre os pacientes e foi encontrada em ambos os fenótipos HIDS e MA (MANDEY *et*

al., 2006; D'OSUALDO *et al*., 2005; VAN DER HILST *et al*., 2008; BADER-MEUNIER *et al*., 2011; TER HAAR *et al*., 2013).

Visto que há uma correlação com genótipo e fenótipo para severidade da doença, foram avaliadas se outras características clínicas como, idade diagnóstico dos sintomas, sintomas durante as febres e frequência de febres por ano, mas não foi encontrada uma relação (CUISSET *et al*., 2001; VAN DER HILST *et al*., 2008). Entretanto, os pacientes MKD são caracterizados pela hiperresponsividade inflamatória devido à falta de produtos isoprenóicos.

2.6 MECANISMOS PATOFISIOLÓGICOS NA MKD

Muitos estudos sobre a patofisiologia da MKD são baseados em modelos celulares *in vitro,* com murinos ou células humanas, utilizando drogas que bloqueiam a via do mevalonato, como no caso dos inibidores de HMG-CoA redutase ou biofosfonatos (MARCUZZI *et al*., 2010; MARCUZZI *et al*., 2011; TRICARICO *et al*., 2014). Nesses modelos, o lipopolissacarídeo (LPS) ou outros componentes bacterianos são usados para mimetizar o estimulo inflamatório. Na última década, estudos reportaram que a redução na disponibilidade dos não- esteróis tem um papel crítico no fenótipo inflamatório da deficiência da mevalonato quinase, o qual é mediada, pelo menos em parte, pela interleucina-1 beta ((IL)-1β) (FRENKEL *et al*., 2002; KUIJK *et al*., 2008a, 2008b; MANDEY *et al*., 2006; PONTILLO *et al*., 2010; MARCUZZI *et al*., 2013). A importância dessa citocina em MKD é apoiada pelo benéfico efeito de drogas direcionadas a IL-1β, como a anakinra, em pacientes com a doença (BODAR *et al*., 2005; BODAR *et al*., 2011; SIMON *et al*., 2013).

Além do modelo celular *in vitro*, dois modelos animais *in vivo* foram testados para MKD. O primeiro utiliza camundongos heterezigotos *knockdown* para MVK, que apresenta um fenótipo semelhante ao MKD, com elevada temperatura corporal, hepatoesplenomegalia e aumento no nível sérico de IgD (HAGER *et al.,* 2007). Com o uso desse modelo foram encontradas diferenças na proliferação celular entre o camundongo tipo selvagem e o *knockdown*. No segundo modelo, os camundongos Balb/c são tratados com um aminobifosfonato, o alendronato, 2 ou 3 dias antes da estimulação inflamatória. Após a análise de marcadores inflamatórios, os autores observaram um aumento significante da citocina IL1-β nos níveis séricos (MARCUZZI *et al*., 2008; KLEINER *et al*., 2013). Além disso, foi utilizada uma

abordagem diferente para aumentar o *pool* de isoprenóides celulares, incluindo Farnesol (FOH), geraniol (GOH) e geranilgeraniol (GGOH) exógenos, e apenas GGOH e GOH obtiveram efeitos anti-inflamatórios (MARCUZZI *et al*., 2008; DE LEO *et al*., 2010).

A comparação do perfil de citocinas neste tipo de modelo com o perfil encontrado nos controles saudáveis e pacientes MKD, mostra de fato, que esses animais tem um perfil pró-inflamatório de citocinas que foi semelhante aos encontrados nos pacientes durante os ataques inflamatórios. Contudo, mostraram uma pequena discrepância entre este modelo e os monócitos humanos. Foi observado um aumento na expressão da interleucina 6 (IL-6) nos pacientes MKD, enquanto que no modelo testado aumentou a expressão de fator de necrose tumoral alfa (TNF-α) (MARCUZZI *et al*., 2013). Este modelo foi importante por introduzir alguns indícios sobre a patogênese da MKD.

Recentemente, alguns artigos relatam o envolvimento de distúrbios mitocondriais na fisiopatologia da MKD (VAN DER BURGH *et al*., 2014; VAN DER BURGH *et al*., 2014; TRICARICO *et al*., 2014; MARCUZZI *et al*., 2015; MARCUZZI *et al*., 2016; CECATTO *et al*., 2017). Análises no modelo com células de murinos, identificaram que a inibição da via mevalonato através dos inibidores de HMG-CoA levou o aumento da morte celular programada via caspase-9, um iniciador, e capase-3, um efetor de caspase, componentes da via mitocondrial intrínseca da apoptose (MARCUZZI *et al*., 2015). Além disso, foi possível identificar que a deficiência da mevalonato quinase leva a formação de mitocôndrias instáveis por defeito na prenilação de RhoA, membro da família das pequenas GTPases, que funcionam como pequenos ativadores de cascatas moleculares de transdução de sinal (TRICARICO *et al*., 2014; VAN DER BURGH *et al*., 2014a; 2014b).

O processo de prenilação proteica, diz respeito as modificações pós-tradução na porção C-terminal de proteínas contendo isoprenóides não-esteróis. As proteínas alvo para prenilação expressam uma sequência curta de quatro aminoácidos na porção C-terminal, chamada de CAAX *box*, onde a modificação está anexada ao C (cisteína), A (alifáticos) ou X (usualmente serina, metionina, glutamina, alanina ou treonina) (CASEY *et al*., 1991; MOORES *et al*., 1991). A prenilação ocorre pelo farnesil (C-15) ou geranilgeranil (C-20) transferase. A porção de prenilação pode ter diferentes funções, como localizador celular e fornecedor de sinal para novas

modificações e estáveis integrações de proteínas na membrana (VAN DER BURGH *et al*., 2014).

Visto que MKD tem um sério impacto no bom desempenho celular, observa-se também uma interferência nas atividades social, educacional e mental dos portadores (VAN DER HILST *et al*., 2008; BADER-MEUNIER *et al*., 2011; BERODY *et al*., 2015; DUREL *et al*., 2016). Os adventos biológicos, contribuíram para que, atualmente, o tratamento da MKD tenha uma abordagem individualmente adaptada ao paciente e endereçados aos sintomas e controle da inflamação, permitindo uma melhora na qualidade de vida. O uso de drogas como os corticoides induzem uma resposta completa de remissão em até 24% dos pacientes (TER HAAR *et al*., 2013). Com crescente compreensão da autoinflamação em MKD, existe um interesse na utilização de anakinra com dose profiláticas diárias, obtendo uma resposta completa de remissão de 30% e parcial de 70% (ROSSI-SEMERANO *et al*., 2015). A anakinra é utilizada como um inibidor da citocina pró-inflamatória IL-1β. Em pacientes MKD, a redução de isoprenóides não-esteróis leva a hipersecreção de IL-1β através da capase-1. A ativação da caspase-1 é mediada pela formação do complexo inflamassoma, que participa da resposta de fase aguda nos ataques de febres recorrentes.

Apesar de todas as contribuições relevantes encontradas nos representantes dos modelos biológicos estudados, a compreensão plena da patofisiologia da doença ainda é ausente. Expandir a avaliação da doença como um todo, com novos modelos biológicos, pode ser um desafio a ser realizado, mas colabora como uma ação multicêntrica nas pesquisas direcionadas a doença.

2.7 *SACCHAROMYCES CEREVISIAE* COMO UM MODELO BIOLÓGICO DA MKD

A levedura *Saccharomyces cerevisiae* é um organismo usado como modelo biológico há várias décadas. O amplo uso deste organismo como modelo eucariótico deve-se ao fato de o mesmo possuir características como, as células se dividem rapidamente através de um processo de brotação. O nome comum "leveduras em brotamento" deriva da notável característica da divisão celular e distingue *S. cerevisiae* da levedura de fissão, *Schizosaccharomyces pombe*, também utilizado como organismo modelo (DUINA *et al*., 2014). As células de S. cerevisiae encontram-se em dois tipos na natureza: células haploides a e diploides α. Em condições precárias em nutrientes, os diploides podem ser induzidos a sofrer

meioses e esporulação, formando quatro esporos haploides, dois de cada tipo de cruzamento. Devido ao seu tamanho microscópico e requisito de crescimento simples, as leveduras são baratas e fáceis de cultivar em laboratório. Além disso, sua sequência genética é conhecida e possui um banco de dados acessível (Quadro 1) (MAGER; WINDERICKX, 2005).

Quadro 1. Plataformas utilizadas para pesquisa em leveduras. Fonte: Adaptado de Mager e Winderickx 2005.

Banco de dados ou pesquisa	Url
Banco de dados geral do genoma e proteoma de leveduras	
Saccharomyces Genome Database (SGD Stanford)	http://www.yeastgenome.org/
Comprehensive Yeast Genome Database (CYGD-MIPS)	http://www.genome.jp/kegg/
Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://mips.gsf.de/genre/proj/yeast/index.jsp
Coleção de leveduras mutantes	
Saccharomyces Genome Deletion Project	http://www-sequence.stanford.edu/group/yeast_deletion_project/
EUROpean Saccharomyces Cerevisiae ARchive for Functional analysis (EUROSCARF)	http://web.uni-frankfurt.de/fb15/mikro/euroscarf/
Pesquisas de homólogos	
Mammalian homology to yeast (SGD)	http://www.yeastgenome.org/mammal/
Clusters of orthologous groups of proteins (COGs)	http://www.ncbi.nlm.nih.gov/COG/
Discover homologs (Homologene)	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?dbZhomologene
Doenças leveduras-humanos	
Mitochondria-related proteins, genes and diseases (MitoP)	http://ihg.gsf.de/mitop2/
Yeast homologs of human disease-associated genes	http://mips.gsf.de/proj/yeast/reviews/human_diseases.html
Análise do proteoma em leveduras	
Yeast Protein Localisation database (YPLdb)	http://genome.tugraz.at/YPL/YPL.html
Yeast GFP Fusion Localization database (yeastgfp)	http://yeastgfp.ucsf.edu/
Database of Interacting Proteins (DIP)	http://dip.doe-mbi.ucla.edu
Molecular Interactions Database (MINT)	http://160.80.34.4/mint/
Análise de expressão em leveduras	
Stanford Microarray database	http://genome-www5.stanford.edu/
Yeast Microarray Global Viewer (yMGV)	http://www.transcriptome.ens.fr/ymgv/
Análise fenotipica em leveduras	
Saccharomyces cerevisiae Morphological Database (SCMD)	http://yeast.gi.k.u-tokyo.ac.jp/
PROfiling of PHEnotypic Characteristics in Yeast (PROPHECY)	http://prophecy.lundberg.gu.se/

Comparando o genoma humano com o das leveduras foi possível observar que 30% dos genes conhecidos envolvidos em alguma doença humana, são ortólogos para leveduras (FOURY, 1997). Grande parte desses genes correspondem a componentes chaves na transdução de sinal ou em processos metabólicos específicos. Dessa forma, pesquisas com leveduras podem fornecer indicativos sobre funções biológicas de alguns genes humanos. Após o completo sequenciamento do genoma humano, mais semelhanças foram identificadas (BERGSTROM *et al*., 2014; LITI; LUIS, 2012; SKELLY *et al*., 2013). A compreensão de processos celulares básicos foi significantemente moldada através de estudos

utilizando como organismo as leveduras, visto que a natureza dos processos celulares básicos em humanos, é conservada em leveduras (KORNBERG, 1974; KORNBERG; THOMAS, 1974; OSTERMANN *et al*,. 1989; BRAIG *et al*. 1994; FENTON *et al*., 1994)

Principais avanços tecnológicos para estudos com leveduras estimularam a utilização desse organismo modelo para fins médicos. Em particular, foram desenvolvidas abordagens *genome-wide* que renderam resultados promissores para elucidação de mecanismos de indução por fármacos (PARTS, 2014). O campo da genômica de leveduras, em particular, engloba as análises mutacionais do genoma para investigar a expressão e a função dos genes. Para este fim, coleções *genome-wide* de leveduras, ambos haploide e diploide, foram construídos (KUMAR; SNYDER, 2001; LITI; LUIS, 2012; PARTS, 2014).

Neste contexto, diversos estudos com *Saccharomyces Cerevisiae* estão direcionados a utilizar este microrganismo como modelo para investigação desde estudos de base para doenças até descobertas terapêuticas, ampliando os conhecimentos sobre os mecanismos patofisiológicos e melhorando de maneira significativa a qualidade de vida dos indivíduos portadores. Na literatura são reportados modelos de doenças relacionadas ao cálcio (VOISSET *et al,* 2014), Parkinson (BREITENBACH *et al*., 2013; WANG *et al*., 2014), Alzheimer (BREITENBACH *et al*., 2013; FRANSSENS *et al*., 2013), doenças associadas ao defeito mitocondrial (KALISZEWSKA *et al*., 2015; LASSERRE et al., 2015). Os defeitos mitocondriais, particularmente, fazem parte de estudos desafiadores, visto que as mitocôndrias realizam processos essenciais, complexos, na qual sua disfunção acarreta doenças humanas severas e não tratáveis.

2.7.1 Implicações do defeito mitocondrial

Diante da complexidade estrutural e funcional das mitocôndrias, a disfunção mitocondrial é implicada em amplo espectro de doenças humanas. As doenças são diversas, pleiotrópicas e desafiadoras no âmbito das pesquisas. Atualmente, são descritos mais de 150 genes distintos relacionados as síndromes de disfunção mitocondrial, nas quais a grande maioria afeta a função energética da mitocôndria, devido a mutações patogênicas (SKLADAL *et al*., 2003). Muito dessas descobertas vem de estudos com a *Saccharomyces cerevisiae,* devido a sua capacidade de sobreviver às mutações que inativam a fosforilação oxidativa, tolerar a perda

completa do DNA mitocondrial (uma propriedade referida como "petite"), e ser passível de manipulação do genoma tanto mitocondrial, como nuclear (MATTIAZZI *et al*., 2004; LASERRE *et al*., 2015). Assim sendo, todas essas características fazem da levedura como um excelente sistema de modelo para estudar a base molecular de doenças mitocondriais.

As mitocôndrias sofrem eventos de fusão e fissão para manter sua estrutura, número, propriedades funcionais e integridade de seu genoma (OSMAN *et al*., 2015). Além disso, têm um mecanismo de avaliação de qualidade que normalmente sequestram, removem e eliminam proteínas e organelas danificadas (ANAND *et al*., 2013; BAKER; HAYNES, 2011; BAKER *et al*., 2011). Sob condições de estresse, a maquinaria mitocondrial torna-se hiperfundida, protegendo as mitocôndrias contra autofagia, o que permite que a célula se degrade e recicle componentes desnecessários ou disfuncionais, mantendo a produção de ATP celular (RAMBOLD *et al*., 2011). Quando ocorre um dano severo, as mitocôndrias tornam-se incapazes de energizar suficientemente a membrana interna para promoção das fusões em longas estruturas, resultando na separação da maquinaria mitocondrial e posterior degradação por meio da autofagia, mais especificamente denominada de mitofagia (KIM *et al*., 2007; SAUVANET *et al*., 2012; TWIG *et al.*, 2008).

A mitofagia, degradação seletiva das mitocôndrias por autofagia, ocorre por meio dos processos autofágicos de macro e microautofagia. A macroautofagia é definida como a via de degradação de componentes citoplasmáticos envolvendo a expansão do fagóforo que leva a formação do autofagossomo, enquanto que a microautofagia é a captação direta do material citoplasmático via invaginação da membrana vacuolar (KLIONSKY, 2005; YORIMITSU; KLIONSKY, 2005; FENG *et al*., 2014). Quando as células de levedura saem de uma fonte de carbono não fermentável, como glicerol, para uma fonte de carbono preferencial, como glicose, uma parte da população de mitocôndrias, que agora é supérflua, é submetida a degradação por meio da mitofagia. Este processo é exacerbado quando as células sofrem simultaneamente privação de nitrogênio (KANKI *et al*., 2009; FENG *et al*., 2014). Este reconhecimento seletivo é mediado pela proteína Atg32 que está localizada na membrana externa da mitocôndria funcionando como um receptor para ligação com outras proteínas Atg (Fig.4). Por participar de uma autofagia seletiva, esta proteína não é necessária na via de transporte do citoplasma para o vacúolo (Cvt) ou autofagia não seletiva (KANKI *et al*., 2009). Embora a autofagia tenha sido

identificada primeiramente em células de mamíferos, a compreensão dos mecanismos moleculares foi expandida através das investigações genéticas em leveduras, visto que é um processo com alto nível de conservação entre este organismo e os humanos.

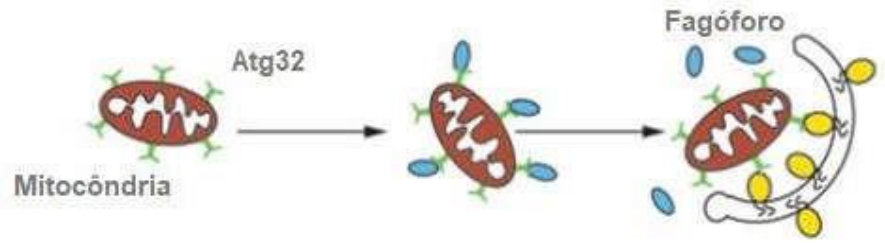

Figura 4. Representação esquemática do mecanismo de autofagia seletiva. Atg32 funciona como um receptor específico para sinalização e iniciação da mitofagia. Fonte: Adaptado de Feng et al., 2014.

Diante das exposições sobre as vantagens do uso de leveduras para investigações biológicas, este trabalho de tese utilizou a levedura *Saccharomyces cerevisiae* como modelo biológico para estudo da deficiência da mevalonato quinase. O equivalente da enzima MK dos humanos é codificada pelo gene *ERG12* em *Saccharomyces cerevisiae* (OULMOUDEN; KARST, 1990). Este gene ortólogo ao MK é essencial para a viabilidade celular e importante na síntese de isoprenóides, além de participar de processos biológicos importantes, como prenilação de proteínas e regulação do ciclo celular desta levedura. Dessa forma, mutações no *ERG12* comprometem a atividade da enzima, afetando a produção de metabólitos importantes no transporte de elétrons e transdução de sinais. Linhagens de *S. cerevisiae* diploide heterozigota para *ERG12* apresentam haploinsuficiência, ou seja, diminuição da velocidade específica de crescimento e auxotrofia para ergosterol. Além disso, os heterozigotos também apresentam morfologia mitocondrial anormal (OULMOUDEN; KARST, 1990), fenótipos muito semelhantes àqueles apresentados por células humanas heterozigotas para o gene *MVK.* Isto nos levou a investigar um possível defeito mitocondrial e suas possíveis implicações em nosso modelo de estudo. Em conjunto, os resultados obtidos indicaram que a linhagem mutante *erg12*-d de *S. cerevisiae* é um excelente modelo biológico para o

estudo da MKD em humanos e apresetaram evidências para o entendimento completo dos problemas biológicos gerados pela MKD que podem ter implicações relevantes para a terapêutica da doença em humanos.

3 MÉTODO

3.1 LINHAGENS

Foram utilizadas duas linhagens diplóides de *S. cerevisiae*: BY4743 (MAT a/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0), contendo o ERG12 intacto, daí em diante denominado de tipo selvagem, e seu isômero YMR208W (GE Dharmacon), com atenuação da expressão de ERG12 (Thermo Scientifics Co., EUA). Esta linhagem mutante, denominada em nosso estudo Erg12-d (Erg12 deficiente), contém ambos os alelos Erg12 com ruptura da região 3 'não traduzida (3' UTR), o que desestabiliza o transcrito correspondente, diminuindo a estabilidade do mRNA e reduzindo a síntese protéica até 10 % em relação ao tipo selvagem.

3.2 EXPERIMENTOS DE CRESCIMENTO

Para os experimentos de crescimento, as células de levedura foram pré-cultivadas em meio de referência sintético YNB (Base Nitrogénio de Levedura, Disco) 1,6 g / l com sulfato de amónio, aminoácidos necessários e glicose a 20 g / l, a 30° C com agitação (150 rpm). Depois, as células foram recuperadas por centrifugação e ressuspendidas em novo meio fresco em uma densidade celular inicial (DO 600) de 0,1 em microplacas (96 poços) para um volume total de 150µL com meio específico. O mesmo procedimento foi realizado para ensaios de crescimento com uma fonte de carbono respirável, glicerol (20 g / l). As culturas foram realizadas na plataforma Synergy HT (BioTek, Suíça) a 30 ° C e velocidade máxima com medidas de absorbância a cada 30 minutos por 24 h. As linhagens parental e a *Erg12*-d também foram cultivadas em meio sintético em glicerol (20 g / l) ou glucose (20 g / l) contendo 5 mM de antioxidante tiol N-acetilcisteína (NAC) (Kwolek-Mirek et al. 2012). Curvas de crescimento foram preparadas a partir da média de dois experimentos biológicos e a taxa de crescimento foi calculada a partir da inclinação da fase de crescimento exponencial.

Experimentos de recuperação de crescimento da BY4743 e erg12-d foram realizados em frascos a 30° C com agitação de 150 rpm. Células de levedura foram cultivadas em meio sintético contendo glicerol. Após 24 h, as células de levedura foram transferidas para meio sintético contendo glicose para DO inicial de 0,1 e incubadas por 24 h. Após esse tempo, as células foram transferidas para meio

sintético contendo glicerol e cultivadas por mais 24 h. A DO final foi registrada para três experimentos independentes (± DP).

3.3 CULTIVOS SUPLEMENTADOS COM ISOPRENÓIDES

As células foram pré-cultivadas em YNB (1,7 g / l) contendo glicose (20 g / l), sulfato de amónio (5 g / l), suplementado com cada aminoácido necessário). 30 ° C e 150 rpm. As linhagens foram re-inoculadas no mesmo meio para a fase exponencial de crescimento fornecendo células para inoculação em poços de 150 µl contendo meio YNB fresco, glicerol (20 g / l), isoprenóides e aminoácidos necessários, e incubados por 24h ou 48h no Synergy HT (BioTek, Suíça). Compostos isoprenóides Farnesol (FOH), geraniol (GOH) e geranylgeranyol (GGOH) foram adicionados nas seguintes concentrações: 0, 25, 50 e 100 µM (MARCUZZI *et al*., 2010).

3.4 DETERMINAÇÃO DE GRUPOS SULFIDRILADOS

Os níveis de sulfidrila ligado à proteína (PB-SH) foram medidos de acordo com o método de Sedlak e Lindsay (1968), subtraindo o teor de sulfidrila não proteico (NP-SH) do teor total de sulfidrilo (T-SH) (Demasi et al. 2006). As células das linhagens BY4743 e Erg12-d foram cultivadas em placas YNB com a presença de 20 g / l de glicose ou 20 g / l de glicerol, durante 72 horas a 30 ° C. Aproximadamente 10^9 células foram coletadas de cada cultura. Os extratos protéicos foram obtidos em EDTA 0,02 M pH 4,7 com a adição de esferas de vidro, seguido de centrifugação a 17.900 g por 15 min. As concentrações de T-SH foram determinadas por níveis de absorção a 412 nm após a incubação de alíquotas de 200 µl de sobrenadantes de extrato protéico com 780 µl de Tris 0,2 M pH 8,2 e 20 µL de DTNB 5 mM por 30 min. Os teores (NP-SH) foram determinados no sobrenadante após precipitação das proteínas com ácido tricloroacético a 5% (concentração final) incubando 450 µl de sobrenadante, 900 µl de 0,4 M Tris pH 8,9 e 26 µl 5 mM DTNB por 5 min (ELSZTEIN *et al.,* 2011). A absorvância foi medida a 412 nm e o teor de sulfidrilo ligado à proteína (PB-SH) foi calculado subtraindo o valor de NP-SH do teor em T-SH. Os resultados são relativos à concentração desses grupos nas células controle (100%) que não foram expostas a nenhum agente e representam a média de três experimentos separados.

3.5 ANÁLISE DE MICROARRANJO

As células foram pré-cultivadas meio sintético YNB sem aminoácidos (1,7 g / l) contendo glucose (20 g / l), sulfato de amónio (5 g / l), suplementado com cada aminoácido necessário a 30 ° C e 150 rpm. Em seguida, as células foram coletadas na fase exponencial, lavadas em solução salina a 0,8%, ressuspendidas em meio sintético de glicose ou glicerol com baixo conteúdo de nitrogênio e incubadas por 4 horas a 30 ° C e 150 r.p.m. As células foram centrifugadas e ressuspensas em 400 mL de tampão AE (50 mM de acetato de sio, 10 mM EDTA, pH 5,3) e 80 mL de soluçãoo a 10% de SDS, misturadas utilizando um vortex e incubadas a 65° C durante 10 min. O RNA total foi extraído a partir de lisados utilizando o kit Maxwell® 16 LEV simply RNA Blood (Promega, EUA). O RNA total foi purificado usando o kit RNAspin Mini RNA Isolation kit (GE HealthCare, EUA) e quantificado no espectrofotômetro NanoDrop ND-2000 UV-Vis (ThermoFisher Scientifics, EUA). A síntese de cDNA, cRNA e marcação foi realizada usando o kit Two-Colour Low Input Quick Amp Labelling (Agilent, EUA) seguindo as instruções do fabricante, com kit de ponta de RNA de duas cores como controle interno (Agilent, EUA) (Lucena et al. 2015). A marcação diferencial com os fluoróforos Cy3 e Cy5 foi nas seguintes combinações: [BY4743 glicerol] x [glucose BY4743]; [Erg12-d glicerol] x [Erg12-d glicose]; [Erg12-d glicerol] x [glicerol BY4743]; [Erg12-d glicose] x [glicose BY4743]. As amostras de cRNA alvo e de referência foram reunidas e utilizadas para hibridizar as lâminas de expressão 8x15k de levedura (Agilent, USA) a 65 ° C durante 17 horas a 10 r.p.m. em um forno de hibridização de microarranjo (Agilent, EUA). Os dados fluorescentes do scanner de microarranjo de resolução de 3 µm (Agilent, EUA) foram extraídos como arquivos .txt usando o software Feature Extraction (Agilent, USA). O teste t relativo (Adj. P <0,05) e o odds ratio de log posterior de expressão diferencial versus expressão não diferencial (B≥3) foram utilizados para as análises de significância estatística de grupos de genes diferencialmente expressos. Os valores do log-FC foram usados para classificar os genes mais- (≥ 0,5 log FC) e menos expressos (≤ -0,5 log FC), um termo que significa a abundância de transcritos sob condições de teste em relação às condições de referência (CUI; CHURCHILL, 2003). Os dados de microarranjo foram depositados no Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) sob o código de acesso GSE98163. A Base de Dados do Genoma de Saccharomyces (SGD) (http://www.yeastgenome.org/), Gene ontology Slim Mapper

(http://www.yestgenome.org/help/analyze/go-slim-mapper) e YeastMine (http: //yeastmine.yeastgenome.org/yeastmine/begin.do) bancos de dados foram utilizados para o reconhecimento de proteínas codificadas e agrupamento GO dos genes.

3.6 PCR EM TEMPO REAL

Células de levedura foram cultivadas em YNB com glicose ou glicerol e coletadas no meio da fase de crescimento exponencial. RNAs totais foram extraídos usando o kit RNeasy Mini (QIAGEN), e a transcrição reversa foi realizada utilizando o Kit de Transcrição Reversa de Alta Capacidade cDNA (Applied Biosystems). Todas as reacções foram corridas em Bio-rad CFX ConnectTM (EUA) utilizando SYBR Green PCR (Applied Biosystems) e os níveis de expressão relativos determinados utilizando o método $\Delta\Delta$Ct com TFC1 (Factor de Transcrição classe C) como referência genética endógena. Os ensaios de expressão de genes estão listados na Tabela 1. As diferenças estatísticas foram avaliadas usando ANOVA * $p < 0,05$.

3.7 MICROSCOPIA DE FLUORESCÊNCIA

Integrao baseada em PCR de um fragmento de ADN que codifica a protea fluorescente verde (GFP) na extremidade 3 'das culas geradas por IDH1 expressando o Idh1-GFP marcado cromossomicamente para a mitofagia (LONGTINE *et al*., 1988). Células expressando proteínas de fusão foram pré-cultivadas em meio YNB contendo glicose ou glicerol por 24h. Além disso, usamos a construção GFP-Atg8 na cepa do tipo selvagem, conforme relatado por Kim et al. 2001 e as células recombinantes foram cultivadas em meio de glicose na presença ou ausência de sinvastatina. As células foram coletadas após incubadas no meio da fase exponencial de crescimento, lavadas em solução salina (0,8%) e usadas para preparar lâminas para observação do microscópio. As lâminas foram visualizadas usando microscópio Leica DMIRB de fluorescência e as imagens foram capturadas usando câmera digital Leica DC 300F, e foram analisadas com o software Leica IM500 Image Manager (Leica, Bensheim, Alemanha).

4 RESULTADOS

4.1 RESPIRATORY DEFICIENCY IN YEAST MEVALONATE KINASE DEFICIENT MAY EXPLAIN MKD-ASSOCIATE METABOLIC DISORDER IN HUMANS

Manuella Maria Silva Santos[1,2], Carolina Elsztein[1,3], Rafael Barros de Souza[1,4], Sérgio de Sá Leitão Paiva Junior[5], Jaqueline de Azevêdo Silva[2,6], Sergio Crovella[2,6] and Marcos Antonio de Morais Junior[1,2*]

Affiliations:

1- Interdepartmental Research Group in Metabolic Engineering, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil
2- Department of Genetics, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil
3- Department of virology/CPqAM, Oswaldo Cruz Fundation, Avenida Moraes Rego, N/S, Recife, PE 50760-901, Brazil
4- Institute for Biologial Sciences, University of Pernambuco. Avenida Agamenon Magalhães, s/n, Recife, PE 50100-010, Brazil
5- Laboratory of Bioinformatics and Evolutionary Biology, Federal Rural University Pernambuco. Rua Dom Manoel de Medeiros, s/n, Recife, PE 52171-900, Brazil
6- Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

***Corresponding Author:**
Marcos Antonio de Morais Junior
Departamento de Genética – Universidade Federal de Pernambuco
A.v. Moraes Rego, 1235, Cidade Universitária, 50670-901 Recife, PE, Brazil, Tel: +55-81-21267817 Fax: +55-81-21268622, marcos.moraisjr@ufpe.com

Abstract

Mevalonate kinase deficiency (MKD) an orphan drug rare disease affecting humans with different clinical presentations, is still lacking information about its pathogenesis; no animal or cell model mimicking the genetic defect, mutations at MVK gene, and its consequences on the mevalonate pathway is available. Trying to clarify the effects of MVK gene impairment on the mevalonate pathway we used a yeast model, the erg12-d mutant strain Saccharomyces cerevisiae (orthologous of MKV) retaining only 10% of mevalonate kinase (MK) activity, to describe the effects of reduced MK activity on the mevalonate pathway. Since shortage of isoprenoids has been described in MKD we checked this observation using a physiologic approach: while normally growing on glucose, erg12-d showed growth deficiency in glycerol, a respirable carbon source, that was not rescued by supplementation with non-sterol isoprenoids, such as farnesol, geraniol nor geranylgeraniol, produced by the mevalonate pathway. Erg12-d whole genome expression analysis revealed specific down regulation of RSF2 gene encoding general transcription factor for respiratory genes, explaining the absence of growth on glycerol. Moreover, we observed the up regulation of genes involved in sulphur amino acids biosynthesis that coincided with the increasing in the amount of proteins containing sulfhydryl groups; up regulation of ubiquinone biosynthesis genes was also detected. Our findings demonstrated that the shortage of isoprenoids is not the main mechanism involved in the respiratory deficit and mitochondrial malfunctioning of MK defective cells, while the scarcity of ubiquinone plays an important role, as already observed in MKD patients.

Keywords: isoprenoids; gene expression; microarray; respiratory metabolism; yeast

Introduction

The impairment of Mevalonate kinase pathway, a crucial metabolic process, part of cholesterol and non-cholesterol isoprenoid metabolism, is at the basis of mevalonate kinase deficiency (MKD) an orphan drug disease affecting humans with heterogeneous clinical presentation and overall still unclear etio-pathologic mechanisms; an involvement of mevalonate pathway has been also observed in the Smith-Lemli-Opitz syndrome, caused by a defect in the post-squalene conversion compounds in cholesterol and peroxisomal disorders, characterized by low plasma cholesterol levels (Layton 1998; Moebius et al. 2000). At present no animal or cell model mimicking the genetic defect, namely mutations at MVK gene, at the basis of the MKD is available. In our study we tried to fill this gap using a yeast model erg-12, the orthologous of human MVK gene, deficient to describe the effects of impaired mevalonate pathway on *S. cerevisiae*.

Mevalonate kinase (MVK) acts one step downstream of HMG-CoA reductase, catalysing the ATP-dependent phosphorylation of mevalonic acid (MVA) to convert in mevalonate 5-phosphate MVAP) (Fig.1) (Fu et al. 2002). This step is largely regulated and MVK is negatively controlled by a feedback loop from geranyl (GPP) and farnesylpyrophosphate (FPP), produced in mevalonate kinase pathway's final steps (Dorsey and Porter 1968; Henneman et al. 2011). The mevalonate pathway is common to most eukaryotic cells being essential for cellular mechanisms as protein prenylation, protein glycosylation and cell cycle regulation by including several non-sterol isoprenoid metabolites (Fu et al. 2002). This pathway is also involved in the control of cell size and growth, autophagy and proteolysis through the action of small GTPase protein Rab11 (Miettinen and Björklund 2016).

Mevalonate kinase deficiency (MKD) caused by mutations at MVK gene, located at 12q24, and responsible for autosomal recessive inborn error of isoprenoid biosynthesis (Moebius et al. 2000; Stenson et al. 2014) is a human disease involving mevalonate pathway impairment. The limited rate from isoprenoids biosynthesis influences the decrease of bioactive molecules that participate in cell growth; protein glycosylation and signal transduction processes as well as dolichol, ubiquinone, may be deficient (van der Burgh et al. 2013).

The MKD is characterized by a shortage in geranylgeranyl pyrophosphate (GGPP), a precursor for isoprenylation of small G proteins, which in turn activates

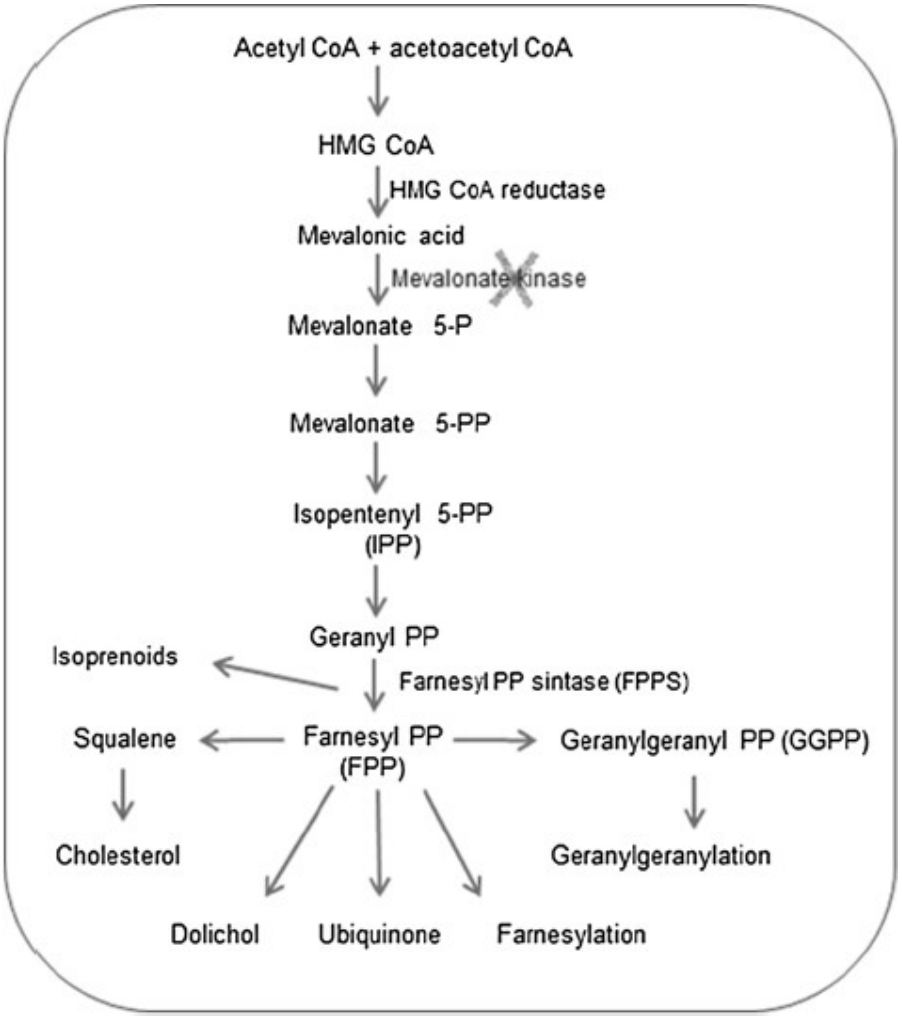

Fig.1 Schematic representation of Mevalonate pathway in *Saccharomyces cerevisiae*. Blockade (gray X) represents the deficiency of enzyme mevalonate kinase activity in the strain erg12-d.

caspase I, stimulating inflammatory events such as recurrent fever and generalized inflammation, due to activation of NLRP3-inflammasome (NLRP: nucleotide-binding oligomerization-domain protein-like receptors) (van der Burgh et al. 2013; Tricarico et al. 2014). MKD has a clinical heterogeneous phenotype, with a milder form known as hyper IgD syndrome (HIDS, OMIM #260920) and a more severe one, the mevalonate aciduria (MA, OMIM #610377). At present, MKD pathogenesis is just partly understood and a specific etiologic therapy for this disease is still unavailable.

Therapies using statins and anti-IL-1, have been used with some success to prevent and cure MKD inflammatory symptoms (Drenth et al. 2001; Bodar et al.

2005), although only supportive therapies are accessible for mevalonic aciduria. The search for new therapies for metabolic disease could take advantage of animal models to mimic human disease's features. Marcuzzi et al. (2008, 2011), developed a mouse model for MKD, showing that the chemical inhibition of the mevalonate pathway through the use of an amino bisphosphonate and statins lead to moderate inflammatory phenotype that could be amplified by bacterial compounds such as muramyl dipeptide (MDP) or lipopolysaccharide (Marcuzzi et al. 2008, 2010, 2011). Recently, MKD cell models employing human monocytes and neuronal cells have been proposed, using natural exogenous isoprenoids, such as geraniol (GOH), Farnesol (FOH), geranylgeraniol (GGOH), as a potential therapeutic approach for MKD (Marcuzzi et al. 2010,2012). Due to their isoprenoid structure, these compounds are able to enter the mevalonate pathway and bypass the biochemical block, thus limiting the shortage of GGPP. Additionally, the isoprenoids have been reported as able to rescue inflammation in the above-mentioned MKD model (Marcuzzi et al. 2008; Frenkel et al. 2002; Mandey et al. 2006).

Aimed at investigating the metabolic pathway in which mevalonate kinase plays a pivotal role, we took advantage of *Saccharomyces cerevisiae* mevalonate kinase (MK) deficiency. In humans, *MVK* gene gene is essential for cell viability and important for isoprenoid synthesis, thus mutations compromising enzyme activity affect the production of important metabolites in electron transport and respiratory metabolism; this causes mitochondrial damage, incomplete autophagy and subsequent cell death (Tricarico et al. 2015). The mevalonate pathway in *S. cerevisiae* is similar to the human one and mevalonate kinase enzyme is encoded by *ERG12* gene (Altmann and Westermann 2005). Some characterization of its deficiency has been reported for the heterozygous yeast strain, as full mutant is unviable, pointing out for mitochondrial abnormalities and respiratory deficiency (Altmann and Westermann 2005). However, there are no direct evidences for the molecular connection between MVK deficiency, isoprenoid shortage and respiratory deficit. Given the phenotypic similarities of MVK heterozygous cells in humans and yeast and due to the methodological facilities for cell manipulation and analysis, the aim of the present study is to identify the key aspect of the MVK deficiency in *S. cerevisiae* by using a mutant that express only residual activity of this enzyme to expand the knowledge. This strain was subject to a combination of wide gene expression analysis and a set of physiological/biochemical data to uncover this

relationship. By doing so, we seek to contribute for the understanding, at least in part, of the etio-pathogenesis of MKD human disease.

Methods

Yeast strain

We used two diploid strains of *S. cerevisiae*: BY4743 (MAT a/ his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0), containing the intact *ERG12*, henceforth denominated the wild type, and its isogenic YMR208W (GE Dharmacon), with attenuation of *ERG12* expression (Thermo Scientifics Co., USA). This mutant strain, named in our study Erg12-d (Erg12 deficient), contains both *Erg12* alleles with disruption of the 3' untranslated region (3' UTR), which destabilizes the corresponding transcript thus decreasing mRNA stability and reducing protein synthesis up to 10% with respect to the wild type.

Growth experiments

For the growth experiments, yeast cells were pre-grown overnight in YNB (Yeast Nitrogen Base, Difco) synthetic reference medium 1.6 g/l with ammonium sulphate, amino acids required and glucose at 20 g/l, at 30 °C with agitation (150 r.p.m.). Afterwards, cells were collected via centrifugation and re-suspended in fresh medium to an initial cell density (OD 600) of 0.1 in sterile microtiter plates (96-well) for a total volume of 150 µL with specific media. The same procedure was performed for growth assays with a respirable carbon source, glycerol (20 g/l). Cultivations were performed in a Synergy HT device (BioTek, Switzerland) at 30 °C and maximal speed with absorbance measurement every 30 minutes for 24 h. Wild-type and Erg12-d strain were also cultivated in synthetic medium in glycerol (20 g/l) or glucose (20 g/l) containing 5mM of thiol antioxidant N-acetylcysteine (NAC) (Kwolek-Mirek et al. 2012). Growth curves were prepared from the average of two biological experiments and the growth rate was calculated from the slope of the exponential growth phase.

Growth recovery experiments of BY4741 (white column) and erg12-d (grey column) were performed in flasks at 30 ^{o}C with 150 rpm agitation. Yeast cells were grown in synthetic medium containing glycerol. After 24 h, yeast cells were transferred to synthetic medium containing glucose for initial OD of 0.1 and incubated

for 24 h. After this time, cells were transferred to synthetic medium containing glycerol and cultivated for another 24 h. Final OD were recorded for three independent experiments (±SD).

Cultivations supplemented with isoprenoids

Cells were pre-cultivated overnight in synthetic yeast nitrogen base (YNB) medium without amino acids (1.7 g/l) containing glucose (20 g/l), ammonium sulphate (5 g/l), supplemented with each required amino acids) at 30°C and 150 r.p.m. The strains were re-inoculated in the same medium for the exponential growth phase providing seed cultures for inoculation into 150 µl plate wells containing fresh YNB medium, glycerol (20 g/l), isoprenoids and required amino acids, then incubated for 24h or 48h on the Synergy HT (BioTek, Switzerland). Isoprenoids compounds Farnesol (FOH), Geraniol (GOH), geranylgeranyol (GGOH) were added at the following concentrations: 0, 25, 50 and 100 µM (Marcuzzi et al. 2010).

Determination of sulfhydryl groups

Protein-bound sulfhydryl (PB-SH) levels were measured in accordance with the method of Sedlak and Lindsay (1968), by subtracting the non-protein sulfhydryl (NP-SH) content from the total sulfhydryl (T-SH) content (Demasi et al. 2006). Cells of the BY4743 and Erg12-d strains were grown on YNB plates the presence of 20 g/l glucose or 20g/l glycerol, for 72 hours at 30°C. Approximately 10^9 cells were collected from each culture. Protein extracts were obtained in 0.02 M EDTA pH 4.7 with the addition of glass beads, followed by centrifugation at 17,900 g for 15 min. The T-SH concentrations were determined by absorption levels at 412 nm after incubating 200 µl aliquots of protein extracts supernatants with 780 µl of 0.2 M Tris pH 8.2 and 20 µl of 5 mM DTNB for 30 min. The (NP-SH) contents were determined in the supernatant after protein precipitation with 5% trichloroacetic acid (final concentration) by incubating 450 µl supernatant, 900 µl 0.4 M Tris pH 8.9 and 26 µl 5 mM DTNB for 5 min (Elsztein et al. 2011). Absorbance was measured at 412 nm and the protein bound sulfhydryl (PB-SH) content was calculated by subtracting the NP-SH value from the T-SH content. The results are relative to the concentration of these groups in the control cells (100%) that were not exposed to any agent, and represent the average of three separate experiments.

Transcriptome-wide expression microarray analyses

Cells were pre-cultivated overnight in synthetic yeast nitrogen base (YNB) medium without amino acids (1.7 g/l) containing glucose (20 g/l), ammonium sulfate (5 g/l), supplemented with each required amino acid at 30°C and 150 r.p.m. Afterwards, cells were collected at the exponential phase, washed in 0.8% saline solution, re-suspended in glucose or glycerol synthetic medium with low nitrogen content, and incubated for 4 hours at 30°C and 150 r.p.m. Collected cells were centrifuged and re-suspended in 400 mL AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.3) and 80 mL of 10% SDS solution, mixed using a vortex and incubated at 65°C for 10 min. The total RNA was extracted from lysates using a Maxwell® 16 LEV simply RNA Blood Kit (Promega, USA). The total RNA was purified using a RNAspin Mini RNA Isolation Kit (GE HealthCare, USA) and quantified on the NanoDrop ND-2000 UV-Vis spectrophotometer (ThermoFisher Scientifics, USA). Synthesis of cDNA, cRNA and labelling were performed using a Two-Colour Low Input Quick Amp Labelling Kit (Agilent, USA) following manufacturer's instructions, with a Two-Colour RNA spike-in kit as the internal control (Agilent, USA) (Lucena et al. 2015). Differential marking with the Cy3 and Cy5 fluorophores was in the following combinations: [BY4743 glycerol] x [BY4743 glucose]; [*Erg12-d* glycerol] x [*Erg12-d* glucose]; [*Erg12-d* glycerol] x [BY4743 glycerol]; [*Erg12-d* glucose] x [BY4743 glucose]. Target and reference cRNA samples were pooled and used to hybridise the yeast gene expression 8x15k spot slides (Agilent, USA) at 65 °C for 17 hours at 10 r.p.m. in a microarray hybridization oven (Agilent, USA). Fluorescent data from a 3 µm-resolution microarray scanner (Agilent, USA) were extracted as .txt files using the software Feature Extraction (Agilent, USA). Relative t test (Adj. $p<0.05$) and log posterior odds ratio of differential expression versus non-differential expression ($B\geq3$) were utilized for the analyses of statistical significance of differentially expressed genes groups. Log FC values used to classify up- (≥ 0.5 log FC) and down-regulated (≤ -0.5 log FC) genes, a term meaning the abundance of transcripts under test conditions relative to the reference conditions (Cui and Churchill 2003). Microarray data were deposited in the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) under the access code GSE98163. The Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/), Gene ontology Slim Mapper (http://www.yestgenome.org/help/analyze/go-slim-mapper) and

YeastMine (http://yeastmine.yeastgenome.org/yeastmine/begin.do) databases were used for the recognition of encoded proteins and GO clustering of the genes.

Results and Discussion

Physiological characterization of the MVK Erg12-d

The results of growth curve showed that the *erg12-d* strain grew poorly when compared to the wild type (wt) strain in glycerol, which requires respiration for its metabolism, while growing normally in glucose (Fig.2). Using glycerol as the only carbon source during growth, this organic compound induces activation of the enzyme glycerol kinase (Gut1p), which promotes its phosphorylation to glycerol 3-phosphate (3-P) (Pavlik et al. 1993). Then, glycerol 3-P is converted to dihydroxyacetone phosphate by the enzyme FAD-dependent glycerol 3-P dehydrogenase (Gut2p) present in the outer mitochondrial membrane. In the presence of glucose, the genes encoding these enzymes are repressed, leading yeast to respiro-fermentative metabolism (Sprague and Cronan 1987). The fact that *erg12-d* did not grow on glycerol indicated that *ERG12* deficiency in yeast, involving mitochondrial functions, is similar to what is observed in humans with mutations in the *MVK* gene. To test whether mutant cells simply stop growing or die in glycerol, yeast cells grown in glycerol were used to inoculate glucose medium. The results showed that mutant cells recovered full growth on glucose after 24 h of poor growth in glycerol, and turned to grow badly when was returned to glycerol medium (Fig. 3). Therefore, whatever the metabolic effect, it was not lethal for *erg12-d* mutant in glycerol, and most probably it has been able to induce a senescent state from which mutant cells can recover as soon glucose is present in the medium.

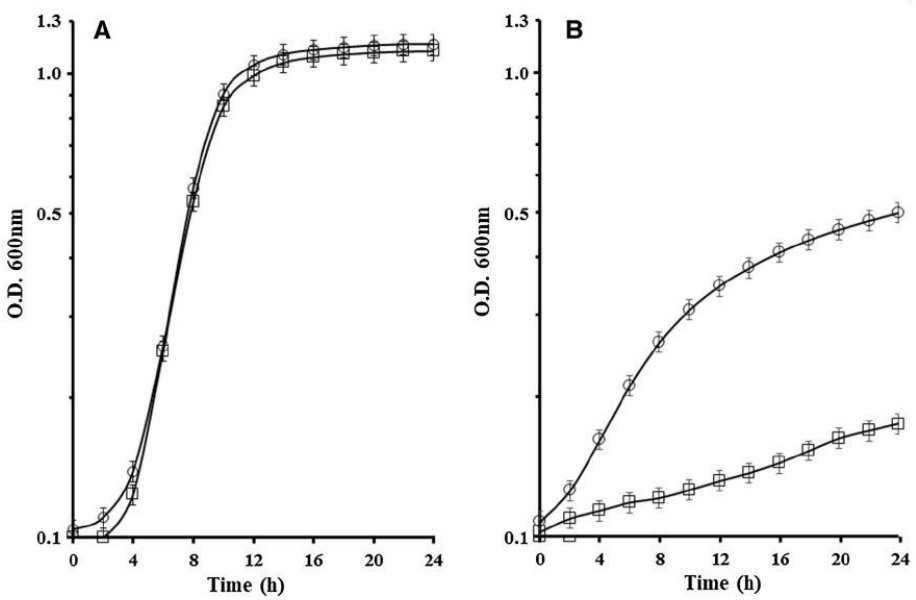

Fig.2 Effect of the carbon source on the growth of *Saccharomyces cerevisiae* wild type BY4743 (○) and Erg12-d mutant (□) strains in synthetic medium containing glucose (panel A) or glycerol (panel B) as carbon source.

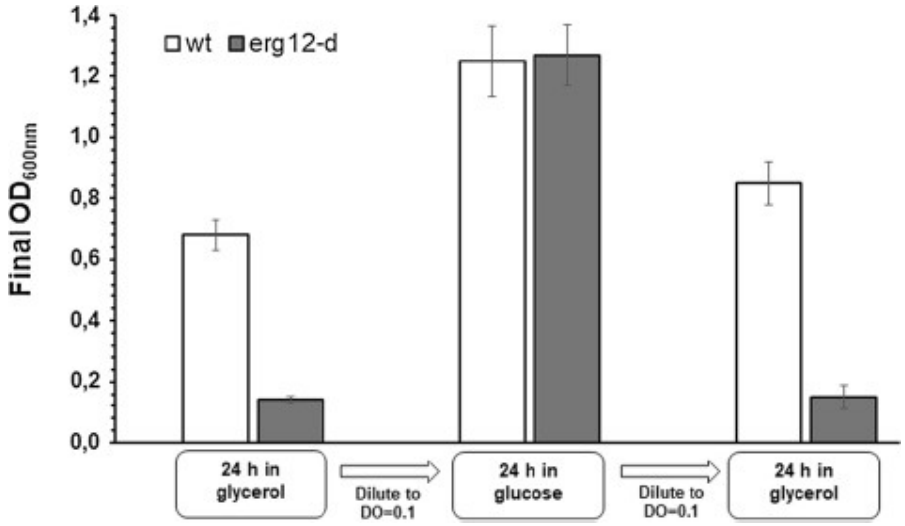

Fig. 3 Growth recovery experiments of BY4741 (white column) and erg12-d (grey column). Yeast cells were grown in synthetic medium containing glycerol. After 24 h, yeast cells were transferred to synthetic medium containing glucose for initial OD of 0.1 and incubated for 24 h. After this time, cells were transferred to synthetic medium containing glycerol and cultivated for another 24 h. Final OD were recorded for three independent experiments (±SD).

It is well reported that human cells with deficiency in MK activity, due to *MVK* mutations, display a reduction in the production of isoprenoids (Tricarico et al 2014), which is also the case for mutant yeast strains (Altmann and Westermann 2005). To

test whether this deficiency is the primary cause of mitochondrial malfunctioning we grew the yeast cells in glycerol-based medium supplemented with exogenous isoprenoids FOH, GOH and GGOH. We observed that concentration starting from 25 µM displayed some cytotoxic effect on the wt strain, and did not recover growth deficiency of the *erg12-d* strain (data not shown). Concentration of 100 µM for both FOH (Fig. 4A) and GOH (Fig. 4B) was very toxic, reducing growth rate of the wt cells from 0.19 h^{-1} to >0.01h^{-1} for both compounds. GGOH was only mildly toxic, reducing growth rate from 0.06 h^{-1} (Fig. 4C). Besides, no effect of any of the terpenoids tested was observed for Erg12-d strain in recovering cell growth to the one of wt level (Fig. 4). Only a slight growth was observed after 48 h of cultivation with GGOH (data not shown). Adverse effects on cell growth and posttranslational protein modifications associated with FOH or GGOH moieties, possibly occur due to partial or complete inhibition of MK activity (DeClue et al 1991). Interestingly, besides the inefficiency of exogenous isoprenoids in recovering the growth of *erg12-d* mutant, different effects in treatment of exogenous isoprenoids were described in MKD mouse model, suggesting a possible role for these compounds in the treatment of MKD in humans (Marcuzzi et al. 2008, 2010, 2011). Machida et al (1998) reported FOH toxic effect of in *S. cerevisiae* cell: the authors showed significant increase of ROS generation after treatment with FOH in a dose-dependent manner. In addition, GOH is effective in inhibiting of pseudohypha formation by *Candida albicans*, the essential aspect of yeast pathogenicity in response to environmental changes (Bard et al. 1988). This compound was shown to disturb the integrity of the cell membrane and to increase its permeability, causing leakage of potassium out of the cell (Zore et al. 2011; Herrero et al. 2008). Our results corroborated these findings, showing the inhibitory effect of exogenous isoprenoids to the yeast cells. Taking in account that *erg12-d* strain has about 10% of MK activity, we suppose that such residual activity could partially supply the cells with isoprenoid requirement, and isoprenoid medium supplementation would lead to the excess of these metabolites in the cells at toxic concentrations. Therefore, the residual concentration of isoprenoids will be enough for some, but not all, isoprenoid-dependent molecular processes.

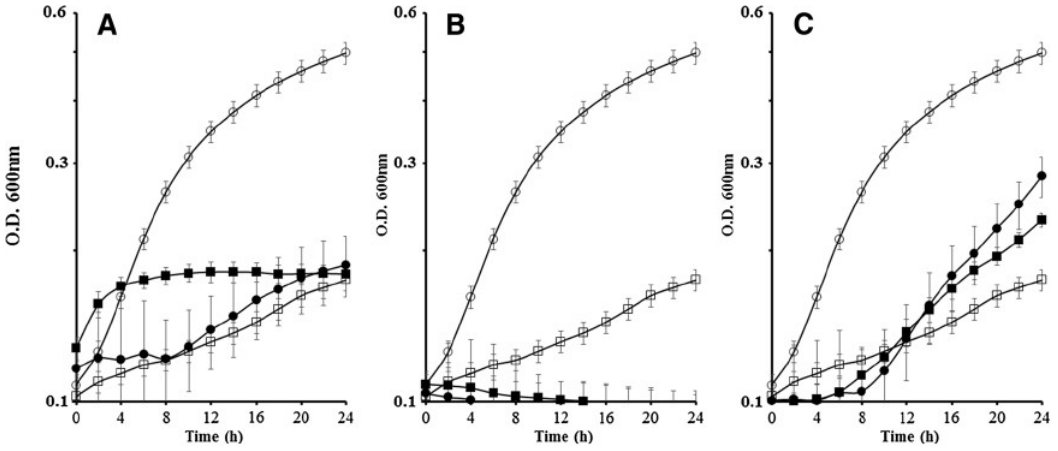

Fig.4 Effect of Farnesol (panel A), Geraniol (GOH) (panel B) or Geranylgeraniol (GGOH) (panel C) on the growth of *Saccharomyces cerevisiae* wild type BY4743 (★) and Erg12-d mutant (✓) strains in synthetic medium containing glycerol as carbon source. Growth on non-supplemented media (○,□) is shown.

Glycerol increase the oxidative stress

Based on the results reported above, we hypothesised that growth defect of *erg12-d* in glycerol could not be only due to isoprenoid shortage. Thus, we turned the attention to the possibility that mitochondrial malfunctioning could be caused by increased production of reactive oxygen species (ROS), and induction of oxidative stress as consequence, during respiratory growth. Cells take advantage of the production of thiols compounds, such as glutathione, and SH-containing proteins, like the small proteins thioredoxins, to detoxify the oxidant compounds produced by ROS (Herrero et al. 2008). So, we performed growth experiments in glycerol medium supplemented with the antioxidant agent NAC. No effect of this compound was observed for both strains when glucose was used as carbon source (Fig. 5A). On the other hand, it was observed a positive effect on the growth of wt strain in glycerol (Fig. 5B). Thus, growth in glycerol indeed leads to the production of ROS by the high respiratory activity, which is not observed in respiro-fermentative state of the cells with glucose (Fig. 5A). Some protective effect by NAC was also observed for the Erg12-d growing in glycerol (Fig. 5B), although it was not enough to restore growth to wt level. Therefore, despite of preventing damages caused by ROS produced by respiration, the presence of NAC was not sufficient to directly rescue the metabolic problem caused by deficiency in MK activity.

Furthermore, we measured the content of thiol compounds in wt and *erg12*-d strains at exponential growth phase. We showed that both strains equally produced more non-proteic thiol compounds (glutathione content) when growing on glycerol

than on glucose (Fig. 6). The production of these compounds is the indicative of oxidative stress in response to respiration. Therefore, the oxidative stress response seemed not to be affected by the deficiency in MK activity. However, it was observed the significant increasing in SH-containing proteins (including thioredoxin) in the Erg12-d cells compared to the wt strain in response to oxidative stress (Fig. 6), indicating a link between the production of sulfhydrylated proteins with the impairment of MVK pathway. In view of fact that MK shortage is connected to respiratory deficiency without connection with oxidative stress, we analysed the global genes expression to understand the direct effect of mevalonate pathway impairment in yeast metabolism.

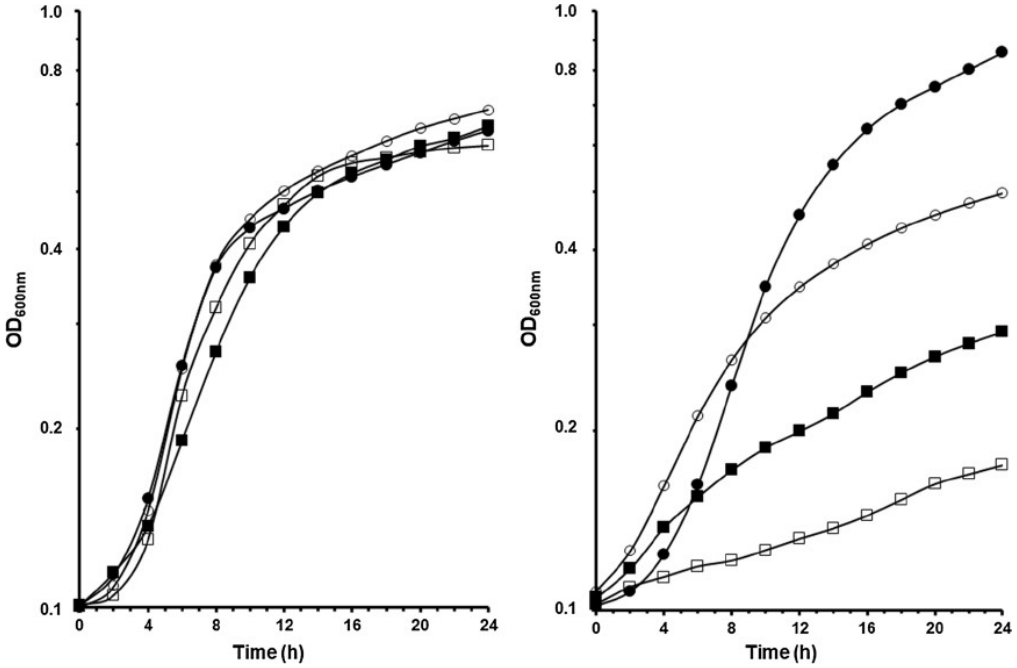

Fig.5 Effect of supplementation of synthetic media containing glucose (panel A) or glycerol (panel B) with 5 mM N-acetylcysteine (NAC) on the growth of *Saccharomyces cerevisiae* wild type BY4743 (★) and Erg12d mutant (✓). Growth on non-supplemented media (○,□) is shown.

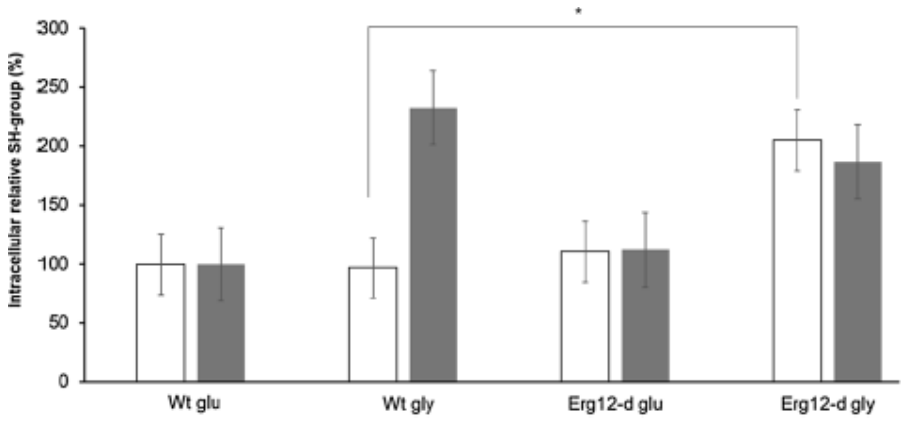

Fig.6 Relative quantification of intracellular content of sulfhydryl groups in soluble proteins (white columns) and non-proteins (grey columns) in the cell extract of wild type BY4743 and Erg12-d mutant strains grown in synthetic medium containing glucose or glycerol as carbon source. Significant difference was observed at *p-value 0.05 (ANOVA: p 0.04).

Overview of gene expression in response to growth on glycerol

Whole genome expression in erg12-d cells were compared to wild type cells both incubated in glucose and glycerol. Cells pre-grown in glucose medium, which displayed similar growth profile (Fig. 2A), were collected at the exponential phase, washed and re-suspended in glucose or glycerol medium. To avoid the effect on growth efficiency caused by MVK defect in glycerol (Fig. 2B), synthetic medium was formulated with low nitrogen content, which limited growth of both cell types. Relative gene expression analyses were performed after four hours of incubation and the differentially expressed genes were grouped according with well-defined metabolic pathways or biological processes by gene ontology (GO) (Lucena et al. 2015). The specificities and overlapping for the four conditions were graphically represented (Fig. 7) and the list of expressed genes can be accessed in the supplementary material (table S1). Following the lack of physiological differences between wild type and erg12-d in glucose (Fig. 2a), we did not observe significant difference in whole genome expression. Therefore, we conclude that whatever effect is caused by the deficiency in MK activity, it does not express relevant phenotype or different gene expression profile when the cells are in respiro-fermentative metabolic state. On the other hand, we observed that in the wild type strain 493 genes were up regulated and 686 were down regulated in glycerol with respect to glucose. As expected, genes of the oxidative stress response, oxidative phosphorylation, autophagy (*ATG8*, *ATG4* and *ATG34*), ubiquinone biosynthesis (*UBX5, UBX2, QCR6* and *QCR7*), and purine

synthesis were up regulated in response to glycerol and respiratory metabolism. In contrast, genes involved in the transcription mechanism, biogenesis of the ribosomes, synthesis and processing of rRNA and tRNA, and the protein synthesis rate were down regulated in glycerol. These findings corroborate the physiological data of lower growth rate of wild type strain in glycerol than in glucose (Fig. 2) (Lempiainen and Shore 2009).

Regarding erg12-d strain, 325 genes were up regulated and 412 were down regulated in glycerol with respect to glucose. Biological processes and metabolic pathway affected by glycerol in the Erg12-d strain were like those reported above for the wild type. However, the defect in growth was more pronounced in the Erg12-d grown in glycerol than in the wild type. Therefore, other genetic differences should exist to explain the impaired growth of Erg12-d cells. A total of 65 genes showed significant changes in their expression in the Erg12-d relative to the wild type when both were incubated with glycerol as carbon source, which might explain the growth difference observed in Fig. 2B. Within these genes, 17 were down regulated and did not reveal any important biological process, and will be no further explored henceforth. Thus, we concentrated the analysis in the group of genes that were up regulated. Our findings revealed that the deficient growth in glycerol is not due to isoprenoid shortage, since the supplementation with these lipids did not recover the growth of mutant erg12-d strain (Fig. 4). In the growth assays with NAC supplementation as an antioxidant agent (Fig. 5) as well as in the measurements of the production of thiol compounds (Fig.6), we observed that there was no further oxidative stress in the Erg12-d compared to wild type. However, there was an increase in SH-containing proteins in the mutant cells (Fig. 6). In this regard, we observed in erg12-d cells up regulation of key genes involved in sulphur amino acids metabolism (*MET6, MET14, MET1, MET10* and *SAM2*) that may account for the increasing of SH-containing proteins, as observed in Fig. 7. This phenomenon might represent the attempt of the yeast cells to restore its metabolic homeostasis in view of the MKV deficiency.

Our results suggest the deficiency in the respiratory metabolism of erg12-d might not cause the exacerbated production of ROS as by-products of complexes I and III of the electron transport chain. These by-products should be detoxified by SH-containing biomolecules (thiols and protein compounds) (Perrone et al. 2008). In this regard, it was observed the down regulation of the gene *RSF2* in erg12-d incubated

in glycerol compared to wild type. *RSF2* encodes a transcription factor involved in the regulation of respiration and growth in glycerol (Lu et al. 2005). Noteworthy, the expression of *RSF2* was not changed in the wild type or in erg12-d in glycerol relative to glucose, meaning that in erg12-d strain the expression of this gene is always lower than in the wild type, irrespective to the carbon source used. We also highlight its activity on the regulation of *OLI1* and *COX4* genes. In our dataset, these two genes were up regulated in wild type-glycerol relative to wild type-glucose, a response absent when the cells were deficient in MK activity. *COX4* encodes the subunit IV of the cytochrome c oxidase, the last step in the phosphorylation chain, and its deletion impairs the respiratory growth (Devenish et al. 2000). In addition, *OLI1* encodes the subunit C of the ATP synthase of the F_0F_1 complex and its deletion leads to growth deficiency in glycerol (Boyer 1997; Nakamoto et al. 1999). Therefore, these results indicate that there is a direct deficiency in respiration in erg12-d.

Since mitochondria are crucial for cellular respiratory machinery, some damages related this organelle, such as organic solvent, telomere-related protein deficiency or biogenesis of molecular components of cellular respiration, cause increase in oxidative stress (Schneider et al. 1995; Nashida et al. 2015; Simonicova et al. 2015). Oxidative phosphorylation is the predominant function of mitochondria responsible to produce ATP. It occurs in a harmonic way by the action of electron carriers, such as Coenzyme Q (CoQ) or ubiquinone, a key factor that contributes to generate the membrane potential, as the case ubiquinone oxidoreductases (Fišar et al. 2016; Li et al. 2016; Bentinger et al. 2010). Ubiquinone transfers electrons between complex I or complex II to complex III of the mitochondrial respiratory chain by getting electrons from NADH or succinate, respectively, and can influence ROS production (Lapuente-Brun et al. 2013). CoQ synthesis occurs in two steps, first the synthesis of isoprenoid and the modification of quinone from the production of FPP, the main regulator of the isoprenoid synthesis (Kawamukai et al. 2015). Several works reported the CoQ deficiency synthesis in humans with mitochondrial dysfunction, is associated with decreased activities of the complexes I-III and II-III and activation of the mitophagy (Desbats et al. 2015; Doimo et al. 2014; Heeringa et al. 2011; Rodríguez-Hernández et al. 2009; Laxman et al. 2013). Mitophagy is part of a specific autophagic mechanism that monitor the mitochondrial quality control and that can be inhibited by the increasing in methionine production (Laxman et al. 2013). The effect of methionine in this process is due to the downstream metabolite of

methionine, S-adenosylmethionine (SAM). SAM is the metabolite methyl donor for several of the metabolic transformations that result in the methylation of lipids, nucleic acid and proteins, playing an important role in cellular growth (Longatti and Tooze 2009). Studies involving shortage of isoprenoids and MKD reported alteration of the autophagic flux due to reduction prenylation of proteins involved in the response to autophagy, leading to the induction of inflammation and apoptosis (Lempiainen and Shore 2009; Tricarico et al. 2015; Miettinen and Björklund 2016). In our experimental dataset we detected the upregulation of *SAM2* gene in erg12-d relative to wt; this gene encodes the SAM synthetase that, among other functions, it is involved in preventing autophagy (Laxman et al. 2013), thus indicating that autophagy, and more specifically mitophagy, should be more exacerbated in erg12-d more than in wild type cells. Thus, we supplemented glycerol-based medium with methionine. However, it did not restore the growth of erg12-d cells (data not shown), indicating that induction of *MET* genes represented a responsive mechanism rather than an essential mechanism to ensure cell growth in full oxidative metabolism.

Our set of findings, compiling physiological profiles and transcriptomics, suggest a reduction in respiration and may be consistent with deficiency in the CoQ synthesis, despite the upregulation of ubiquinone synthesis genes. Thus, we draw the hypothesis that in MK deficiency the metabolic flux toward CoQ synthesis may be impaired by the decreasing in the flow of the mevalonate kinase pathway, with consequent reduction in the production of the intermediaries of the CoQ biosynthesis. James et al (2005) reported that MitoQ10 is ineffective as electron carrier for respiration in yeast and cannot revert the deficiency of *Δcoq2* mutant to synthesize CoQ. We also supplemented glycerol-based medium with MitoQ10 and confirmed that this compound is not capable of reverting growth deficiency of erg12-d mutant (data not shown). In yeast, this ubiquinone is composed by six isoprenyl units (CoQ6), not ten (CoQ10) as in humans, and unfortunately there is no commercially available CoQ6 for testing. All these results indicate that this deficiency leads to a respiratory deficit that is perceived as mitochondrial dysfunction, triggering mitophagy, with the induction of the preventive mechanism of SAM production through the up regulation of *MET* genes. Mitophagy involves numerous molecular mediators called autophagy-related (ATG) proteins and the prenylation of those proteins is the key regulatory mechanisms in this process (Longatti and Tooze 2009). Mevalonate kinase deficiency leads to reduction in CoQ6 production and

mitochondrial dysfunction. Besides, it may cause deficiency in protein prenylation that has been associated with defective mitophagy (Tricarico et al. 2015) resulting in the accumulation of damaged mitochondria. A work involving patients with mevalonate kinase deficiency reported a drastic decrease in plasma CoQ10 concentration (Hubner et al. 1993), while the reduction of the level of protein prenylation in MKD in neuroblastoma cells observed alteration of the autophagic flow and cell death (Tricarico et al. 2017). Thus, it is possible that progression of autophagosomes formation induced by scarcity of ubiquinone is hampered by the limitation in the production of isoprenoids.

Transcriptomic analysis of the biological yeast model studied indicated a deficit in the production of ubiquinone, due to the overexpression of its own genes. It causes respiratory deficiency that might be followed by an accumulation of mitochondrial damage. The mitochondrial dysfunction leads to activation of the mitophagy signalling pathway and in response to mitochondrial degradation, sulphur amino acids, such as methionine, are synthesized to detoxify cells. However, it is possible that the mitophagic mechanism is not completed due to the lack of protein prenylation caused by the shortage of FPP and GGPP for farnesylation and geranylgeranylation, respectively, leading to accumulation of damaged mitochondria (Fig. 8).

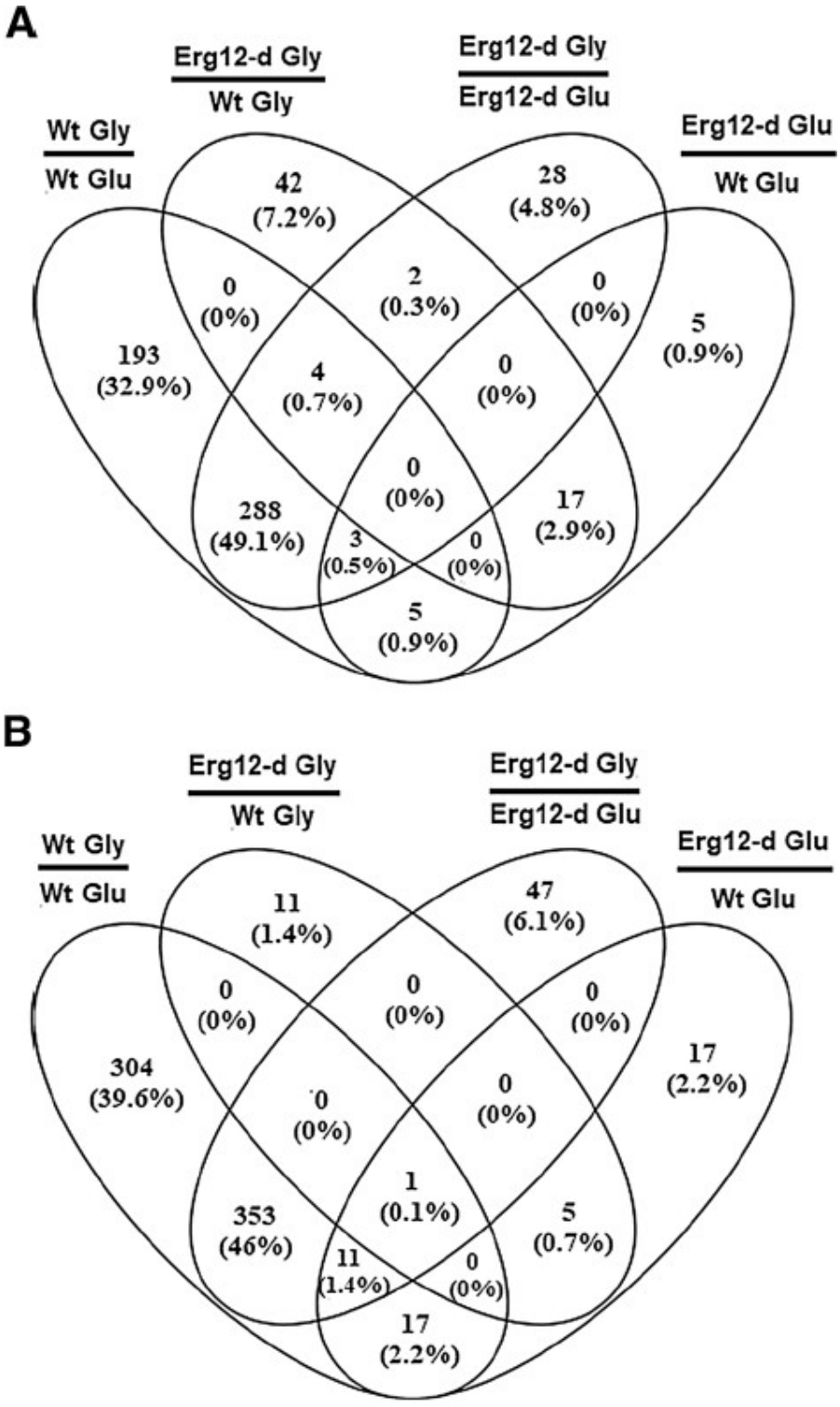

Fig.7 Venn diagram representing the overlaps of differentially expressed genes in four conditions: Erg12-d glycerol relative to wild type glycerol, wild type glycerol relative to wild type Glucose, Erg12-d glycerol relative to Erg12-d glucose and Erg12-d glucose relative to wild type glucose. Percentage of upregulated (panel A) and downregulated genes (panel B) were shown.

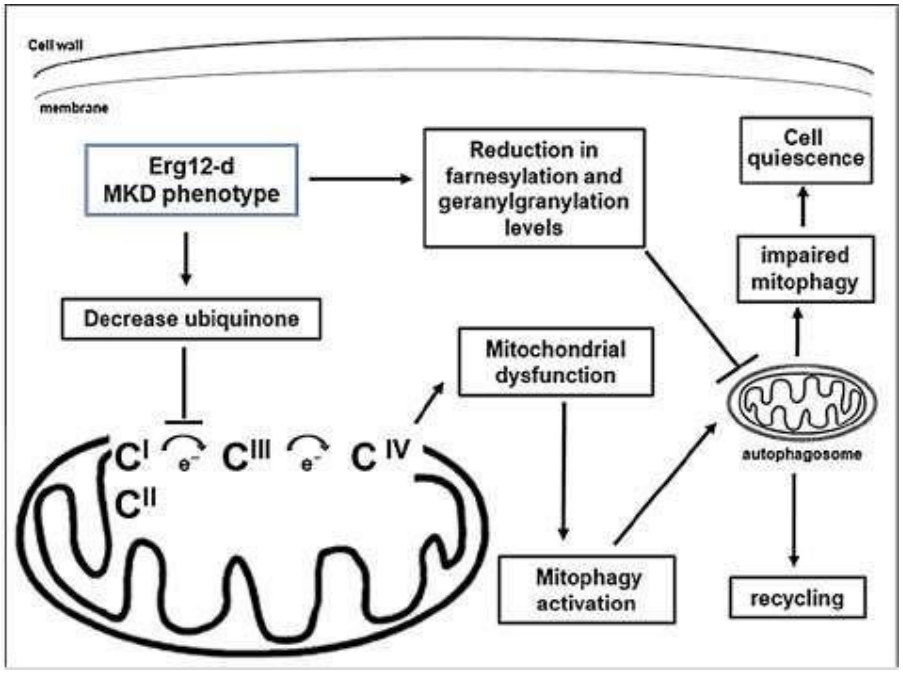

Fig.8 Model of the interaction among the molecular and cellular processes upon the deficiency in mevalonate kinase activity. Arrows represent induction while lines with a bar at one end represent inhibition of the target metabolic process.

Conclusion

Our findings, obtained using a *S. cerevisiae* erg-12, orthologous of human MVK gene, deficient model demonstrated that the shortage of isoprenoids is not the main, or the sole, mechanism involved in the respiratory deficit and mitochondrial malfunctioning of MK defective cells. We also showed that the scarcity of ubiquinone plays an important role in respiratory deficiency and its consequence on cell metabolism, thus confirming previous results already observed in MKD patients. So, in the investigation of possible mechanisms involved in the pathogenesis of MKD, we should also look at the ubiquinone synthesis, opening novel possibilities to design potential drugs to rescue also this impaired biochemical pathway, thus not limiting our action on isoprenoids supplementation.

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4.2 INCOMPLETE MITOPHAGY IN THE MEVALONATE KINASE DEFICIENT *SACCHAROMYCES CEREVISIAE* MIGHT EXPLAIN INFLAMMATORY DISEASE IN HUMANS

Running title: uncompleted mitophagy in MKD yeast

Manuella Maria Silva Santos[1,2], Dámian Gatica Mizala[3], Jaqueline de Azêvedo Silva[2,3], Sergio Crovella[2,3] Daniel Jay Klionsky[4], and Marcos Antonio de Morais Junior[1,2 *]

Affiliations:

1- Interdepartmental Research Group in Metabolic Engineering, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

2- Department of Genetics, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

3- Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

4- Life Sciences Institute and Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA

***Corresponding Author:**

Marcos Antonio de Morais Junior

Departamento de Genética – Universidade Federal de Pernambuco

A.v. Moraes Rego, 1235, Cidade Universitária, 50670-901 Recife, PE, Brazil, Tel: +55-81-21267817 Fax: +55-81-21268622, marcos.moraisjr@ufpe.com

Abstract

Mutations in the gene coding mevalonate kinase (MVK) are responsible for mevalonate kinase deficiency (MKD), an autosomal recessive disorder in humans which limits the isoprenoid biosynthesis. In previous work we used MVK deficient strain of *Saccharomyces cerevisiae* as a biological model to unveil the basis of the human disorder. As reported, MKD strain showed reduced growth rate in glycerol, which was not rescued by isoprenoids supplementation. The genetics and physiological analyses resulted in a mechanistic model in which mitophagy could be initiated but not terminated, resulting in accumulation of malfunctioning mitochondria. In the present work, we consolidated the evidences for that perturbance by analyzing the relative expression of autophagy-related genes (ATG) and using GFP labelling of mitochondrial protein Idh1 as cellular marker. The results confirmed the above-mentioned assumption that MKD phenotype indeed results in incomplete mitophagy that lead to accumulation of damaged mitochondria. This could be one of the bases of unspecific inflammatory response observed in human MKD patients.

Key words: *Saccharomyces cerevisiae*, autophagy; mevalonate kinase; mitochondrial disorder; vacuolar targeting

INTRODUCTION

The impairment of the mevalonate pathway has been observed in several metabolic diseases, such as Mevalonate Kinase deficiency (MKD; OMIM #610377). MKD described as an autosomic recessive inborn error of metabolism, is a rare pediatric and autoinflammatory disease caused by mutations at mevalonate kinase gene (MVK), encoding mevalonate kinase (MK) enzyme (Clayton 1998; Moebius et al. 2000; Posada et al. 2010; van der Burgh et al. 2013). At present, the difficulties of handling animal or cell models mimicking MKD stimulate alternative approaches, like the yeast erg12-d model, a strain of *Saccharomyces cerevisiae* with deficiency in the production of MVK enzyme used in our recent work (Santos et al. 2018). In that work, we reported that the erg12-d mutant showed a decreased growth rate in glycerol as a carbon source, indicating a respiratory deficit. The shortage of the intermediates of the MVK metabolic pathway possibly associates with a decrease in ubiquinone synthesis, which is essential in respiratory chain. That respiratory deficiency might be aggravated by the downregulation of *RSF2* gene in erg12-d using glycerol as carbon source compared to the wild type. *RSF2* encodes a transcription factor involved in the regulation of respiration and growth in glycerol (Lu et al. 2005). Furthermore, it was observed the upregulation of several genes related to the autophagy/mitophagy process in response to growth in glycerol, which led us to hypothesize that the mitochondrial defect in mevalonate impairment might be activating the autophagic process (Santos et al. 2018).

The mitochondrial integrity is regulated through a fine process of quality control termed autophagy, that is highly conserved from yeast to mammals, being important for the maintenance of cellular homeostasis (Feng et al, 2014). This

process participates as the responsible pathway for mitochondrial recycling through specific recognition of the organelle' signaling (mitophagy) (Mijaljica et al, 2007; Zhang et al, 2007). Mitophagy is a selective macro-micro-autophagy trigged by selective recognition of damaged mitochondria and mobilization of cytosolic particles for the formation of double-membrane vesicles termed autophagosomes (Feng et al, 2014). This mechanism involves a series of autophagy-specific proteins (Atg) involved in the stages of the mitophagic process, from activation to degradation of a defective mitochondria (Tsukada et al, 1993; Thumm et al, 1994; Klionsky et al 2003). In this context, the goal of the present work was to evaluate genes that participate in the mitophagy activation and autophagosome formation, besides monitoring the mitophagy in the erg12-d model in *Saccharomyces cerevisiae*.

RESULTS AND DISCUSSION

In a recent study we observed that our *S. cervisiae* erg12-d model yeast growing on glucose and glycerol, fermentable and non-fermentable carbon sources, respectively, indicated deficiency in respiration as the major problem caused by shortage in the MVK pathway (Santos et al. 2018). In that work, we detected the down-regulation of *OLI1* mRNA, encoding the subunit C of the ATP synthase in the F0F1 complex in erg12-d mutant relative to its parental strain (Santos et al., 2018). Interestingly, in yeast, alterations of F0F1-ATPase biogenesis triggers autophagy (Priault et al, 2005). Our previous results led us to hypothesize that mitophagy is induced but might not be completed in erg12-d mutant defective in MVK activity (Santos et al. 2018).

To test that hypothesis, we performed gene expression analyses specifically for genes related to mitophagy and formation of the autophagosome in erg12-d mutant and its parental strain cultivated in glycerol relative to their expression in glucose. The results showed that all tested genes, but *ATG41*, exhibited significantly upregulation in the mutant strain (Fig. 1). First, we evaluated the relative expression of *ATG32* gene that encodes a mitochondrial membrane protein, essential for selective degradation of the mitochondrion by specific autophagic process called mitophagy (Kanki et al, 2009; Okamoto et al, 2009). The protein Atp32 (Atg32p) is not required for the other nonselective autophagic mechanisms, such as Cvt pathway, and works as a receptor to recruit the mitochondrion to the pre-autophagosomal structure (PAS), and also binds to Atg8p to complete vesicle formation (Kanki et al, 2009). The present work detected an eight-fold increase in the expression of *ATG32* in erg12-d mutant in glycerol, while *ATG32* expression remained unaltered in the parental strain in the same condition (Fig. 1). This result indicated that the severe deficiency in respiration caused by shortage of MVK activity observed in erg12-d mutant triggers an intense signal for mitophagic process, indicating a close relation between MKD and mitophagy.

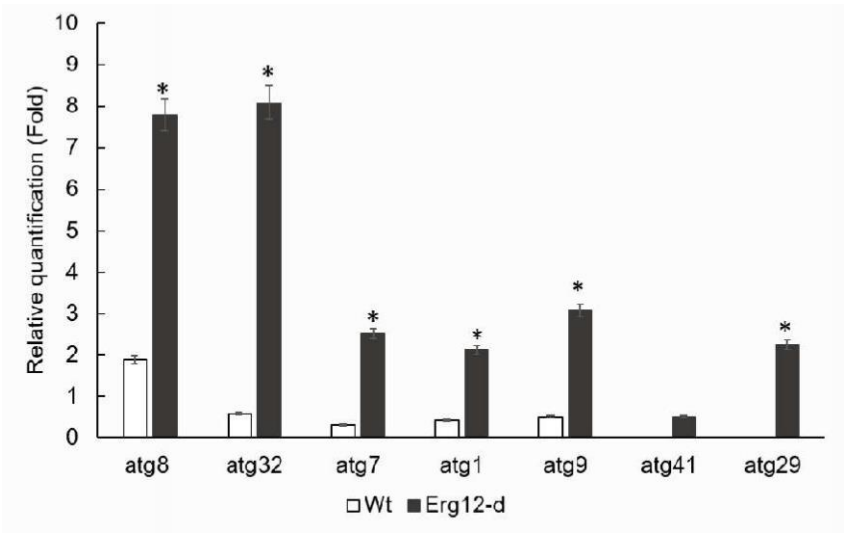

Figure 1. Relative expression of genes involved in the autophagic mechanism. Relative quantification represents the expression of the genes studied in yeasts cultivated in glycerol (20g / L) relative to those grown in glucose from the wt (white bars) and Erg12-d (black bars) strains. The data represent the average of at least 3 independent experiments. (*) represents the significant difference at $p<0.001$.

Furthermore, we tested the expression of *ATG1* gene, encoding the kinase that acts as major regulator of the protein complex involved in general autophagy (Kamada et al 2000, Kijanska et al 2010, Köfinger et al 2015). This complex includes others Atg proteins such as Atg13p, a regulatory subunit, and the Atg17p-Atg31p-Atg29p complex that function as a scaffold, in addition to mediating the retrieval of Atg9 from PAS (Reggiori et al 2004). It was also included the analysis of readouts of *ATG29* gene as part of this complex, as well as *ATG9 and ATG41* genes, encoding for proteins that are involved in the formation and expansion of the PAS complex. Both *ATG29 and ATG9* were upregulated by two and three times, respectively, in the mutant strain grown in glycerol over glucose (Fig. 1). The complex the Atg17p-Atg31p-Atg29p is the first target to the PAS when autophagy is initiated, and afterwards recruits other Atg proteins such as Atg1 and Atg13 to the complex (Suzuki et al 2007, Kawamata et al 2008). Deletion of *ATG29* leads to dramatic decrease in

autophagy activity, showing its importance in autophagic process (Kawamata et al 2005). In addition, *ATG9* encodes an integral membrane protein essential for double-membrane vesicle formation that transits between the PAS and peripheral sites (Suzuki et al 2001; Reggiori et al 2004). When this gene is absent, the recruitment of Atg components to the PAS fails, indicating that this factor plays a key role in organizing this specialized site (Reggiori et al 2005, Mari et al 2010). The upregulation of *ATG1* and *ATG9* is indicative that erg12-d mutant is also able to signaling for the initiation of PAS complex.

The protein Atg9 colocalizes peri-mitochondrially with Atg41p and this last protein acts in determining the frequency of autophagosome formation, and its absence reduces the number of autophagic bodies (Yao et al 2015). In our results, the expression of *ATG41* in glycerol was not significantly different to the one observed in glucose (Fig. 1), thus suggesting that the Erg12-d mutant is not capable of triggering the increment of autophagic bodies that is normally observed in glycerol-growing cells, necessary for the completion of mitophagy. In addition, the expression of the *ATG8* gene was upregulated by eight times in glycerol relative to glucose (Figure 1). Its protein is a highly conserved lipid-conjugated ubiquitin-like protein (Ubl), which participates in the Ubl conjugation systems required for the formation of the double-membrane vesicles responsible for the delivery of cytoplasmic material to lysosomes (Nakatogawa et al., 2007; Xie et al., 2008, Nair et al, 2012). Atg8p works along with atg7p, a noncanonical E1-enzyme that recognizes and activates Atg8p (Xie et al 2008). However, it still unclear how this recognition mechanism works, although structural and biochemical data indicate that Atg8 is first recognized by the C-terminal tail of Atg7p (Ichimura et al 2000). Our results showed that *ATG7* was upregulated only by three times (Fig. 1), lower than expected from the reported

cooperation between Atg7 and Atg8. Thus, the expression of *ATG7* was not increased as expected that might reflect negatively on autophagosome expansion, implying incomplete recycling and mitochondrial degradation. Overall, these gene expression profile indicated that mitophagy and PAS complex are initiated but not completed in erg12-d mutant, and the accumulation of old damaged mitochondria by the impaired respiration turn mutant yeast cells to quiescent state in glycerol as carbon source.

In view of the above discussed results we monitored the process of the activation of mitophagy using the isocitrate dehydrogenase enzyme (Idh1p) target with GFP at its C-terminus. This method takes advantage of the observation that GFP present in fusion constructs is relatively stable within the vacuole lumen and is often released as an intact protein after delivery to the vacuole (Shintani and Klionsky, 2004). It has been reported that Idh1p co-localizes with Atg32p in the vacuole upon induction of autophagy, and that the completion of mitophagic processing ensures vacuolar GFP accumulation (Kanki and Klionsky 2008; Kanki et al 2009). On the other hand, both processing and vacuolar accumulation are blocked when mitophagy is impaired by, for example, absence of Atg1p (Kanki and Klionsky 2008). When the yeast cells were cultivated in glucose it was clearly observed the vacuolar-dotted fluorescence likely corresponding to autophagic bodies containing mitochondria as a cargo in both yeast strain (Fig. 2A). Considering that both normal growth and our findings indicated that MKD phenotype in yeast is specific to exclusive respiratory/oxidative metabolism. On the other hand, no dotted-concentrated fluorescence in the vacuoles, actually only a diffused-pale fluorescence, was observed when erg12-d cells were cultivated in glycerol (Fig. 2B).

Finally, we cultivated wild type cells harboring fusions of Idh1p and Atg8p with GFP in glucose medium supplemented with simvastatin. This molecule is part of statins family that acts as metabolic inhibitor of the MVK pathway by impairing HMG-CoA reductase, the first enzyme of the mevalonate pathway (Sirtori 2014). Thus, it might mimic the genetic inhibition of MVK in the erg12-d strain. No significant difference in growth was observed among wild type, erg12-d and wild type treated with simvastatin (Fig. 3A), as previously reported for wild type and erg12-d cultivated in glucose medium (Santos et al. 2018). Regarding GFP labelling, it was observed only some fluorescent dots of Idh1-GFP in the presence of simvastatin, whereas only a very few points were observed in the GFP-Atg8 construct (Fig. 3B-C). It pointed in the same direction indicated by gene expression analysis above, in which mitophagy process could be genetically trigged but not mechanistically completed when MVK pathway is not functional, leading to defective mitophagy and accumulated damaged/malfunctioning mitochondria, as previously proposed (Tricarico et al. 2015; Santos et al 2018). This failure in completing mitophagy should be caused by the reduced activity in protein prenylation due to shortage of isoprenoids derivative in MKD cells (Tricarico et al. 2015; Miettinen and Björklund 2016; Tricarico et al. 2017; Santos et al 2018). In a recent review, Miettinen and Björklund (2016) presented evidences that defect in MVK pathway trigs the mechanism of autophagy but blocks the completion of the process in human cells, and presents the consequences of such physiological failure to tissue toxicity and general inflammatory reactions, besides other the clinical symptoms.

CONCLUSION

In view of the present data, it can be concluded that the deficiency in the mevalonate kinase pathway indeed lead to respiratory failure resulting in mitochondrial malfunctioning that triggers mitophagy. However, the completion of the mitophagic process might depend on the correct level of protein prenylation, which is also impaired by the restricted supply of isoprenoids. This physiological failure might lead to accumulation of dysfunctional mitochondria that could be the cellular cause of unspecific inflammatory reactions observed for MKD patients.

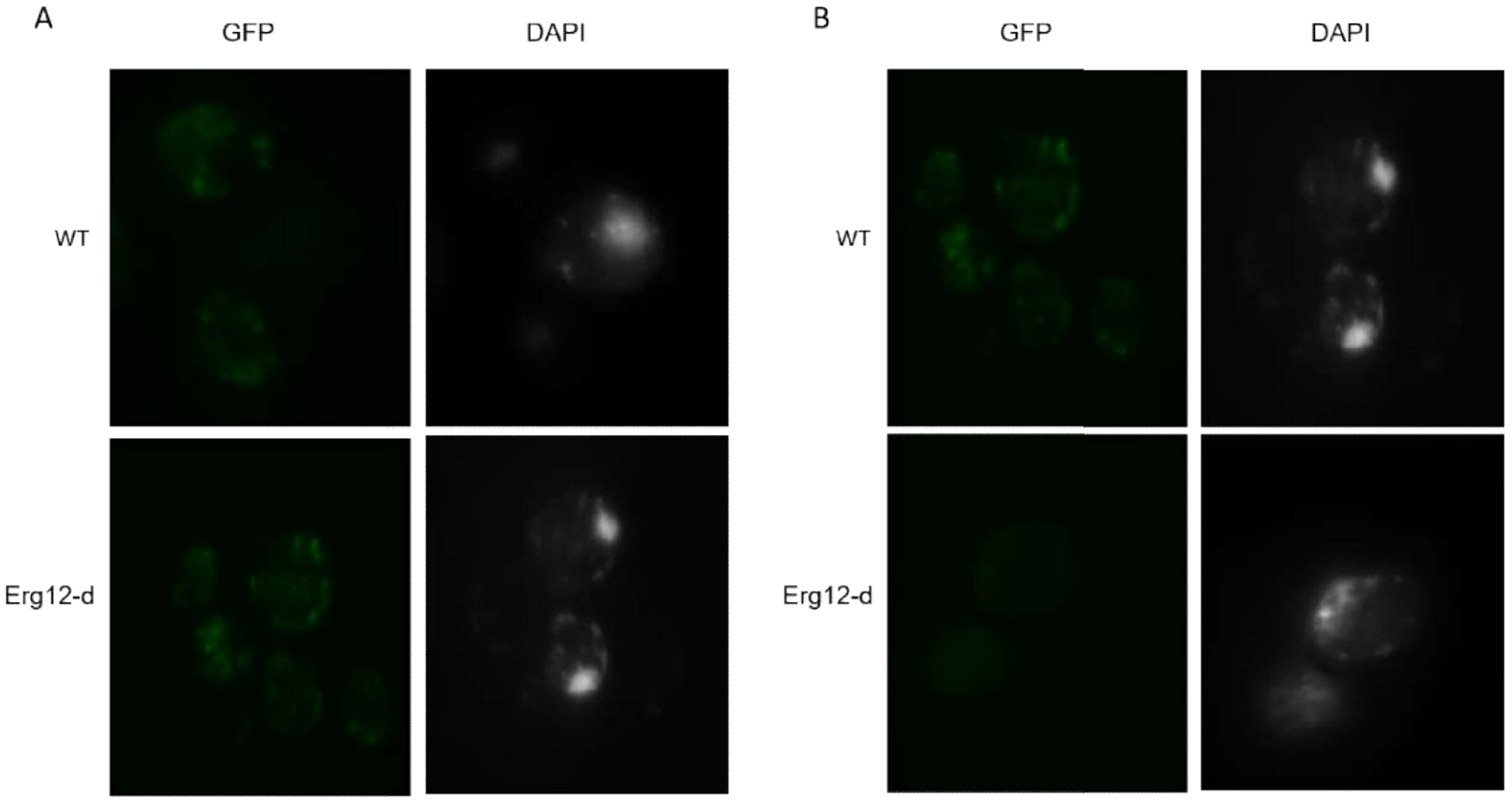

Figure 2. Monitoring mitophagy using C-terminal GFP-tagged mitochondrial protein processing. Wild type (WT) and Erg12-d strains expressing Idh1-GFP were pre-cultured in synthetic medium with glucose for 24 hours. The localization of GFP was visualized by fluorescence microscopy after cultivation in YNB with glucose (A) or glycerol (B) at 4 hours.

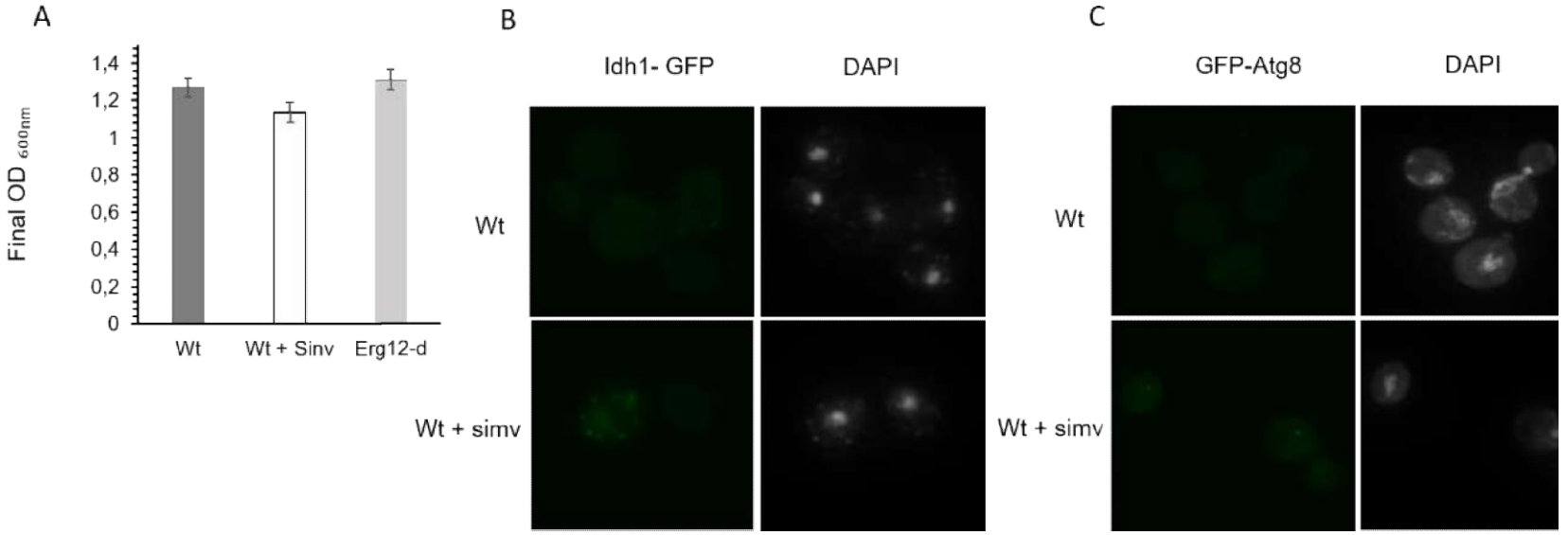

Figure 3. Growth experiments with simvastatin. (A) Yeast cells were grown in synthetic medium containing glucose with without simvastatin (simv) 50µM for 24 h. (B) Wild type (WT) strain expressing Idh1-GFP (C) Wild type expressing GFP-At Final OD were recorded for three independent experiments (± SD).

MATERIALS AND METHODS

Yeast strain

We used two diploid strains of *S. cerevisiae*: BY4743 (MAT a/ his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0), containing the intact *ERG12*, henceforth denominated the parental strain, and its isogenic YMR208W strain with attenuation of *ERG12* expression, hence forth denominated erg12-d, that result only 10% of the parental mevalonate kinase activity (Thermo Scientifics Co., USA).

Media and Cultures

Yeast cells were grown in Yeast Nitrogen Base (YNB without amino acids and ammonium sulphate (1.7 g L^{-1}), glucose (20 g L^{-1}), ammonium sulphate (5 g L^{-1}) and auxotrophic amino acids whenever needed. Deficient condition was induced through the grown in YNB containing glycerol (20 g L^{-1}) at 30 °C with agitation (150 rpm). Simvastatin were obtained from Sigma Chemical Co. Aldrich (St. Louis, MO, USA) and dissolved in saline solution at a concentration of 10mM and diluted to working concentration (50 μM).

Real time-PCR

Yeast cells were grown in YNB with glucose or glycerol and collected in the middle of exponential growth phase. Total RNAs were extracted using the RNeasy Mini kit (QIAGEN), and reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All reactions were run on Bio-rad CFX Connect™ (USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the relative expression levels determined using the ΔΔCt method with *TFC1* (Transcription Factor class C) as endogenous gene reference. The genes

expression assays are listed in Table 1. Statistical differences were assayed using ANOVA * p < 0 .05.

Table 1. Primers used in qPCR.

Gene name	Sequence (5' 3')
ATG1_Forward	ATCTAAGATGGCCGCACATATG
ATG1_Reverse	AGGGTAGTCACCATAGGCATTC
ATG7_Forward	ATGAGCATTGTCCAGCATGTAG
ATG7_Reverse	GACCTCCTGCTTTATGACTGAC
ATG8_Forward	GAAGGCCATCTTCATTTTTGTC
ATG8_Reverse	TTCTCCTGAGTAAGTGACATAC
ATG9_Forward	CGTACTAACAGAGTCTTTCCTTG
ATG9_Reverse	CTAAGACACCACCCTTATTGAG
ATG29_Forward	ATGAGGCGTTACAACATTTGC
ATG29_Reverse	TCGTCATCTGAACTACCGCAC
ATG32_Forward	GGGCAAAATGAATACTTTTGTCTTGCATGC
ATG32_Reverse	CCCAGTGCCAAAATCCGATTAGATTCATC
ATG41_Forward	CGAGTACTGAAGACGATTGCAT
ATG41_Reverse	TGCGACATTGGCAAAGGCAT
TFC1_Forward	GCTGGCACTCATATCTTATCGTTTCACAATGG
TFC1_Reverse	GAACCTGCTGTCAATACCGCCTGGAG

Fluorescence Microscopy

PCR-based integration of a DNA fragment encoding green fluorescent protein (GFP) at the 3' end of IDH1 generated cells expressing chromosomally tagged Idh1-GFP for the mitophagy (Longtine et al, 1988). Cells expressing fusion proteins were pre-cultivated in YNB medium containing glucose or glycerol for 24h. In addition, we used the GFP-Atg8 construct in the wild type strain as reported by Kim et al. 2001 and the recombinant cells were cultivated in glucose medium in the presence or absence of simvastatin. The cells were collected after incubated in the middle of the exponential growth phase, washed in saline (0.8%) and used to prepare slides for microscope observation. The slides were visualized using *fluorescence Leica* DMIRB *microscope* and the images were captured using *Leica DC 300F* digital

camera*, and were analyzed with the Leica IM500 Image Manager software (Leica,* Bensheim, Germany).

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Competing interests

The authors declare no competing or financial interests.

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5 CONCLUSÃO

Os resultados deste trabalho mostraram que a levedura *Saccharomyces cerevisiae* foi um excelente organismo modelo para o estudo da deficiência da mevalonato quinase. Dessa forma pudemos concluir que a falta de isoprenóides não é o mecanismo principal para o mau funcionamento de células com defeito em Erg12. Na verdade, a escassez de ubiquinona, devido a deficiência na via mevalonato, desempenha um papel importante na deficiência respiratória e metabolismo celular. Afetando diretamente o funcionamento mitocondrial, ativando mecanismos de monitoramento da qualidade mitocondrial, como a mitofagia.

No entanto, concluímos que o processo mitofágico pode depender do nível de prenilação de proteínas, que é prejudicado pela restrita oferta de isoprenóides. O que pode desencadear um acúmulo de mitocôndrias não funcionais, desencadeando o processo inflamatório relatado em pacientes com MKD.

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APÊNDICE A – ARTIGO PUBLICADO NA REVISTA CURRENT GENETICS

Current Genetics
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ORIGINAL ARTICLE

Respiratory deficiency in yeast mevalonate kinase deficient may explain MKD-associate metabolic disorder in humans

Manuella Maria Silva Santos[1,2] · Carolina Elsztein[1,3] · Rafael Barros De Souza[1,4] · Sérgio de Sá Leitão Paiva Junior[5] · Jaqueline Azevêdo Silva[2,6] · Sergio Crovella[2,6] · Marcos Antonio De Morais Junior[1,2]

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Abstract
Mevalonate kinase deficiency (MKD) an orphan drug rare disease affecting humans with different clinical presentations, is still lacking information about its pathogenesis; no animal or cell model mimicking the genetic defect, mutations at MVK gene, and its consequences on the mevalonate pathway is available. Trying to clarify the effects of MVK gene impairment on the mevalonate pathway we used a yeast model, the erg12-d mutant strain *Saccharomyces cerevisiae* (orthologous of MKV) retaining only 10% of mevalonate kinase (MK) activity, to describe the effects of reduced MK activity on the mevalonate pathway. Since shortage of isoprenoids has been described in MKD, we checked this observation using a physiologic approach: while normally growing on glucose, erg12-d showed growth deficiency in glycerol, a respirable carbon source, that was not rescued by supplementation with non-sterol isoprenoids, such as farnesol, geraniol nor geranylgeraniol, produced by the mevalonate pathway. Erg12-d whole genome expression analysis revealed specific downregulation of RSF2 gene encoding general transcription factor for respiratory genes, explaining the absence of growth on glycerol. Moreover, we observed the upregulation of genes involved in sulphur amino acids biosynthesis that coincided with the increasing in the amount of proteins containing sulfhydryl groups; upregulation of ubiquinone biosynthesis genes was also detected. Our findings demonstrated that the shortage of isoprenoids is not the main mechanism involved in the respiratory deficit and mitochondrial malfunctioning of MK-defective cells, while the scarcity of ubiquinone plays an important role, as already observed in MKD patients.

Keywords Isoprenoids · Gene expression · Microarray · Respiratory metabolism · Yeast

Introduction

The impairment of mevalonate kinase pathway, a crucial metabolic process, part of cholesterol and non-cholesterol isoprenoid metabolism, is at the basis of mevalonate kinase deficiency (MKD) an orphan drug disease affecting humans with heterogeneous clinical presentation and

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✉ Marcos Antonio De Morais Junior
marcos.moraisjr@ufpe.com

[1] Interdepartmental Research Group in Metabolic Engineering, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

[2] Department of Genetics, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Cidade Universitária, Recife, PE 50760-901, Brazil

[3] Department of virology/CPqAM, Oswaldo Cruz Fundation, Avenida Moraes Rego, N/S, Recife, PE 50760-901, Brazil

[4] Institute for Biologial Sciences, University of Pernambuco, Avenida Agamenon Magalhães, s/n, Recife, PE 50100-010, Brazil

[5] Laboratory of Bioinformatics and Evolutionary Biology, Federal Rural University Pernambuco, Rua Dom Manoel de Medeiros, s/n, Recife, PE 52171-900, Brazil

[6] Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

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overall still unclear etio-pathologic mechanisms; an involvement of mevalonate pathway has been also observed in the Smith–Lemli–Opitz syndrome, caused by a defect in the postsqualene conversion compounds in cholesterol and peroxisomal disorders, characterized by low plasma cholesterol levels (Clayton 1998; Moebius et al. 2000). At present, no animal or cell model mimicking the genetic defect, namely mutations at MVK gene, at the basis of the MKD is available. In our study we tried to fill this gap using a yeast model erg-12, the orthologous of human MVK gene, deficient to describe the effects of impaired mevalonate pathway on *S. cerevisiae*.

Mevalonate kinase (MVK) acts one step downstream of HMG-CoA reductase, catalysing the ATP-dependent phosphorylation of mevalonic acid (MVA) to convert in mevalonate 5-phosphate (MVAP) (Fig. 1) (Fu et al. 2002). This step is largely regulated and MVK is negatively controlled by a feedback loop from geranyl (GPP) and farnesylpyrophosphate (FPP), produced in mevalonate kinase pathway's final steps (Dorsey and Porter 1968; Henneman et al. 2011). The mevalonate pathway is common to most eukaryotic cells being essential for cellular mechanisms as protein prenylation, protein glycosylation and cell cycle regulation by including several non-sterol isoprenoid metabolites (Fu et al. 2002). This pathway is also involved in the control of cell size and growth, autophagy and proteolysis through the action of small GTPase protein Rab11 (Miettinen and Björklund 2016).

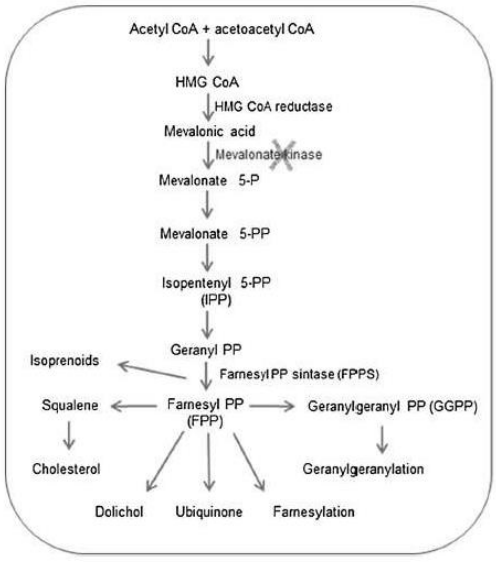

Fig. 1 Schematic representation of Mevalonate pathway in *Saccharomyces cerevisiae*. Blockade (gray cross) represents the deficiency of enzyme mevalonate kinase activity in the strain erg12-d

Mevalonate kinase deficiency (MKD) caused by mutations at MVK gene, located at 12q24, and responsible for autosomal-recessive inborn error of isoprenoid biosynthesis (Moebius et al. 2000; Stenson et al. 2014) is a human disease involving mevalonate pathway impairment. The limited rate from isoprenoids biosynthesis influences the decrease of bioactive molecules that participate in cell growth; protein glycosylation and signal transduction processes as well as dolichol, ubiquinone, may be deficient (van der Burgh et al. 2013).

The MKD is characterized by a shortage in geranylgeranyl pyrophosphate (GGPP), a precursor for isoprenylation of small G proteins, which in turn activates caspase I, stimulating inflammatory events such as recurrent fever and generalized inflammation, due to activation of NLRP3-inflammasome (NLRP: nucleotide-binding oligomerization-domain protein-like receptors) (van der Burgh et al. 2013; Tricarico et al. 2014). MKD has a clinical heterogeneous phenotype, with a milder form known as hyper IgD syndrome (HIDS, OMIM #260920) and a more severe one, the mevalonate aciduria (MA, OMIM #610377). At present, MKD pathogenesis is just partly understood and a specific etiologic therapy for this disease is still unavailable.

Therapies using statins and anti-IL-1, have been used with some success to prevent and cure MKD-inflammatory symptoms (Drenth et al. 2001; Bodar et al. 2005), although only supportive therapies are accessible for mevalonic aciduria. The search for new therapies for metabolic disease could take advantage of animal models to mimic human disease's features. Marcuzzi et al. (2008, 2011), developed a mouse model for MKD, showing that the chemical inhibition of the mevalonate pathway through the use of an amino bisphosphonate and statins lead to moderate inflammatory phenotype that could be amplified by bacterial compounds such as muramyl dipeptide (MDP) or lipopolysaccharide (Marcuzzi et al. 2008, 2010, 2011). Recently, MKD cell models employing human monocytes and neuronal cells have been proposed, using natural exogenous isoprenoids, such as geraniol (GOH), Farnesol (FOH), geranylgeraniol (GGOH), as a potential therapeutic approach for MKD (Marcuzzi et al. 2010, 2012). Due to their isoprenoid structure, these compounds are able to enter the mevalonate pathway and bypass the biochemical block, thus limiting the shortage of GGPP. Additionally, the isoprenoids have been reported as able to rescue inflammation in the above-mentioned MKD model (Marcuzzi et al. 2008; Frenkel et al. 2002; Mandey et al. 2006).

Aimed at investigating the metabolic pathway in which mevalonate kinase plays a pivotal role, we took advantage of *Saccharomyces cerevisiae* mevalonate kinase (MK) deficiency. In humans, *MVK* gene gene is essential for cell

viability and important for isoprenoid synthesis, thus mutations compromising enzyme activity affect the production of important metabolites in electron transport and respiratory metabolism; this causes mitochondrial damage, incomplete autophagy and subsequent cell death (Tricarico et al. 2015). The mevalonate pathway in *S. cerevisiae* is similar to the human one and mevalonate kinase enzyme is encoded by *ERG12* gene (Altmann and Westermann 2005). Some characterization of its deficiency has been reported for the heterozygous yeast strain, as full mutant is unviable, pointing out for mitochondrial abnormalities and respiratory deficiency (Altmann and Westermann 2005). However, there are no direct evidences for the molecular connection between MVK deficiency, isoprenoid shortage and respiratory deficit. Given the phenotypic similarities of MVK heterozygous cells in humans and yeast and due to the methodological facilities for cell manipulation and analysis, the aim of the present study is to identify the key aspect of the MVK deficiency in *S. cerevisiae* using a mutant that express only residual activity of this enzyme to expand the knowledge. This strain was subject to a combination of wide gene expression analysis and a set of physiological/biochemical data to uncover this relationship. By doing so, we seek to contribute for the understanding, at least in part, of the etio-pathogenesis of MKD human disease.

Methods

Yeast strain

We used two diploid strains of *S. cerevisiae*: BY4743 (MAT a/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0), containing the intact *ERG12*, henceforth denominated the wild type, and its isogenic YMR208W (GE Dharmacon), with attenuation of *ERG12* expression (Thermo Scientifics Co., USA). This mutant strain, named in our study Erg12-d (Erg12 deficient), contains both *Erg12* alleles with disruption of the 3′ untranslated region (3′ UTR), which destabilizes the corresponding transcript thus decreasing mRNA stability and reducing protein synthesis up to 10% with respect to the wild type.

Growth experiments

For the growth experiments, yeast cells were pre-grown overnight in YNB (Yeast Nitrogen Base, Difco) synthetic reference medium 1.6 g/l with ammonium sulphate, amino acids required and glucose at 20 g/l, at 30 °C with agitation (150 rpm). Afterwards, cells were collected via centrifugation and re-suspended in fresh medium to an initial cell density (OD 600) of 0.1 in sterile microtiter plates (96-well) for a total volume of 150 µL with specific media. The same procedure was performed for growth assays with a respirable carbon source, glycerol (20 g/l). Cultivations were performed in a Synergy HT device (BioTek, Switzerland) at 30 °C and maximal speed with absorbance measurement every 30 min for 24 h. Wild-type and Erg12-d strain were also cultivated in synthetic medium in glycerol (20 g/l) or glucose (20 g/l) containing 5 mM of thiol antioxidant N-acetylcysteine (NAC) (Kwolek-Mirek et al. 2012). Growth curves were prepared from the average of two biological experiments and the growth rate was calculated from the slope of the exponential growth phase.

Growth recovery experiments of BY4741 (white column) and erg12-d (grey column) were performed in flasks at 30 °C with 150 rpm agitation. Yeast cells were grown in synthetic medium containing glycerol. After 24 h, yeast cells were transferred to synthetic medium containing glucose for initial OD of 0.1 and incubated for 24 h. After this time, cells were transferred to synthetic medium containing glycerol and cultivated for another 24 h. Final OD were recorded for three independent experiments (± SD).

Cultivations supplemented with isoprenoids

Cells were pre-cultivated overnight in synthetic yeast nitrogen base (YNB) medium without amino acids (1.7 g/l) containing glucose (20 g/l), ammonium sulphate (5 g/l), supplemented with each required amino acids at 30 °C and 150 r.p.m. The strains were re-inoculated in the same medium for the exponential growth phase providing seed cultures for inoculation into 150 µl plate wells containing fresh YNB medium, glycerol (20 g/l), isoprenoids and required amino acids, then incubated for 24 or 48 h on the Synergy HT (BioTek, Switzerland). Isoprenoids compounds farnesol (FOH), geraniol (GOH), geranylgeranyol (GGOH) were added at the following concentrations: 0, 25, 50 and 100 µM (Marcuzzi et al. 2010).

Determination of sulfhydryl groups

Protein-bound sulfhydryl (PB-SH) levels were measured in accordance with the method of Sedlak and Lindsay (1968), by subtracting the nonprotein sulfhydryl (NP-SH) content from the total sulfhydryl (T-SH) content (Demasi et al. 2006). Cells of the BY4743 and Erg12-d strains were grown on YNB plates the presence of 20 g/l glucose or 20 g/l glycerol, for 72 h at 30 °C. Approximately, 10^9 cells were collected from each culture. Protein extracts were obtained in 0.02 M EDTA pH 4.7 with the addition of glass beads, followed by centrifugation at 17,900*g* for 15 min. The T-SH concentrations were determined by absorption levels at 412 nm after incubating 200 µl aliquots of protein extracts supernatants with 780 µl of 0.2 M Tris pH 8.2 and 20 µl of 5 mM DTNB for 30 min. The (NP-SH) contents were

determined in the supernatant after protein precipitation with 5% trichloroacetic acid (final concentration) by incubating 450 µl of supernatant, 900 µl of 0.4 M Tris pH 8.9 and 26 µl of 5 mM DTNB for 5 min (Elsztein et al. 2011). Absorbance was measured at 412 nm and the protein-bound sulfhydryl (PB-SH) content was calculated by subtracting the NP-SH value from the T-SH content. The results are relative to the concentration of these groups in the control cells (100%) that were not exposed to any agent, and represent the average of three separate experiments.

Transcriptome-wide expression microarray analyses

Cells were pre-cultivated overnight in synthetic yeast nitrogen base (YNB) medium without amino acids (1.7 g/l) containing glucose (20 g/l), ammonium sulfate (5 g/l), supplemented with each required amino acid at 30 °C and 150 r.p.m. Afterwards, cells were collected at the exponential phase, washed in 0.8% saline solution, re-suspended in glucose or glycerol synthetic medium with low nitrogen content, and incubated for 4 h at 30 °C and 150 r.p.m. Collected cells were centrifuged and re-suspended in 400 mL of AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.3) and 80 mL of 10% SDS solution, mixed using a vortex and incubated at 65 °C for 10 min. The total RNA was extracted from lysates using a Maxwell® 16 LEV simply RNA Blood Kit (Promega, USA). The total RNA was purified using a RNAspin Mini RNA Isolation Kit (GE HealthCare, USA) and quantified on the NanoDrop ND-2000 UV–Vis spectrophotometer (ThermoFisher Scientifics, USA). Synthesis of cDNA, cRNA and labelling were performed using a Two-Colour Low Input Quick Amp Labelling Kit (Agilent, USA) following manufacturer's instructions, with a Two-Colour RNA spike-in kit as the internal control (Agilent, USA) (Lucena et al. 2015). Differential marking with the Cy3 and Cy5 fluorophores was in the following combinations: [BY4743 glycerol] × [BY4743 glucose]; [*Erg12-d* glycerol] × [*Erg12-d* glucose]; [*Erg12-d* glycerol] × [BY4743 glycerol]; [*Erg12-d* glucose] × [BY4743 glucose]. Target and reference cRNA samples were pooled and used to hybridise the yeast gene expression 8 × 15 k spot slides (Agilent, USA) at 65 °C for 17 h at 10 r.p.m. in a microarray hybridization oven (Agilent, USA). Fluorescent data from a 3 µm-resolution microarray scanner (Agilent, USA) were extracted as .txt files using the software Feature Extraction (Agilent, USA). Relative *t* test (Adj. $p < 0.05$) and log posterior odds ratio of differential expression versus non-differential expression ($B \geq 3$) were utilized for the analyses of statistical significance of differentially expressed gene groups. Log FC values used to classify up- (≥ 0.5 log FC) and downregulated (≤ -0.5 log FC) genes, a term meaning the abundance of transcripts under test conditions relative to the reference conditions. Microarray data were deposited in the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) under the access code GSE98163. The Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/), Gene ontology Slim Mapper (http://www.yestgenome.org/help/analyze/go-slim-mapper) and YeastMine (http://yeastmine.yeastgenome.org/yeastmine/begin.do) databases were used for the recognition of encoded proteins and GO clustering of the genes.

Results and discussion

Physiological characterization of the MVK Erg12-d

The results of growth curve showed that the *erg12-d* strain grew poorly when compared to the wild-type (wt) strain in glycerol, which requires respiration for its metabolism, while growing normally in glucose (Fig. 2). Using glycerol as the only carbon source during growth, this organic compound induces activation of the enzyme glycerol kinase (Gut1p), which promotes its phosphorylation to glycerol 3-phosphate (3-P) (Pavlik et al. 1993). Then, glycerol 3-P is converted to dihydroxyacetone phosphate by the enzyme FAD-dependent glycerol 3-P dehydrogenase (Gut2p) present in the outer mitochondrial membrane. In the presence of glucose, the genes encoding these enzymes are repressed, leading yeast to respiro-fermentative metabolism (Sprague and Cronan 1977). The fact that *erg12-d* did not grow on glycerol indicated that *ERG12* deficiency in yeast, involving mitochondrial functions, is similar to what is observed in humans with mutations in the *MVK* gene. To test whether mutant cells simply stop growing or die in glycerol, yeast cells grown in glycerol were used to inoculate glucose medium. The results showed that mutant cells recovered full growth on glucose after 24 h of poor growth in glycerol, and turned to grow badly when returned to glycerol medium (Fig. 3). Therefore, whatever the metabolic effect, it was not lethal for *erg12-d* mutant in glycerol, and most probably it has been able to induce a senescent state from which mutant cells can recover as soon as glucose is present in the medium.

It is well reported that human cells with deficiency in MK activity, due to *MVK* mutations, display a reduction in the production of isoprenoids (Tricarico et al. 2014), which is also the case for mutant yeast strains (Altmann and Westermann 2005). To test whether this deficiency is the primary cause of mitochondrial malfunctioning, we grew the yeast cells in glycerol-based medium supplemented with exogenous isoprenoids FOH, GOH and GGOH. We observed that concentration starting from 25 µM displayed some cytotoxic effect on the wt strain, and did not recover growth deficiency of the *erg12-d* strain (data not shown). Concentration of 100 µM for both FOH (Fig. 4a) and GOH (Fig. 4b) was very toxic, reducing growth rate of the wt cells from 0.19 h^{-1} to > 0.01 h^{-1} for

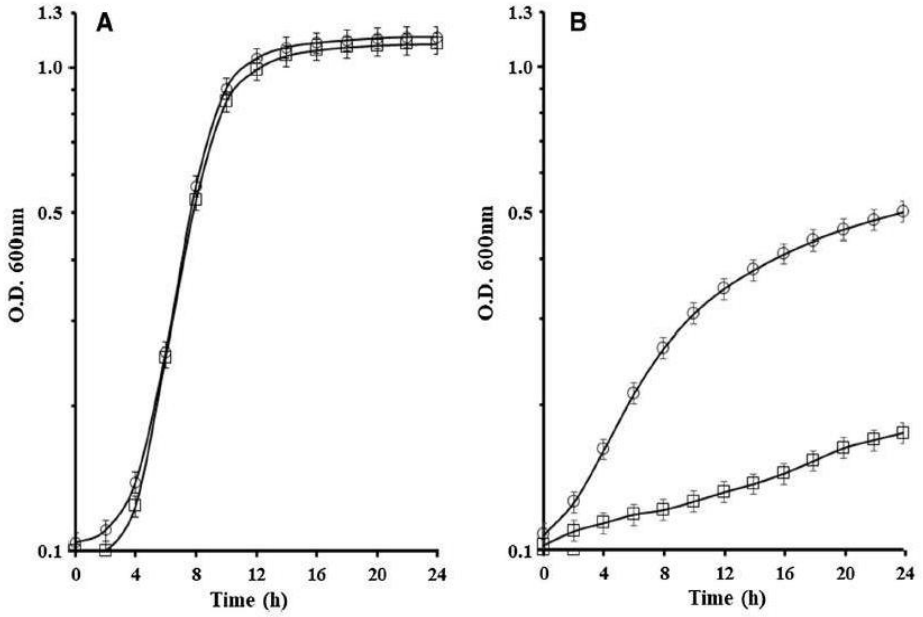

Fig. 2 Effect of the carbon source on the growth of *Saccharomyces cerevisiae* wild type BY4743 (open circle) and Erg12-d mutant (open square) strains in synthetic medium containing glucose (panel A) or glycerol (panel B) as carbon source

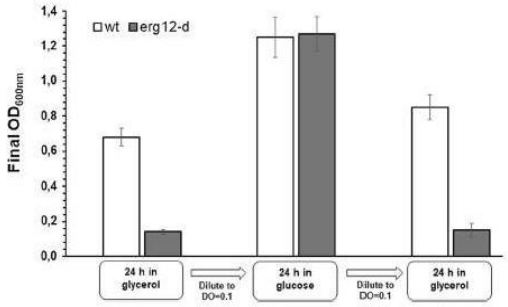

Fig. 3 Growth recovery experiments of BY4741 (white column) and erg12-d (grey column). Yeast cells were grown in synthetic medium containing glycerol. After 24 h, yeast cells were transferred to synthetic medium containing glucose for initial OD of 0.1 and incubated for 24 h. After this time, cells were transferred to synthetic medium containing glycerol and cultivated for another 24 h. Final OD were recorded for three independent experiments (± SD)

both compounds. GGOH was only mildly toxic, reducing growth rate from 0.06 h^{-1} (Fig. 4c). Besides, no effect of any of the terpenoids tested was observed for Erg12-d strain in recovering cell growth to the one of wt level (Fig. 4). Only a slight growth was observed after 48 h of cultivation with GGOH (data not shown). Adverse effects on cell growth and posttranslational protein modifications associated with FOH or GGOH moieties, possibly occur due to partial or complete inhibition of MK activity (DeClue et al. 1991). Interestingly, besides the inefficiency of exogenous isoprenoids in recovering the growth of *erg12-d* mutant, different effects in treatment of exogenous isoprenoids were described in MKD mouse model, suggesting a possible role for these compounds in the treatment of MKD in humans (Marcuzzi et al. 2008, 2010, 2011). Machida et al. (1998) reported FOH toxic effect of in *S. cerevisiae* cell: the authors showed significant increase of ROS generation after treatment with FOH in a dose-dependent manner. In addition, GOH is effective in inhibiting of pseudohypha formation by *Candida albicans*, the essential aspect of yeast pathogenicity in response to environmental changes (Bard et al. 1988). This compound was shown to disturb the integrity of the cell membrane and to increase its permeability, causing leakage of potassium out of the cell (Zore et al. 2011; Herrero et al. 2008). Our results corroborated these findings, showing the inhibitory effect of exogenous isoprenoids to the yeast cells. Taking into account that *erg12-d* strain has about 10% of MK activity, we suppose that such residual activity could partially supply the cells with isoprenoid requirement, and isoprenoid medium supplementation would lead to the excess of these metabolites in the cells

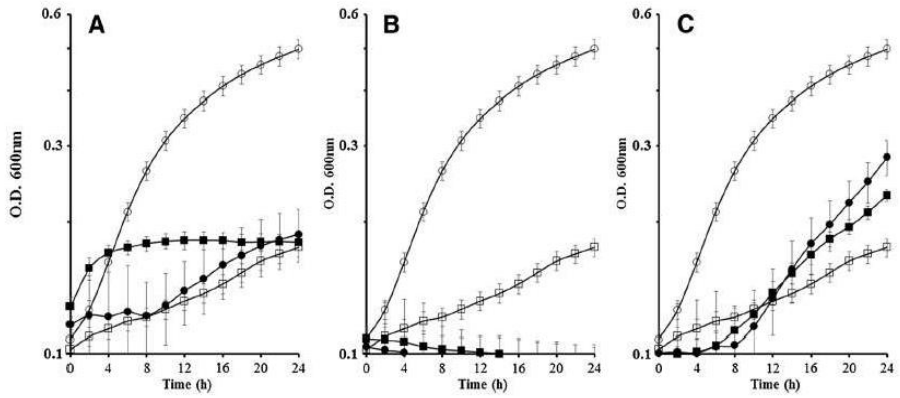

Fig. 4 Effect of Farnesol (panel **a**), Geraniol (GOH) (panel **b**) or Geranylgeraniol (GGOH) (panel **c**) on the growth of *Saccharomyces cerevisiae* wild type BY4743 (filled circle) and Erg12-d mutant (filled square) strains in synthetic medium containing glycerol as carbon source. Growth on non-supplemented media (open circle, open square) is shown

at toxic concentrations. Therefore, the residual concentration of isoprenoids will be enough for some, but not all, isoprenoid-dependent molecular processes.

Glycerol increases the oxidative stress

Based on the results reported above, we hypothesised that growth defect of *erg12-d* in glycerol could not be only due to isoprenoid shortage. Thus, we turned the attention to the possibility that mitochondrial malfunctioning could be caused by increased production of reactive oxygen species (ROS), and induction of oxidative stress as consequence, during respiratory growth. Cells take advantage of the production of thiols compounds, such as glutathione, and SH-containing proteins, like the small protein thioredoxins, to detoxify the oxidant compounds produced by ROS (Herrero et al. 2008). So, we performed growth experiments in glycerol medium supplemented with the antioxidant agent NAC. No effect of this compound was observed for both strains when glucose was used as carbon source (Fig. 5a). On the other hand, it was observed a positive effect on the growth of wt strain in glycerol (Fig. 5b). Thus, growth in glycerol indeed leads to the production of ROS by the high respiratory activity, which is not observed in respiro-fermentative state of the cells with glucose (Fig. 5a). Some protective effect by NAC was also observed for the Erg12-d growing in glycerol (Fig. 5b), although it was not enough to restore growth to wt level. Therefore, despite preventing damages caused by ROS produced by respiration, the presence of NAC was not sufficient to directly rescue the metabolic problem caused by deficiency in MK activity.

Furthermore, we measured the content of thiol compounds in wt and *erg12*-d strains at exponential growth phase. We showed that both strains equally produced more non-proteic thiol compounds (glutathione content) when growing on glycerol than on glucose (Fig. 6). The production of these compounds is indicative of oxidative stress in response to respiration. Therefore, the oxidative stress response seemed not to be affected by the deficiency in MK activity. However, it was observed the significant increase in SH-containing proteins (including thioredoxin) in the Erg12-d cells compared to the wt strain in response to oxidative stress (Fig. 6), indicating a link between the production of sulfhydrylated proteins with the impairment of MVK pathway. In view of fact that MK shortage is connected to respiratory deficiency without connection with oxidative stress, we analysed the global gene expression to understand the direct effect of mevalonate pathway impairment in yeast metabolism.

Overview of gene expression in response to growth on glycerol

Whole genome expression in erg12-d cells were compared to wild type cells both incubated in glucose and glycerol. Cells pre-grown in glucose medium, which displayed similar growth profile (Fig. 2a), were collected at the exponential phase, washed and re-suspended in glucose or glycerol medium. To avoid the effect on growth efficiency caused by MVK defect in glycerol (Fig. 2b), synthetic medium was formulated with low nitrogen content, which limited growth of both cell types. Relative gene expression analyses were performed after four hours of incubation and the differentially expressed genes were grouped according with well-defined metabolic pathways or biological processes by gene ontology (GO) (Lucena et al. 2015). The specificities

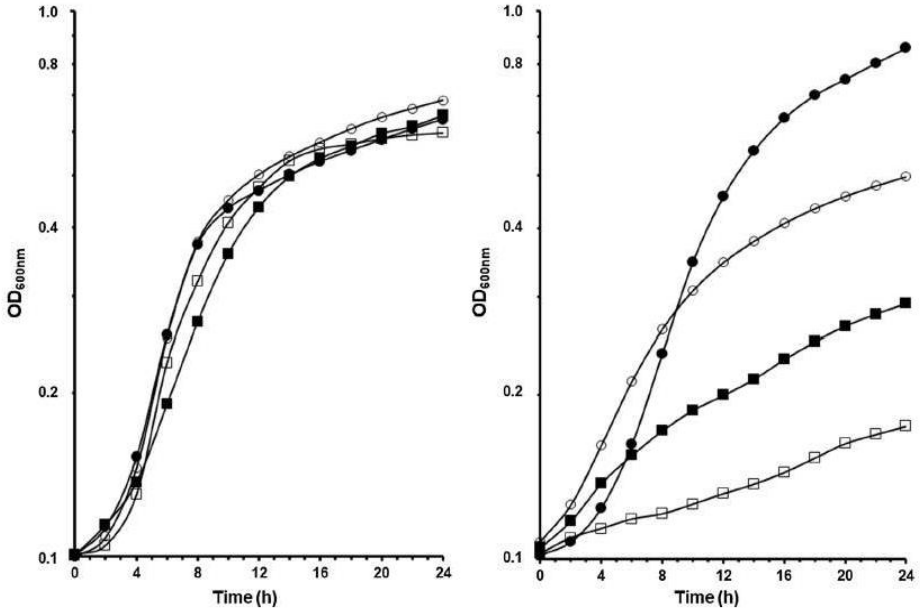

Fig. 5 Effect of supplementation of synthetic media containing glucose (panel **a**) or glycerol (panel **b**) with 5 mM of *N*-acetylcysteine (NAC) on the growth of *Saccharomyces cerevisiae* wild type BY4743 (filled circle) and Erg12-d mutant (filled square). Growth on non-supplemented media (open circle, open square) is shown

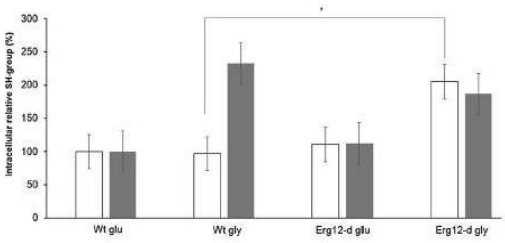

Fig. 6 Relative quantification of intracellular content of sulfhydryl groups in soluble proteins (white columns) and non-proteins (grey columns) in the cell extract of wild type BY4743 and Erg12-d mutant strains grown in synthetic medium containing glucose or glycerol as carbon source. Significant difference was observed at $*p$ value < 0.05 (ANOVA: $p < 0.04$)

and overlapping for the four conditions were graphically represented (Fig. 7) and the list of expressed genes can be accessed in the supplementary material (Table S1). Following the lack of physiological differences between wild type and erg12-d in glucose (Fig. 2a), we did not observe significant difference in whole genome expression. Therefore, we conclude that whatever effect is caused by the deficiency in MK activity, it does not express relevant phenotype or different gene expression profile when the cells are in respiro-fermentative metabolic state. On the other hand, we observed that in the wild type strain, 493 genes were upregulated and 686 were downregulated in glycerol with respect to glucose. As expected, genes of the oxidative stress response, oxidative phosphorylation, autophagy (*ATG8, ATG4* and *ATG34*), ubiquinone biosynthesis (*UBX5, UBX2, QCR6* and *QCR7*), and purine synthesis were upregulated in response to glycerol and respiratory metabolism. In contrast, genes involved in the transcription mechanism, biogenesis of the ribosomes, synthesis and processing of rRNA and tRNA, and the protein synthesis rate were downregulated in glycerol. These findings corroborate the physiological data of lower growth rate of wild-type strain in glycerol than in glucose (Fig. 2) (Lempiainen and Shore 2009).

Regarding erg12-d strain, 325 genes were upregulated and 412 were downregulated in glycerol with respect to glucose. Biological processes and metabolic pathway affected by glycerol in the Erg12-d strain were like those reported above for the wild type. However, the defect in growth was more pronounced in the Erg12-d grown in glycerol than in the wild type. Therefore, other genetic differences should exist to explain the impaired growth of Erg12-d cells. A total of 65 genes showed significant changes in their expression in the Erg12-d relative to the wild type when both were

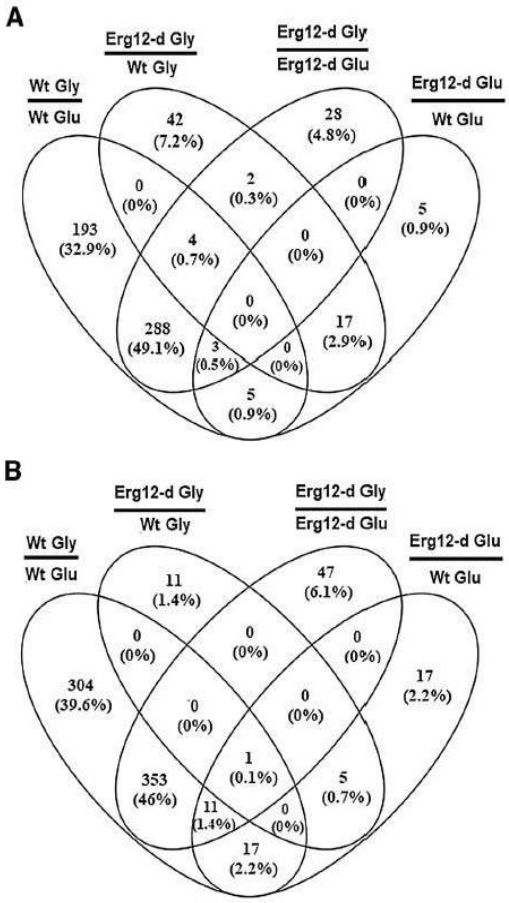

Fig. 7 Venn diagram representing the overlaps of differentially expressed genes in four conditions: Erg12-d glycerol relative to wild type glycerol, wild type glycerol relative to wild type Glucose, Erg12-d glycerol relative to Erg12-d glucose and Erg12-d glucose relative to wild type glucose. Percentage of upregulated (panel **a**) and downregulated genes (panel **b**) were shown

incubated with glycerol as carbon source, which might explain the growth difference observed in Fig. 2b. Within these genes, 17 were downregulated and did not reveal any important biological process, and will be no further explored henceforth. Thus, we concentrated the analysis in the group of genes that were upregulated. Our findings revealed that the deficient growth in glycerol is not due to isoprenoid shortage, since the supplementation with these lipids did not recover the growth of mutant erg12-d strain (Fig. 4). In the growth assays with NAC supplementation as an antioxidant agent (Fig. 5) as well as in the measurements of the production of thiol compounds (Fig. 6), we observed that there was no further oxidative stress in the Erg12-d compared to wild type. However, there was an increase in SH-containing proteins in the mutant cells (Fig. 6). In this regard, we observed in erg12-d cells upregulation of key genes involved in sulphur amino acids metabolism (*MET6, MET14, MET1, MET10* and *SAM2*) that may account for the increasing of SH-containing proteins, as observed in Fig. 7. This phenomenon might represent the attempt of the yeast cells to restore its metabolic homeostasis in view of the MKV deficiency.

Our results suggest the deficiency in the respiratory metabolism of erg12-d might not cause the exacerbated production of ROS as by-products of complexes I and III of the electron transport chain. These by-products should be detoxified by SH-containing biomolecules (thiols and protein compounds) (Perrone et al. 2008). In this regard, it was observed the downregulation of the gene *RSF2* in erg12-d incubated in glycerol compared to wild type. *RSF2* encodes a transcription factor involved in the regulation of respiration and growth in glycerol (Lu et al. 2005). Noteworthy, the expression of *RSF2* was not changed in the wild type or in erg12-d in glycerol relative to glucose, meaning that in erg12-d strain the expression of this gene is always lower than in the wild type, irrespective to the carbon source used. We also highlight its activity on the regulation of *OLI1* and *COX4* genes. In our dataset, these two genes were upregulated in wild type-glycerol relative to wild type-glucose, a response absent when the cells were deficient in MK activity. *COX4* encodes the subunit IV of the cytochrome c oxidase, the last step in the phosphorylation chain, and its deletion impairs the respiratory growth (Devenish et al. 2000). In addition, *OLI1* encodes the subunit C of the ATP synthase of the F_0F_1 complex and its deletion leads to growth deficiency in glycerol (Boyer 1997; Nakamoto et al. 1999). Therefore, these results indicate that there is a direct deficiency in respiration in erg12-d.

Since mitochondria are crucial for cellular respiratory machinery, some damages related this organelle, such as organic solvent, telomere-related protein deficiency or biogenesis of molecular components of cellular respiration, cause increase in oxidative stress (Schneider et al. 1995; Nishida et al. 2015; Simonicova et al. 2015). Oxidative phosphorylation is the predominant function of mitochondria responsible to produce ATP. It occurs in a harmonic way by the action of electron carriers, such as Coenzyme Q (CoQ) or ubiquinone, a key factor that contributes to generate the membrane potential, as the case ubiquinone oxidoreductases (Fišar et al. 2016; Li et al. 2016; Bentinger et al. 2010). Ubiquinone transfers electrons between complex I or complex II to complex III of the mitochondrial respiratory chain by getting electrons from NADH or succinate, respectively, and can influence ROS production (Lapuente-Brun et al. 2013). CoQ synthesis occurs in two steps, first the synthesis of isoprenoid and the modification of quinone from the production of FPP, the main regulator of the isoprenoid

synthesis (Kawamukai et al. 2015). Several works reported the CoQ deficiency synthesis in humans with mitochondrial dysfunction, is associated with decreased activities of the complexes I-III and II-III and activation of the mitophagy (Desbats et al. 2015; Doimo et al. 2014; Heeringa et al. 2011; Rodríguez-Hernández et al. 2009; Laxman et al. 2013). Mitophagy is part of a specific autophagic mechanism that monitor the mitochondrial quality control and that can be inhibited by the increasing in methionine production (Laxman et al. 2013). The effect of methionine in this process is due to the downstream metabolite of methionine, *S*-adenosylmethionine (SAM). SAM is the metabolite methyl donor for several of the metabolic transformations that result in the methylation of lipids, nucleic acid and proteins, playing an important role in cellular growth (Longatti and Tooze 2009). Studies involving shortage of isoprenoids and MKD reported alteration of the autophagic flux due to reduction prenylation of proteins involved in the response to autophagy, leading to the induction of inflammation and apoptosis (Lempiainen and Shore 2009; Tricarico et al. 2015; Miettinen and Björklund 2016). In our experimental dataset, we detected the upregulation of *SAM2* gene in erg12-d relative to wt; this gene encodes the SAM synthetase that, among other functions, it is involved in preventing autophagy (Laxman et al. 2013), thus indicating that autophagy, and more specifically mitophagy, should be more exacerbated in erg12-d more than in wild type cells. Thus, we supplemented glycerol-based medium with methionine. However, it did not restore the growth of erg12-d cells (data not shown), indicating that induction of *MET* genes represented a responsive mechanism rather than an essential mechanism to ensure cell growth in full oxidative metabolism.

Our set of findings, compiling physiological profiles and transcriptomics, suggest a reduction in respiration and may be consistent with deficiency in the CoQ synthesis, despite the upregulation of ubiquinone synthesis genes. Thus, we draw the hypothesis that in MK deficiency the metabolic flux toward CoQ synthesis may be impaired by the decreasing in the flow of the mevalonate kinase pathway, with consequent reduction in the production of the intermediaries of the CoQ biosynthesis. James et al. (2005) reported that MitoQ10 is ineffective as electron carrier for respiration in yeast and cannot revert the deficiency of *Δcoq2* mutant to synthesize CoQ. We also supplemented glycerol-based medium with MitoQ10 and confirmed that this compound is not capable of reverting growth deficiency of erg12-d mutant (data not shown). In yeast, this ubiquinone is composed by six isoprenyl units (CoQ6), not ten (CoQ10) as in humans, and unfortunately there is no commercially available CoQ6 for testing. All these results indicate that this deficiency leads to a respiratory deficit that is perceived as mitochondrial dysfunction, triggering mitophagy, with the induction of the preventive mechanism of SAM production through the up regulation of *MET* genes. Mitophagy involves numerous molecular mediators called autophagy-related (ATG) proteins and the prenylation of those proteins is the key regulatory mechanisms in this process (Longatti and Tooze 2009). Mevalonate kinase deficiency leads to reduction in CoQ6 production and mitochondrial dysfunction. Besides, it may cause deficiency in protein prenylation that has been associated with defective mitophagy (Tricarico et al. 2015) resulting in the accumulation of damaged mitochondria. A work involving patients with mevalonate kinase deficiency reported a drastic decrease in plasma CoQ10 concentration (Hubner et al. 1993), while the reduction of the level of protein prenylation in MKD in neuroblastoma cells observed alteration of the autophagic flow and cell death (Tricarico et al. 2017). Thus, it is possible that progression of autophagosomes formation induced by scarcity of ubiquinone is hampered by the limitation in the production of isoprenoids.

Transcriptomic analysis of the biological yeast model studied indicated a deficit in the production of ubiquinone, due to the overexpression of its own genes. It causes respiratory deficiency that might be followed by an accumulation of mitochondrial damage. The mitochondrial dysfunction leads to activation of the mitophagy signalling pathway and in response to mitochondrial degradation, sulphur amino acids, such as methionine, are synthesized to detoxify cells. However, it is possible that the mitophagic mechanism is not completed due to the lack of protein prenylation caused by the shortage of FPP and GGPP for farnesylation and geranylgeranylation, respectively, leading to accumulation of damaged mitochondria (Fig. 8).

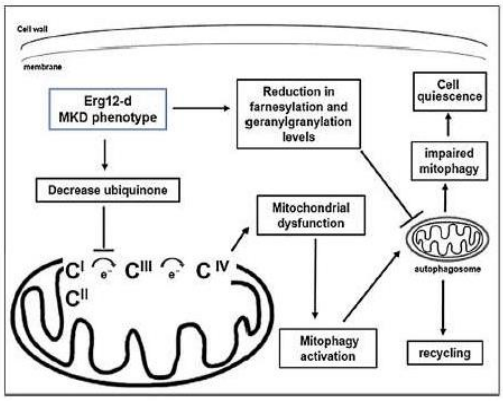

Fig. 8 Model of the interaction among the molecular and cellular processes upon the deficiency in mevalonate kinase activity. Arrows represent induction while lines with a bar at one end represent inhibition of the target metabolic process

Conclusion

Our findings, obtained using a *S. cerevisiae* erg-12, orthologous of human MVK gene, deficient model demonstrated that the shortage of isoprenoids is not the main, or the sole, mechanism involved in the respiratory deficit and mitochondrial malfunctioning of MK-defective cells. We also showed that the scarcity of ubiquinone plays an important role in respiratory deficiency and its consequence on cell metabolism, thus confirming previous results already observed in MKD patients. So, in the investigation of possible mechanisms involved in the pathogenesis of MKD, we should also look at the ubiquinone synthesis, opening novel possibilities to design potential drugs to rescue also this impaired biochemical pathway, thus not limiting our action on isoprenoids supplementation.

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APÊNDICE B – ARTIGO PUBLICADO NA GENETIC AND MOLECULAR RESEARCH

Association of *TNF-α*, *CTLA4*, and *PTPN22* polymorphisms with type 1 diabetes and other autoimmune diseases in Brazil

N.A.C. Tavares[1], M.M.S. Santos[1,3], R. Moura[1,3], J. Araújo[2], R.L. Guimarães[1,3], S. Crovella[1,3] and L.A.C. Brandão[1,4]

[1]Laboratório de Imunopatologia Keiso Asami, Universidade Federal de Pernambuco, Recife, PE, Brasil
[2]Unidade de Endocrinologia Pediátrica do Hospital das Clínicas, Universidade Federal de Pernambuco, Recife, PE, Brasil
[3]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil
[4]Departamento de Patologia, Universidade Federal de Pernambuco, Recife, PE, Brasil

Corresponding author: N.A.C. Tavares
E-mail: nathaliadealencar@gmail.com

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ABSTRACT. Type 1 diabetes mellitus (T1D) is a complex disorder characterized by an autoimmune response against human pancreatic beta-cells. Patients with T1D can also develop a response toward one or more other factors, such as in autoimmune thyroiditis (AITD) and celiac disease (CD). In the presence of T1D + AITD, the patient is diagnosed with autoimmune polyglandular syndrome type III (APSIII); patients with APSIII may also present with CD. These diseases have a strong genetic component and share many susceptibility genes, suggesting potentially overlapping pathogenic pathways. Polymorphisms in the *TNF-α* (rs1800629), *CTLA-4* (rs231775), and *PTPN22* (rs2476601) genes have been previous associated with T1D; however, there is no consensus regarding their role in

T1D and scarce literature focusing on AIDT and/or CD. Thus, we analyzed these genetic variants in 205 Northeast Brazilian patients with T1D and with/without AITD and/or CD, and in 308 healthy controls. The *PTPN22* gene variants were associated with T1D susceptibility and APSIII [odds ratio (OR) = 2.57 and 2.77, respectively]. *CTLA4* rs231775 and *TNF-α* rs1800629 were not associated with T1D onset in the Brazilian population. However, when comparing APSIII individuals in the T1D only group, we observed an association of the *TNF-α* SNP in the allelic (P = 0.0442; OR = 0.44) and dominant models (P = 0.0387; OR = 0.40). This study reinforces the importance of *CTLA-4* and other variants in unraveling the pathogenic mechanisms of T1D in different populations and in understanding their relationships with the development of other T1D-related autoimmune diseases.

Key words: Type 1 diabetes mellitus; Autoimmune disease; *CTLA-4*; *PTPN22*; *TNF-α*

INTRODUCTION

Type 1 diabetes mellitus (T1D) is caused by an autoimmune reaction with both genetic and environmental factors and promotes the destruction of insulin-producing pancreatic beta cells (Sugihara, 2012). The genetic component is crucial to T1D onset and has been the subject of intensive study during the last four decades (Noble and Erlich, 2012). These studies have revealed the human leukocytes antigen (HLA) encoding genes as the main locus associated with T1D onset, corresponding to over 40% of disease susceptibility (Steck and Rewers, 2011; Noble and Erlich, 2012). However, more than 40 loci have been described that also contribute to T1D pathogenesis (Bergholdt et al., 2012; Sugihara, 2012). Among these, three genetic variants in the *CTLA-4* (rs231775), *PTPN22* (rs2476601), and *TNF-α* (rs1800629) genes have been previously associated not only with T1D, but also with different autoimmune disorders including autoimmune thyroid disease (AITD) (Luo et al., 2012; Pan and Xing, 2012; Pastuszak-Lewandoska et al., 2012), celiac disease (CD) (Ueda et al., 2003; Eyre et al., 2010), systemic lupus erythematosus (SLE) (Pradhan et al., 2010), and rheumatoid arthritis (RA) (Nong et al., 2011), albeit in different populations.

Some diabetic patients also develop AITD and/or CD, at a higher frequency than in the general population (Witek et al., 2012): the prevalence of CD + T1D ranges between 4.4-11.1% versus 0.5% in the common population (Camarca et al., 2012) and 15-30% of patients with T1D also have AITD (Van den Driessche et al., 2009). In the presence of both T1D + AITD, a patient is diagnosed with autoimmune polyglandular syndrome type III (APSIII) (Horie et al., 2012; Wémeau et al., 2013). Furthermore, patients with APSIII can also present with CD. It is logical therefore to suggest that different autoimmune disorders potentially share common pathogenetic pathways and that the *TNF-α*, *CTLA-4*, and *PTPN22* genes might be involved in these common underlying mechanisms.

The tumor necrosis factor alpha (*TNF-α*) gene, located on chromosome 6p21.3, encodes a proinflammatory cytokine involved in different biological activities (i.e., proliferation, differentiation, and death) and associated with the destruction of pancreatic β-cells (Duffy, 2007; Feng, et al., 2009). The cytotoxic T-lymphocyte associated protein 4 gene (*CTLA-4*), on chromosome

2q33, has been shown to be a negative regulator of T-cell activation during immune response (Kosmaczewska et al., 2001; Karman et al., 2012). The protein tyrosine phosphatase, non-receptor type 22 (lymphoid) gene (*PTPN22*), on chromosome 1p13.2, encodes the lymphoid-specific phosphatase (Lyp) and is involved in the prevention of spontaneous T-cell activation (Vang et al., 2007; Burn et al., 2011). *CTLA-4* and *PTPN22* deficiencies might therefore induce the proliferation of auto-reactive lymphocytes in autoimmune-mediated diabetes (Kosmaczewska et al., 2001; Burn et al., 2011).

The present study aimed to analyze the genetic association of three single nucleotide polymorphisms (SNPs) in the *CTLA-4* (rs231775), *PTPN22* (rs2476601), and *TNF-α* (rs1800629) genes with susceptibility to the development of T1D in Brazilian patients presenting with or without APSIII, as well as other autoimmune diseases, together or isolated.

MATERIAL AND METHODS

Subjects

We enrolled 205 patients with T1D diagnosed according to the clinical criteria established by the American Diabetes Association (2012). The mean age was 13.22 years (standard deviation (SD) ± 4.87) with 107/205 (52%) females and the average age at T1D onset was 7.33 years (SD ± 4.07). All patients were followed up at one of the three major pediatric endocrinology centers of the Public Health Service of Recife, Brazil (Instituto de Medicina Integral Professor Fernando Figueira - IMIP, Hospital da Restauração, or Hospital das Clínicas - UFPE).

In the control group, we enrolled 308 healthy individuals with no clinical evidence or family history of autoimmune diseases. The mean age was 27.5 years (SD ± 11.23), with 215/308 (70%) females. Healthy individuals were selected from the same geographical region as the patient group.

A free and informed consent was obtained from both patients and controls as well as from each person responsible for the patient and healthy individuals. The ethics committee from IMIP approved the study (IMIP No. 1717/2010).

Diagnosis of autoimmune polyglandular syndrome

Four milliliter of peripheral blood samples collected in order to extract DNA and diagnose the presence of antibodies against thyroperoxidase (Anti-TPO) and transglutaminase (anti-tTg). Plasma samples (approximately 2 mL) were isolated from the whole blood stored at -80°C and the rest of the sample proceeded to extraction and posterior storage at -20°C.

For Anti-TPO, were performed a chemi-luminescence assay (Immulite anti-TPO Ab assay kit, Diagnostic products Co, Los Angeles, CA, USA). Patients with positive anti-TPO (titer exceeding 35 IU/mL, accordingly to the manufacturer suggestion) were considered as having AITD.

The presence of anti-tTg was determined by using an ELISA Eu-tTG kit (Eurospital, Trieste, Italy) following manufacturer instructions. Patients presenting with 10 AU (absorbance units) for anti-tTg antibodies were considered positive for CD.

The frequency of AITD in the patients with T1D was 21.4% (44/205); the percentage of patients with CD was 6.3% (13/205); patients with T1D characterized by both AITD and CD were 2.4% (5/205). The frequency of patients with T1D only was 69.9% (143/205).

DNA extraction and single nucleotide polymorphism (SNP) genotyping

Genomic DNA was extracted from using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), according to standard laboratory protocols. DNA quality and quantity was evaluated using Nanodrop spectrophotometer model 2000c (Thermoscientific, Waltham, MA, USA).

Three SNPs were selected for analysis, one per candidate gene. For *TNF-α* we selected a SNP located in the promoter region at position -308G/A (rs1800629); for *CTLA4* we analyzed the +49A/G polymorphism (rs231775) situated at exon 1; and for *PTPN22*, the non-synonymous SNP at the position +1858G/A (rs2476601) in exon 14 was considered.

All SNPs were genotyped using fluorescent allele-specific probes (TaqMan®, Life Technologies, Carlsbad, CA, USA) with TaqMan® Universal PCR Master Mix and using the ABI-7500 Real-Time PCR platform following the standard PCR protocol (Life Technologies). The TaqMan® assays and its respective SNP identifications are: C___7514879_10 for rs1800629; C___2415786_20 for rs231775; and C__16021387_20 for rs2476601.

Statistical analysis

Allele and genotype frequencies of *CTLA4*, *TNF-α*, and *PTPN22* SNPs were obtained by direct counting. The chi-square test with Yates' continuity correction was used to determine the association between the SNP distribution and the susceptibility to T1D and to clinical features as well as to evaluate conformation with Hardy-Weinberg equilibrium. All tests were performed using R software (www.cran.us.rproject.org) through the SNPassoc package for R (R 2012). P values < 0.05 were considered statistically significant. However, for tests of gene interaction after the application of Bonferroni correction, P values < 0.016 were considered statistically significant.

RESULTS

Association of CTLA4, PTPN22, and TNF-α SNPs and susceptibility to T1D

The distributions of *CTLA4* (rs231775), *PTPN22* (rs2476601), and *TNF-α* (rs1800629) SNPs are reported in Table 1. The genotype frequencies in all studied groups were in Hardy-Weinberg equilibrium. The results of the association tests for the three SNPs are shown in Table 2.

The *PTPN22* +1858A allele (rs2476601) was significantly more frequent in patients with T1D (8%) than in healthy subjects (3%) (P = 0.0018; odds ratio (OR) = 2.52). Using the dominant model, the +1858 G/A+A/A genotypes were also statistically more frequent in patients with T1D+ (15%) than in healthy subjects (6%) (P = 0.0023; OR= 2.57).

Using the allelic model, a similar result was observed when comparing patients presenting only T1D vs HC (P = 0.0055; OR = 2.48) and patients with APSIII vs HC individuals (P = 0.0256; OR = 2.61); We also verified an association for the PTPN22 +1858A allele by the dominant model with a P value = 0.0083 and OR = 2.48 for the T1D only vs HC comparison and P = 0.0225 (OR = 2.77) for the APSIII vs HC comparison.

No association was found in any comparison or model for the *CTLA-4* rs231775 (A/G) SNP in the *CTLA4* gene. We also did not observe an association between the *TNF-α* rs1800629 (G/A) SNP and the development of T1D, even when comparing patients presenting only T1D *vs* HC. However, when comparing individuals with APSIII with the T1D only group, we observed an association of this SNP in the allelic (P = 0.0442; OR = 0.44) and dominant models (P = 0.0387; OR = 0.40).

Age-at-diagnosis and gene-gene interactions

Table 1. *TNF-α* (rs1800629), *CTLA4* (rs231775), and *PTPN22* (rs2476601) genotype and allele frequencies of healthy individuals and patients with type 1 diabetes mellitus stratified according to the insurgence of autoimmune polyglandular syndrome type III (APSIII).

	HC		T1D		T1D only		APSIIII	
	N	Frequency	N	Frequency	N	Frequency	N	Frequency
TNF-α rs1800629								
G	532	0.89	358	0.87	243	0.85	115	0.93
A	68	0.11	52	0.13	43	0.15	9	0.07
GG	235	0.78	158	0.77	104	0.73	54	0.87
GA	62	0.21	42	0.20	35	0.24	7	0.11
AA	3	0.01	5	0.02	4	0.03	1	0.02
CTLA-4 rs231775								
A	394	0.65	255	0.63	179	0.63	76	0.61
G	216	0.35	153	0.38	105	0.37	48	0.39
AA	127	0.42	82	0.40	59	0.42	23	0.37
AG	140	0.46	91	0.45	61	0.43	30	0.48
GG	38	0.12	31	0.15	22	0.15	9	0.15
PTPN22 rs2476601								
G	596	0.97	378	0.92	264	0.92	114	0.92
A	20	0.03	32	0.08	22	0.08	10	0.08
GG	288	0.94	174	0.85	122	0.85	52	0.84
GA	20	0.06	30	0.15	20	0.14	10	0.16
AA	0	0.00	1	0.00	1	0.01	0	0.00

HC = healthy controls; T1D = type 1 diabetes; Freq. = frequency.

Table 2. Association between the three polymorphisms studied and type 1 diabetes (T1D) with or without autoimmune polyglandular syndrome (APSIII or T1D only) versus healthy individuals (HC).

Comparison	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
TNF-α (rs1800629)		G *vs* A			GG *vs* GA			GG *vs* GA			GG *vs* GA+AA	
HC *vs* T1D	1.14	(0.77-1.67)	0.5810	1.01	(0.65-1.57)	0.9371	2.48	(0.58-10.52)	0.3641	1.08	(0.70-1.65)	0.8215
HC *vs* T1D only	1.38	(0.92-2.09)	0.1477	1.28	(0.79-2.05)	0.3770	3.01	(0.66-13.70)	0.2785	1.36	(0.86-2.15)	0.2373
HC *vs* T1D+APSIII	0.61	(0.30-1.26)	0.2380	0.49	(0.21-1.13)	0.1292	1.45	(0.15-14.22)	0.7464	0.54	(0.24-1.18)	0.1640
T1D only *vs* APSIII	0.44	(0.21-0.94)	0.0442	0.39	(0.16-0.92)	0.0453	0.48	(0.05-4.41)	0.8573	0.40	(0.17-0.90)	0.0387
CTLA4 (rs231775)		A *vs* G			AA *vs* AG			AA *vs* GG			AA *vs* AG+GG	
HC *vs* T1D	1.09	(0.84-1.42)	0.5397	1.01	(0.61-1.44)	0.9494	1.26	(0.73-2.19)	0.4880	1.06	(0.74-1.52)	0.8162
HC *vs* T1D only	1.07	(0.80-1.43)	0.7051	0.94	(0.61-1.44)	0.8559	1.25	(0.68-2.29)	0.5817	1.00	(0.67-1.50)	0.9322
HC *vs* T1D+APSIII	1.15	(0.77-1.71)	0.5516	1.18	(0.65-2.14)	0.6855	1.31	(0.56-3.06)	0.6949	1.21	(0.69-2.13)	0.6019
T1D only *vs* APSIII	1.08	(0.70-1.66)	0.8241	1.26	(0.66-2.42)	0.5923	1.05	(0.42-2.61)	0.8962	1.21	(0.65-2.23)	0.6590
PTPN22 (rs2476601)		G *vs* A			GG *vs* GA			GG *vs* GA			GG *vs* GA+AA	
HC *vs* T1D	2.52	(1.42-4.48)	0.0018	2.48	(1.23-4.54)	0.0036	ND	ND	0.8011	2.57	(1.42-4.64)	0.0023
HC *vs* T1D only	2.48	(1.33-4.63)	0.0055	2.36	(1.23-4.54)	0.0142	ND	ND	0.6608	2.48	(1.30-4.74)	0.0083
HC *vs* T1D+APSIII	2.61	(1.19-5.73)	0.0256	2.77	(1.23-6.25)	0.0225	ND	ND	ND	2.77	(1.23-6.25)	0.0225
T1D only *vs* APSIII	1.05	(0.48-2.29)	0.9431	1.17	(0.51-2.68)	0.8695	0	ND	0.6562	1.12	(0.49-2.54)	0.9579

The results for the allelic, co-dominant and dominant models are shown from left to right, respectively. OR = odds ratio; CI = confidence intervals.

Age-at-diagnosis of T1D and gene-gene interaction associations among rs1800629, rs231775, and rs2476601 SNPs were also evaluated. No association with gene-gene interaction and T1D was found for any loci tested (P > 0.05). However, the mean age-at-diagnosis was significantly different according to the genotypes of *TNF-α* and *PTPN22*. In the over-dominant model the mean ages-at-diagnosis were 7.24 and 8.95 for the *TNF-α* G/G+A/A *vs* G/A genotypes, respectively (P = 0.014). The mean ages-at-diagnosis for the G/G and G/A+A/A in the dominant model for the *PTNP22* SNP were 7.87 and 6.10, respectively (P = 0.025). However, after Bonferroni

correction, these associations were no longer statistically significant.

DISCUSSION

T1D is a multifactorial autoimmune disorder with an HLA-specific main locus that has been shown to be responsible for 40% of the susceptibly to T1D. However, previous genome wide association studies have demonstrated associations of other loci from non-HLA regions with T1D onset susceptibility. These non-HLA loci might modulate and modify the course of disease, i.e., disease progression, clinical manifestation, and the onset of other autoimmune disorders. In this study we analyzed three SNPs in three non-HLA classical loci previously implicated in the susceptibility to develop T1D or other associated autoimmune diseases: *CTLA-4* (rs231775), *PTPN22* (rs2476601), and *TNF*-α (rs1800629).

Feng et al. (2009) reported in their meta-analysis that the association of the polymorphism rs1800629, which represents a G>A transition at the -308 position in *TNF-α*, with T1D is primarily found in Asian populations. The -308A allele might increase *TNF-α* protein production *in vitro* (Lee et al., 2005) and could be associated with the onset of T1D. In our study, we did not observe any association of this SNP with T1D development. We are aware that *TNF-α* and the rs1800629 genetic variant considered in this study are in strong linkage disequilibrium with the class II HLA region, although it is not clear whether *TNF-α* polymorphisms at the promoter region have an independent role in the predisposition to T1D or if they show association through a "hitchhiking effect" (Deja et al., 2006; Feng et al., 2009). However, we observed a marginal association between the *TNF-α* -308 variant and the development of APSIII, as compared to individuals exhibiting only T1D. This finding suggests that this cytokine might be involved in the common pathways underlying the development of multiple autoimmune diseases. Fourati et al. (2012) found an association of the rs1800629 SNP with APSIII in Tunisian patients, although they compared APSIII individuals with healthy controls. Therefore, as the published results for *TNF-α* are weak and controversial, more replica studies are needed.

The genetic associations between the *CTLA4* polymorphic rs231775 variant have been previously investigated in different ethnic groups, but with inconsistent findings (Si et al., 2012; Chen et al., 2013). In a meta-analysis, Chen et al. (2013) evaluated 52 studies and concluded that a modest association between the +49A>G polymorphism with T1D risk was indicated, with a related ethnic component. However, in a case-control study performed in a cohort of Turkish children with T1D, no association was observed between this polymorphism and increased susceptibility to T1D or with the clinical and laboratory characteristics of the patients with T1D ($P > 0.05$) (Çelmeli et al., 2013). Furthermore, in another study by Rodríguez et al. (2014), no significant association was found for *CTLA4* in the development of T1D in a Colombian population. These results are in agreement with those found in our population.

We also found no association for the +49 A>G polymorphism in the APSIII comparisons. However, Villano et al. (2009) observed an association of this SNP with APSIII when considering only individuals with simultaneous T1D and AITD, although these results have not been replicated in a Japanese cohort (Horie et al., 2012). In contrast, in our APSIII group we enrolled not only individuals with T1D and AITD, but also individuals with T1D and CD and even patients with simultaneous T1D, AITD and CD as well.

The 1858A+ variant of the *PTPN22* gene, also known as R620W, was associated with T1D in our study population from Brazil. In fact, the +1858A allele was more frequent in T1D+ patients

(OR = 2.52; CI=1.42-4.48) than in healthy subjects. These findings are in agreement with the meta-analysis performed by Lee et al. (2007), wherein the authors demonstrated that the +1858A allele conferred susceptibility to RA, SLE, Graves' disease (GD), as well as T1D, supporting evidence of an association of the *PTPN22* gene with subgroups of autoimmune diseases. Furthermore, in a recent study the same authors confirmed that the rs2476601 *PTPN22* polymorphism was associated with T1D susceptibility in Europeans (Lee and Song, 2012). For a Colombian population, as well as in our study, an association was found for the *PTPN22* gene and development of T1D (Rodríguez et al., 2014). Tang et al. (2012) indicated that T1D is associated with the *PTPN22* +1858G/A gene polymorphism, and that association with this promoter polymorphism was likely dependent on ethnicity.

In our study, we also described an association of the *PTPN22* +1858G/A variant and APSIII onset, when compared with HC individuals. Similar studies from Villano et al. (2009) and Dultz et al. (2009) also found significant results.

Overall, such positive results indicate that an individual carrying these alleles is at risk to develop both T1D and AITD, and furthermore, they suggest that T1D and CD might also share similar pathways and pathogenic mechanisms.

No association with age-at-diagnosis and gene-gene interaction between the three SNPs and T1D was observed in the Brazilian population in our study. The reports of age-at-diagnosis interaction effects at non-HLA loci (Howson et al., 2012) as well as the study of gene-gene interactions of T1D-associated regions are contradictory (Payne et al., 2007), supplementary studies focusing on these fields should be performed.

In conclusion, our study, even with the limitation of a small number of patients analyzed (which numbers are even lower when considering individuals with the combined diagnoses of T1D, AITD, and CD), suggests an association between a *CTLA4* SNP (rs231775) and the susceptibility to develop T1D and other autoimmune diseases in Brazilian patients. However, divergent results of association between the *CTLA4* gene and T1D have been found across various studies: these apparent discrepancies might be attributed to several factors, including differences in genetic background (Marron et al., 1997; Ikegami et al., 2006), possible linkage to HLA susceptibility haplotypes, and patient selection (Ikegami et al., 2006).

Unlike this study, due to the higher prevalences, most previous studies only investigated potential associations of *PTPN22*, *CTLA4*, and *TNF*-α with T1D, AITD, and CD as individual diseases. Our findings provide new insights into the genetic components associated with the susceptibility to T1D in Brazilian patients, and reinforce the importance of discriminating whether a patient presents with other autoimmune diseases that might be correlated with T1D.

Conflicts of interest

The authors declare no conflicts of interest.

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APÊNDICE C – ARTIGO PUBLICADO NA GENETIC AND MOLECULAR RESEARCH

Meta-analysis of *STAT4* and *IFIH1* polymorphisms in type 1 diabetes mellitus patients with autoimmune polyglandular syndrome type III

J. de Azevêdo Silva[1], N.A.C. Tavares[1], M.M.S. Santos[1,2], R. Moura[1,2], R.L. Guimarães[1,2], J. Araújo[3], S. Crovella[1,2] and L.A.C. Brandão[2,4]

[1]Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife, PE, Brasil
[2]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil
[3]Hospital das Clínicas, Unidade de Endocrinologica Pediátrica, Universidade Federal de Pernambuco, Recife, PE, Brasil
[4]Departamento de Patologia, Universidade Federal de Pernambuco, Recife, PE, Brasil

Corresponding author: J. de Azevêdo Silva
E-mail: j.azvedo@gmail.com

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ABSTRACT. Type 1 diabetes mellitus (T1D) is an organ-specific autoimmune disease characterized by T-cell mediated self-destruction of insulin-producing β cells in the pancreas. T1D patients are prone to develop other glandular autoimmune disorders, such as autoimmune thyroid disease that occurs simultaneously with autoimmune polyglandular syndrome type III (APSIII). Signal transducer and activator of transcription 4 (STAT4) is a well-known regulator of proinflammatory cytokines, and interferon-induced with helicase C domain 1 (IFIH1) is activated in the interferon type I response. Both genes have been examined separately in autoimmune diseases and,

in this study, we assessed their joint role in T1D and APSIII. We conducted a case-control study, enrolling 173 T1D patients and 191 healthy controls from northeastern Brazil, to assess the distribution of the rs7574865 and rs3024839 SNPs in *STAT4* and the rs3747517 and rs1990760 SNPs in *IFIH1* in T1D and APSIII patients. Additionally, we conducted a meta-analysis with the rs7574865 SNP in *STAT4* (1392 T1D patients and 1629 controls) and the rs1990760 SNP in *IFIH1* (25092 T1D patients and 28544 controls) to examine their association with T1D. Distribution of *STAT4* and *IFIH1* allelic frequencies did not show statistically significant differences between T1D patients and controls in our study population; however, the meta-analysis indicated that SNPs in *STAT4* and *IFIH1* are associated with T1D worldwide. Our findings indicate that although *STAT4* and *IFIH1* SNPs are not associated with T1D in a Brazilian population, they might play a role in susceptibility to T1D on a larger worldwide scale.

Key words: STAT4; IFIH1; Autoimmune polyglandular syndrome type III; SNP; Type 1 diabetes

INTRODUCTION

Type 1 diabetes mellitus (T1D) is an organ-specific autoimmune disease characterized by T cell-mediated attack of the insulin-producing β cells in the pancreas, leading to insulin deficiency (Gillespie, 2006). T1D is a multifactorial disease caused by genetic and environmental factors, as well as their interaction, that play a key role in the development of the disease. In up to one-quarter of T1D patients, an unbalanced immune system leads to autoimmune polyglandular syndrome type III (APSIII), which is characterized by the simultaneous occurrence of autoimmune thyroid disease (AITD) and sex bias (adult females are preferentially affected) (Kordonouri et al., 2002; Dittmar and Kahaly, 2010). Furthermore, patients with APSIII may also be diagnosed with celiac disease (CD). Despite the genetic variation in human leukocyte antigen (HLA) and its involvement in T1D development, new genes have been identified as potentially important in disease's susceptibility and modulation outside HLA range (Gillespie, 2006; Liang et al., 2012).

Signal transducer and activator of transcription 4 (STAT4) is a latent cytoplasmic transcription factor activated by phosphorylation in response to proinflammatory cytokines, such as interleukin (IL)-12, IL-15, and IL-23 (Levy and Darnell, 2002). STAT4 is involved in T helper 1 (Th1) cell regulation and is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at inflammation sites. Additionally, STAT4 mediates IL-12 signaling, which modulates Th1 cell differentiation and proliferation, interferon-γ (INF-γ) production, and development of T helper 17 (Th17) cells (Kobayashi et al., 2008; Zervou et al., 2008). Since Th1 cells are critical effectors of chronic inflammation disorders, STAT4 could play a pivotal role in the pathogenesis of immune diseases (Kobayashi et al., 2008; Zervou et al., 2008; Bi et al., 2013; Zheng et al., 2013). In fact, single nucleotide polymorphisms (SNPs) within *STAT4* (chromosome location: 2q32.2-q32.3) have been reported to be associated with increased risk for several autoimmune diseases (Liang et al., 2012), including rheumatoid arthritis (RA) (Stark et al., 2009), systemic lupus erythematosus (SLE) (Kobayashi et al., 2008), and Sjögren's syndrome (SS) (Palomino-Morales et al., 2010).

Viral infections have been implicated as triggers in autoimmune disorders in T1D (Jun and Yoon, 2001; Salminen et al., 2003). Interferon-induced with helicase C domain 1 (IFIH1; gene located at chromosome 2q24), also known as MDA5, activates the type I interferon (IFN-I) pathway and pro-inflammatory cytokines by its CARD domains after detecting double-stranded RNA viruses (Chistiakov, 2010). Enterovirus infections, particularly coxsackievirus B4 strains, are known to be T1D-associated; therefore, IFIH1 may play an important role in the development of T1D and its autoimmune-related disorders (Jaïdane et al., 2009). Interestingly, during IFN-I activation, IFIH1 and STAT4 share a common pathway and, since both genes are known to be associated with T1D (Zheng et al., 2013), one can hypothesize that defects in both genes may increase susceptibility to disease compared to defects in just one gene, resulting in a cumulative effect of genetic mutations.

In this study, we investigated the single nucleotide polymorphisms (SNPs) rs7574865 (G > T) and rs3024839 (T > C) in *STAT4* and rs3747517 (C > T) and rs1990760 (C > T) in *IFIH1* and their link to T1D and APSIII susceptibility in a northeast Brazilian population. Additionally, we performed a meta-analysis for the rs7574865 and rs1990760 SNPs in T1D predisposition.

MATERIAL AND METHODS

Patients and control subjects

We performed a case-control study in T1D patients from Pernambuco State in northeast Brazil. We enrolled 173 T1D patients ranging in age from 0 to 18 years at diagnosis, with an age of 7.3 ± 4.1 (means ± SD) years at disease onset. The patients attended one of three pediatric endocrinology departments in the public healthcare system in Recife, Brazil (Instituto de Medicina Integral Professor Fernando Figueira, Hospital da Restauração and Hospital das Clínicas). A consent form was obtained from all patients (or their legal representative) enrolled in this study. T1D patients were diagnosed according to American Diabetes Association (ADA) criteria and classified as T1D from clinical and pathological presentation (Gabir et al., 2000).

From the T1D patient group, 47 (27.2%) were diagnosed with APSIII. AITD was diagnosed using antibodies against thyroperoxidase (anti-TPO) and detection was performed using chemiluminescence (Immulite anti-TPO, Diagnostic Products Co., Los Angeles, CA, USA) following the manufacturer instructions. The individuals positive for TPO (titer exceeding 35 IU/mL, according to indications provided by the manufacturer) were considered as presenting AITD. The control group consisted of 191 healthy unrelated blood donors from the same geographic region with no history of autoimmune or chronic disease. The age of the control group ranged from 16 to 72 years and the means ± SD age was 38.8 ± 14.7 years. This study was carried out with advanced approval from the local Ethics Committee (IMIP Nos. 762/2006 and 1717/2010).

Genotyping

Genomic DNA was obtained from whole blood and the extraction protocol was performed according to the manufacturer instructions (Wizard Genomic DNA Purification Kit; Promega, Madison, MA, USA). The DNA was stored at -20°C until analysis. The SNPs assessed in this study

have frequently been described in the literature: rs7574865 (G > T) from intron 3, rs3024839 (T > C) from exon 4 in *STAT4*, rs3747517 (C > T) in exon 13, and rs1990760 (C > T) in exon 15 of *IFIH1*. Genotyping was performed using commercially available Taqman probes and the ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The allelic discrimination protocol was performed as recommended by the manufacturer and analyzed using SDS 2.3 software (Applied Biosystems).

Statistics and meta-analysis

Association analyses were performed by the chi-squared (χ^2) test with continuity correction, and the odds ratio (OR) and 95% confidence intervals (CI) were calculated using the Fisher test. For the meta-analysis, we searched peer-reviewed articles published between 2003 and 2015 (last search performed in July 2015) using logical equations with the following key words on PubMed and Web of Knowledge: "[(IFIHI or MDA5) and T1D]" or "(STAT4 and T1D)". We selected only case-control studies with the allele counts available for both SNPs analyzed (rs7574865 and rs1990760). The meta-analysis tests were carried out using the "Metafor" package (Viechtbauer, 2010). When the P value from the Cochran Q test for heterogeneity was lower than 0.1, the DerSimonian-Laird's estimator was used for the random-effect or fixed-effect models when necessary. The Haploview version 4.2 software was used for calculation of haplotype associations. Power analyses were performed using the G*Power 3.1.3 software (http://www.psycho.uniduesseldorf.de).

RESULTS

The allele and genotype frequencies of the SNPs in *STAT4* and *IFIH1* in patients (T1D + AITD + CD, T1D only, and APSIII) and healthy controls are shown in Table 1. All polymorphisms were in Hardy-Weinberg equilibrium in all groups except the T1D only group. Distribution of *STAT4* and *IFIH1* genotype and allele frequencies did not show statistically significant differences between patients and controls, indicating no association of the SNPs with development of T1D or APSIII regardless of the genetic model used. These results are shown in Table 2. Of note, the examined SNPs did not show linkage disequilibrium.

Furthermore, we performed a meta-analysis for the rs7574865 and rs1990760 SNPs from *STAT4* and *IFIH1*, respectively. Including the present study, we gathered seven publications with data on the rs7574865 SNP (Lee et al., 2008; Martínez et al., 2008a; Zervou et al., 2008; Howson et al., 2011; Park et al., 2011; Bi et al., 2013) and ten for the rs1990760 SNP (Smyth et al., 2006, 2008; Martínez et al., 2008b; Aminkeng et al., 2009; Jermendy et al., 2009; Liu et al., 2009; Schulte et al., 2010; Yang et al., 2012; Bouças et al., 2013; Zurawek et al., 2015). The total number of cases and controls was 1392 and 1629 for the rs7574865 SNP and 25,092 and 28,544 for the rs1990760 SNP, respectively.

The forest plots of the meta-analyses of the rs7574865 and rs1990760 SNPs are shown in Figures 1 and 2, respectively. The rs7574865 (OR = 1.37; 95%CI = 1.23-1.52; P < 0.0001) and rs1990760 (OR = 0.85; 95%CI = 0.81-0.89; P < 0.0001) SNPs were both associated with T1D, although some moderate heterogeneity was detected (I^2 = 43.35%; $P_{|Q|}$ = 0.0478) for the rs1990760 SNP.

Table 1. Allele and genotype frequencies of the *STAT4* (rs7574865 and rs3024839) and *IFIH1* (rs3747517 and rs1990760) SNPs in type 1 diabetes mellitus patients (T1D only), T1D patients with autoimmune thyroid disease (AITD) and celiac disease (CD), autoimmune polyglandular syndrome type III (APSIII) patients, and healthy controls (HC).

SNP	HC		T1D + AITD + CD		T1D only		APSIII	
	N	Freq.	N	Freq.	N	Freq.	N	Freq.
rs7574865								
G	294	0.77	260	0.75	197	0.78	63	0.68
T	88	0.23	86	0.25	57	0.22	29	0.32
GG	112	0.59	98	0.57	76	0.60	22	0.48
GT	70	0.37	64	0.37	45	0.35	19	0.41
TT	9	0.05	11	0.06	6	0.05	5	0.11
rs3024839								
T	353	1.00	331	1.00	245	1.00	86	1.00
C	1	0.00	1	0.00	1	0.00	0	0.00
TT	176	0.99	165	0.99	122	0.99	43	1.00
TC	1	0.01	1	0.01	1	0.01	0	0.00
CC	0	0.00	0	0.00	0	0.00	0	0.00
rs3747517								
C	230	0.68	249	0.72	179	0.72	70	0.74
T	110	0.32	95	0.28	71	0.28	24	0.26
CC	77	0.45	87	0.51	63	0.50	24	0.51
CT	76	0.45	75	0.44	53	0.42	22	0.47
TT	17	0.10	10	0.06	9	0.07	1	0.02
rs1990760								
C	202	0.61	201	0.60	150	0.61	51	0.57
T	130	0.39	135	0.40	96	0.39	39	0.43
CC	59	0.36	67	0.40	52	0.42	15	0.33
CT	84	0.51	67	0.40	46	0.37	21	0.47
TT	23	0.14	34	0.20	25	0.20	9	0.20

Table 2. Odds ratios, 95% confidence intervals, and P values from the association analysis between the *STAT4* (rs7574865 and rs3024839) and *IFIH1* (rs3024839 and rs1990760) variants and type 1 diabetes (T1D) and autoimmune polyglandular syndrome type III (APSIII).

Comparison	11 x 01 x 00			01 x 00			11 x 00			1 x 0
	P value	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
rs7574865										
HC *vs* T1D	0.7737	1.04	(0.68-1.61)	0.9302	1.40	(0.56-3.51)	0.6320	1.11	(0.79-1.55)	0.6258
HC *vs* T1D only	0.9753	0.95	(0.59-1.52)	0.9184	0.98	(0.34-2.87)	0.8095	0.97	(0.66-1.41)	0.9371
HC *vs* APSIII	0.1864	1.38	(0.7-2.73)	0.4507	2.83	(0.86-9.25)	0.1570	1.54	(0.93-2.54)	0.1188
T1D only *vs* APSIII	0.2038	1.46	(0.71-2.98)	0.3947	2.88	(0.8-10.34)	0.1910	1.59	(0.94-2.7)	0.1127
rs3024839										
HC *vs* T1D	ND	1.07	(0.09-23.29)	0.5067	ND	ND	ND	1.07	(0.07-17.12)	0.5073
HC *vs* T1D only	ND	1.44	(0.09-23.29)	0.6444	ND	ND	ND	1.44	(0.09-23.15)	0.6449
HC *vs* APSIII	ND	0.00	ND	0.4415	ND	ND	ND	0.00	ND	0.4420
T1D only *vs* APSIII	ND	0.00	ND	0.5812	ND	ND	ND	0.00	ND	0.5817
rs3747517										
HC *vs* T1D	0.2983	0.87	(0.53-1.38)	0.6264	0.52	(0.22-1.2)	0.1821	0.80	(0.57-1.11)	0.2046
HC *vs* T1D only	0.5700	0.85	(0.53-1.38)	0.6000	0.65	(0.27-1.55)	0.4438	0.83	(0.58-1.18)	0.3480
HC *vs* APSIII	0.2176	0.93	(0.48-1.8)	0.9589	0.19	(0.02-1.49)	0.1519	0.72	(0.43-1.2)	0.2539
T1D only *vs* APSIII	0.4346	1.09	(0.55-2.16)	0.9433	0.29	(0.04-2.43)	0.4108	0.86	(0.5-1.48)	0.6929
rs1990760										
HC *vs* T1D	0.1037	0.70	(0.37-1.04)	0.1805	1.30	(0.69-2.45)	0.5124	1.04	(0.77-1.42)	0.8486
HC *vs* T1D only	0.0688	0.62	(0.37-1.04)	0.0941	1.23	(0.63-2.43)	0.6645	0.99	(0.71-1.39)	0.9569
HC *vs* APSIII	0.5943	0.98	(0.47-2.06)	0.8848	1.54	(0.59-4.01)	0.5259	1.19	(0.74-1.9)	0.5512
T1D only *vs* APSIII	0.5036	1.58	(0.73-3.43)	0.3298	1.25	(0.48-3.24)	0.8351	1.19	(0.73-1.95)	0.5567

0 represents the less frequent allele and 1 is the most frequent; ND = not determined; OR = odds ratio; CI = confidence interval; HC = healthy control.

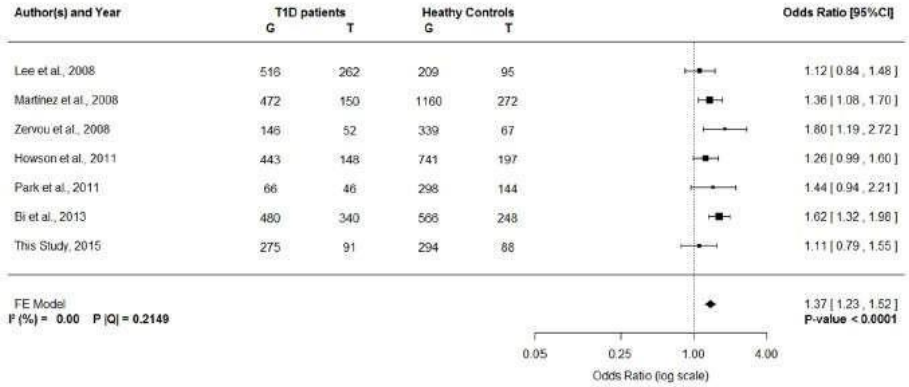

Figure 1. Forest plot from the meta-analysis for the SNP rs7574865 within the *STAT4*.

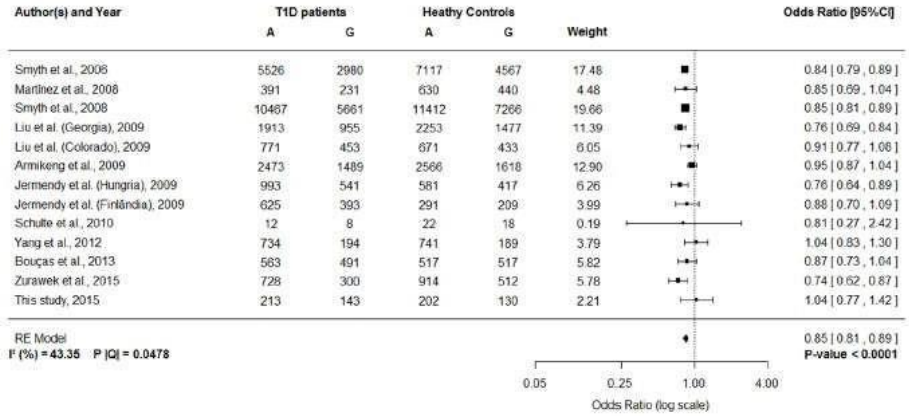

Figure 2. Forest plot from the meta-analysis for the SNP rs7574865 within the *IFIH1*.

DISCUSSION

Several T1D patients present simultaneously with autoimmune disorders and organ commitment. APSIII is the most common autoimmune disorder in T1D patients and is found more frequently in adults; it has low incidence in children. The individual pathogenic mechanisms underlying T1D, AITD, and CD remain unknown. Furthermore, it is unclear whether the development of these diseases is due to a shared common etiopathological mechanism or if it is just a consequence of the presence of one autoimmune disorder functioning as a trigger for the insurgence of another. The regulatory pathway of T cells might be a molecular target to understand the pathogenesis of APSIII due to the involvement of T cell activation in T1D, AITD and CD mechanisms. In addition, the role of viral infection and its related pathways may also be important in understanding the link between T1D, AITD, APSIII, and CD.

In this study, we assessed the role of *STAT4* and *IFIH1* variants in T1D and APSIII susceptibility. *STAT4* is involved in Th1 regulation and its inhibition prevents the development of

autoimmune diabetes in non-obese diabetic (NOD) mice. Moreover, genetic variants of *STAT4* are associated with autoimmune disorders in several populations, making STAT4 an emerging therapeutic target (Yang et al., 2004; Bi et al., 2013; Zheng et al., 2013). *IFIH1* SNPs were first associated with T1D in autoimmune disease and have since been associated with other autoimmune systemic disorders. Upon viral infection, IFIH1 activates the IFN-I pathway and the release of IFN-I can activate STAT4, followed by the Th1 gene expression profile in mature dendritic cells (Kariuki et al., 2008). Herein, we investigated the individual and combined influence of *STAT4* and *IFIH1* variants on T1D and APSIII development. In addition, we examined if *STAT4* and *IFIH1* SNPs are associated with development of T1D in a meta-analysis study.

The rs7574865 SNP is one of the most frequently examined polymorphisms in the *STAT4* gene and its function is related to gene expression on a transcriptional level and splice variation (Liang et al., 2012). The T allele of this particular SNP has been associated with multiple autoimmune disorders but the association is dependent on the population examined (Lee et al., 2010). In the present study, the SNPs examined from *STAT4* and *IFIH1* did not show any correlation (individually or combined) with incidence of T1D and/or APSIII. Although the rs7574865 SNP is within intron 3, it displays a linkage disequilibrium with other SNPs that have a possible functional consequence (Zheng et al., 2013). The rs3024839 SNP is an intragenic missense mutation (A > G resulting in isoleucine > valine substitution) with probable functional consequences in STAT4. Although *STAT4* SNPs have been frequently studied as potential indicators for autoimmune diseases, the results are still unclear, indicating that STAT4 might play varying roles in these disorders. Our results agree with the meta-analysis performed by Zheng et al. (2013), which revealed that the *STAT4* rs7574865 polymorphism is associated with several autoimmune diseases, including SLE, RA, scleroderma, systemic sclerosis (SSc), and primary SS, but is not associated with T1D, ulcerative colitis, and Crohn's disease. Interestingly, SLE, RA, SSc, and SS are considered systemic disorders, whereas T1D is characterized as an organ-specific manifestation, which suggests that mutations in *STAT4* are primarily related to systemic rather than organ-specific disorders.

On the other hand, the study performed by Zervou et al. (2008) assessed the link between the rs7574865 SNP and risk of T1D in Crete, where there is a genetically homogenous population, and the results indicated that there was an association. Moreover, this polymorphism was strongly associated with T1D in a northeastern Chinese population (Bi et al., 2013). Additionally, the study performed by Fourati et al. (2012) assessed the possible role of non-HLA genes in APSII, which includes Addison's disease and AITD and/or T1D, in a Tunisian population. Their results indicated that the rs7574865 SNP in *STAT4* was associated with APSII but not with T1D or AITD alone, suggesting that *STAT4* is involved with the co-occurrence of autoimmune endocrinopathies in APSII individuals.

IFIH1 is a helicase that senses viral dsRNA and, when activated, supports the transcription of IFN-I and IFN-induced genes (Robinson et al., 2011). Since IFIH1 acts during viral infections, we hypothesized in a previous publication that a defective mechanism in virus recognition might be caused by a defective IFIH1 (Moura et al., 2013). However, in the present study, we did not find any association between the rs3747517 (A > G resulting in histidine > arginine substitution in exon 13) or rs1990760 (C > T resulting in alanine > threonine substitution in exon 15) SNPs in IFIH1 and T1D or APSIII. Despite this, on including our data in the meta-analysis study, the rs1990760 SNP in *IFIH1* was found to be associated with T1D. The overexpression of IFIH1 in murine models is related to a chronic state of IFN-I production (Crampton et al., 2012). In multiple sclerosis (MS), which is an autoimmune disease, *IFIH1* and Toll-like receptor 7 (*TLR7*) are overexpressed. TLR7

is associated with the IFN-I response and, consequently, the IFN signature (Hundeshagen et al., 2012). The T allele in the rs1990760 SNP in *IFIH1* is associated with increased expression of IFIH1 in peripheral blood mononuclear cells and sensitivity to IFN-α (Rönnblom et al., 2011). Therefore, both STAT4 and IFIH1 exert some control in the IFN-I pathway, and their malfunction leads to an altered immune response.

In conclusion, we did not find any association of SNPs in *STAT4* or *IFIH1* with T1D development in a northeast Brazilian population. However, the meta-analysis showed an association between the rs7574865 and rs1990760 SNPs in *STAT4* and *IFIH1*, respectively, with T1D, even when our negative associations were included.

Conflicts of interest

The authors declare no conflict of interest.

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APÊNDICE D – ARTIGO PUBLICADO NA INDIAN ACADEMY OF SCIENCES

RESEARCH NOTE

Polymorphism in ficolin-1 (*FCN1*) gene is associated with an earlier onset of type 1 diabetes mellitus in children and adolescents from northeast Brazil

ZILMA PEREIRA DOS ANJOSA[1], MANUELLA MARIA SILVA SANTOS[2], NATASSIA JAVORSKI RODRIGUES[3], GLAUCIA ALYNE NUNES DE LACERDA[3], JAQUELINE ARAUJO[4], JAQUELINE DE AZEVÊDO SILVA[3], NATHÁLIA DE ALENCAR CUNHA TAVARES[3], RAFAEL LIMA GUIMARÃES[2,3], SERGIO CROVELLA[2,3,*] and LUCAS ANDRÉ CAVALCANTI BRANDÃO[1,3]

[1]*Department of Pathology,* [2]*Department of Genetics,* [3]*Keizo Asami Immunopathology Laboratory (LIKA), and* [4]*Pediatric Endocrinology Unity of Clinical Hospital, Federal University of Pernambuco, Recife, Pernambuco 50670-901 Brazil*

[Anjosa Z. P. D., Santos M. M. S., Rodrigues N. J., Lacerda G. A. N. D., Araujo J., Silva J. D. A., Tavares N. D. A. C., Guimarães R. L., Crovella S. and Brandão L. A. C. 2016 Polymorphism in ficolin-1 (*FCN1*) gene is associated with an earlier onset of type 1 diabetes mellitus in children and adolescents from northeast Brazil. *J. Genet.* **95**, 1031–1034]

Introduction

The lectin pathway is activated by the mannose-binding lectin (MBL) and ficolins (FCN), both have already been associated with several autoimmune disorders. Here, we assessed *FCN1* and *FCN2* functional SNPs in 203 type 1 diabetes mellitus (T1D) patients with celiac disease (CD) and autoimmune thyroiditis (AITD). We identified that the *FCN1* rs1071583 SNP was correlated with earlier age of T1D diagnosis and the combination analysis from the SNPs (rs2989727 and rs1071583) assessed in *FCN1* and *FCN2* was associated with T1D lower susceptibility. This is the first finding that suggested the genetic role of *FCN1* and *FCN2* in northeastern Brazilian children and adolescents with T1D.

The lectin pathway of complement system acts in the elimination of pathogens, being able to phagocyte and induce inflammation response. The lectin pathway is activated by two different lectins, the mannose-binding lectin (MBL) and ficolins. So far, three humans ficolins are described: M-ficolin (ficolin-1), L-ficolin (ficolin-2) and H-ficolin (ficolin-3), encoded by the *FCN1*, *FCN2* and *FCN3* genes, respectively (Messias-Reason *et al.* 2009b; Hu *et al.* 2013). Several studies suggested the role of ficolins and MBL in the development of autoimmune disorders due to their ability to promote the apoptotic bodie's clearance, to increase the inflammation and to avoid viral infection (Atkinson *et al.* 2004; Vander Cruyssen *et al.* 2007; Messias-Reason *et al.* 2009a). In this study, we assessed the possible influence of *FCN1* and *FCN2* functional SNPs in T1D development and the insurgence of related AITD and CD.

Although *FCN1* and *FCN2* genes are located at the same chromosome region, 9q34 presents a differential expression pattern. *FCN1* is mainly expressed by leukocytes in intracellular secretory granules and is secreted in the interstitium and plasma. *FCN2* is expressed in liver cells and ficolin-2 protein once produced is secreted in the plasma (Garred *et al.* 2009).

Therefore, our hypothesis is that decrease in ficolins production/function in interstitium or plasma, related to the presence of such SNPs, located at regulatory region of *FCN1* and *FCN2* genes, may be responsible for apoptotic bodies' accumulation with a future autoimmune response in pancreatic tissues. Moreover, these SNPs in *FCN1* and *FCN2* could be involved with the autoantibody production in the AITD and CD in T1D patients.

Material and methods

Patients and healthy individuals

The study was carried out at three pediatric endocrinology reference services in Pernambuco, Brazil (Instituto de Medicina Integral Professor Fernando Figueira, Hospital da Restauração e Hospital das Clínicas), from March 2010 to December 2013. Children and adolescents were diagnosed with T1D according to American Diabetes Association criteria (2012). Ethics committee (Instituto de Medicina Integral Professor Fernando Figueira, number 1717/2010) approved the project and patients assigned a written free and informed consent.

We enrolled 204 T1D subjects (median age 13.5 years) diagnosed with T1D and 193 healthy individuals (median age 30 years), without clinical signs or family history of T1D and not related to the patient group, as controls. Both T1D

*For correspondence. E-mail: crovelser@gmail.com.

Keywords. type 1 diabetes; ficolins; autoimmunity; single-nucleotide polymorphism; complement system.

patients and healthy individuals were born and reared in the same geographical region of Recife, Pernambuco, Brazil. As Brazilians are considered an admixed population, due to recent events of European and African migrations, we previously studied the ethnical composition of Recife population to avoid spurious association: we used 12 genetic ancestries in formative markers (AIMs) described by Kosoy *et al.* (2009), demonstrating a contribution of 59.7%, 23% and 17.3% of European, African and Native Amerindian ancestry, respectively (Coelho *et al.* 2015).

Diagnosis of autoimmune thyroid and celiac disease

Antibodies to thyroid peroxidase (anti-TPO) and antitransglutaminase (anti-tTG) were determined by chemio-luminescence (anti-TPO Ab, diagnostic products IMMULITE, Los Angeles) and by ELISA kit I-tTG (Eurospital, Trieste, Italy), respectively, following manufacturer's instructions.

DNA extraction

Genomic DNA was extracted from peripheral whole blood using Wizard Genomic DNA purification kit (Promega, Madison, USA) according to the standard laboratory protocols.

Polymorphism selection and genotyping

After *in silico* and literature search, we selected five SNPs: −1981 (rs2989727) and 7919 (rs1071583) located at promoter region and in exon 9 of *FCN1* gene, respectively, and −4 (rs17514136), +5839 (rs3124954) and +6424 (rs7851696) located at 5′UTR, intron 1 and exon 8 of *FCN2* gene, respectively. SNP in 5′UTR region of *FCN2* have been associated with variations in serum concentration of the protein, whereas the polymorphism located in exon 8, which encodes the domain similar to fibrinogen has been associated with an increase in the ability to link the acetylated residues of the protein (Luz *et al.* 2013). The rs3124954 represents a haploblock tagged by rs3128624 of *FCN2* gene.

The SNPs at promoter and exon 9 of *FCN1* could regulate both the expression and synthesis of ficolin-1 and were previously associated with autoimmune disorder (Vander Cruyssen *et al.* 2007). The rs1071583 of *FCN1* is synonymous SNP that changes dramatically the frequency of codon usage in the translation process, there by altering the rate of bioavailable protein and is associated with other autoimmune events (http://www.kazusa.or.jp/codon/).

Genotyping was performed using commercially available TaqMan probes (Applied Biosystem, Foster City, USA). The rs2989727 and rs1071583, by C_26745032_10 and C_1819018_1_, respectively. The rs17514136, rs3124954 and rs7851696, by C_25765134_10, C_27462209_20 and C_29220549_20), respectively. TaqMan reactions were set up based on the manufacture's protocol using 7500 Real-Time PCR instrument (Applied Biosystem).

Statistical analysis

Allelic discrimination was performed as suggested by the manufacturer (TaqMan® Genotyping Software), and analysed using Genotyping Transposer software. Using the R statistical package (https://www.r-project.org), we obtained the chi-square test to correlate polymorphism distribution with the susceptibility to develop T1D and its clinical features as well as to evaluate the Hardy–Weinberg equilibrium (HWE). Linkage disequilibrium (LD) within *FCN1* and *FCN2* SNPs and haplotype constructions were assessed using the Haploview and SNPstats softwares (ver. 4.2). Odds ratio (OR) and 95% confidence intervals (CI) were also computed. All analyses, *P*-value <0.05 were considered statistically significant. Power analyses were performed using G*Power 3.1.9.2 software (http://www.psycho.uniduesseldorf.de). The association with the age of T1D onset was performed using the package SNPassoc, implemented in statistical software R (ver. 3.0.0, http://www.r-project.org).

Results

In this study, a total of five SNPs, two SNPs of *FCN1* and three of *FCN2* genes were analysed in 204 T1D patients and 193 healthy subjects, classified according to the presence of other autoimmune disorder, namely CD and AITD.

Allele and genotype frequencies of *FCN1* gene polymorphisms (rs2989727 and rs1071583) were in HWE in both T1D patients and healthy individuals groups. The *FCN2* SNPs frequencies were also in HWE, except for rs7851696 (| 6424 G>T variant) in both groups. No statistical differences were found in the SNPs distribution of T1D patients, CD and AITD subgroups and healthy individuals ($P > 0.05$) in all genetic models, i.e. codominant, dominant, recessive, overdominant and log-additive (data not shown). Only rs1071583 at *FCN1* was associated with age-at-diagnosis of T1D (*P*: 0.016; Akaike information criterion (AIC): 1095; dif: −2.20; 95% CI: −3.997 to −0.419; table 1). This association shows the genotype T/T as major susceptibility risk factor for an earlier insurgence of diabetes when compared to C/C and C/T genotypes. Patients with T/T genotypes could develop T1D around two years earlier than the others patients. No statistical association with the sex distribution was obtained for all five loci ($P > 0.05$, data not shown).

Since SNPs may act in combination to increase the risk of disease, the haplotypes of the studied *FCNs* SNPs were investigated and their frequencies in T1D and healthy control groups were compared. In spite of lack of strong LD between rs2989727 and rs1071583 SNPs ($D' = 0.2$), individuals carrying the C allele at both SNPs showed some protection against T1D onset (*P* value: 0.0003, OR: 0.53, 95% CI = 0.38–0.75) as shown in table 2.

Discussion

T1D is a multifactorial autoimmune disease caused by one or more environmental factors, such as viruses and bacterial toxins, interacting with the genetic profile of the individual

Table 1. Association between age of onset of type 1 diabetes and polymorphism in rs1071583 of *FCN1* gene.

	rs1071583	*N*	Av.	SD	Dif.	LL	UL	*P* value	AIC
Codominant	C/C	91	7.86	0.42	0.002	–	–	0.05	1097
	C/T	81	7.52	0.48	−0.34	−1.549	0.87		
	T/T	22	5.45	0.63	−2.37	−4.25	−0.49		
Dominant	C/C	91	7.86	0.42	0.00	–	–	0.19	1100
	C/T–T/T	103	7.09	0.41	−0.77	−1.92	0.37		
Recessive	C/C–C/T	172	7.70	0.32	0.00	–	–	0.02	1095
	T/T	22	5.49	0.63	−2.21	−3.98	−0.42		
Overdominant	C/C–T/T	113	7.40	0.37	0.00	–	–	0.84	1101
	C/T	81	7.52	0.48	0.12	−1.41	1.29		
Log additivate	0.1.2	–	–	–	−0.91	−1.75	−0.06	0.04	1097

N, number of individuals; Av., average for a certain genotype; SD, standard deviation of average; Dif., the difference between the averages of certain genotype with genotype reference; LL, lower limit of the CI of difference; UL, upper limit of the CI of difference; AIC, Akaike information criterion.

Table 2. Frequencies of haplotypes in *FCN1* gene in T1D patients and healthy controls.

FCN1	rs2989727	rs1071583	HC	T1D	OR (95% CI)	*P*
1	T	C	0.2459	0.5050	1.00	–
2	C	T	0.1422	0.3216	0.94 (0.65–1.35)	0.72
3	C	C	0.4004	0.1734	0.53 (0.38–0.75)	3×10^{-4}
4	T	T	0.2116	0	0.00 (ND)	1

*Global haplotype association $P<0.0001$; HC, healthy control; T1D, type 1 diabetes.

(Kyvik *et al.* 1995; Tsutsumi *et al.* 2003; Hansen *et al.* 2004). In the context of infectious triggering of T1D, it is known that ficolins can activate the lectin pathway of complement after binding to various microbial ligands such as mannose (Ohta *et al.* 1990), lipoteichoic acid (Lynch *et al.* 2004), GlcNAc (Matsushita *et al.* 1996), lipopolysaccharide (Neth *et al.* 2000; Zhao *et al.* 2002). Thus, it can be assumed that the reduction or deficiency of serum and interstitium ficolin-1/2 in children and adolescents could be a risk factors for T1D onset.

Further, as ficolins are involved in the cleaning of apoptotic bodies, its deficiency may also be associated with a poor removal of apoptotic cells resulting in the spread of autoantigens and immune system activation (Boniotto *et al.* 2005; Runza *et al.* 2008).

The knowledge of ficolin in autoimmune diseases remains scarce; in fact, in the literature, we found only one genetic association study with autoimmune disorder. Vander Cruyssen *et al.* (2007) described the association of rs2989727 and rs1071583 in *FCN1* with the development of rheumatoid arthritis. Both SNPs were included in the present study, but no association with T1D development was found. Further, these SNPs were not involved in the insurgence of CD or AITD or both. As Vander Cruyssen *et al.* (2007) demonstrated a strong LD between the SNPs rs2989727 and rs1071583, we also performed LD analysis and found that the combination of alleles C–C (for the rs2989727 and rs1071583, respectively) were associated with protection to T1D development, even if these SNPs were not in LD in our studied population. Unfortunately, Vander Cruyssen *et al.* (2007) did not perform the haplotype association, limiting our discussion.

The MBL and ficolin proteins may share the same molecular function and both take part in similar innate immune pathways as synonymous proteins, i.e., absence of one protein could be masked by the presence the other. Thus, we hypothesize that individuals carrying low levels of FCN1 or FCN2 could present normal or even high levels of MBL, thus creating a balance in the complement activation and apoptotic clearance.

Despite these major functions and similarities between MBL and ficolins, the ficolin-1 is the unique to be released by leukocytes that infiltrates the interstitium during the inflammation, such as is observed in the triggering T1D, which the beta-cells are infiltrated by reactive T cells generating an inflammatory process termed insulitis. We believe that the absence of ficolin-1 in the microenvironment of the inflammatory process in pancreas tissues will not be balanced by the MBL presence. However, according to our results this hypotheses was not confirmed, corroborating Munthe-Fog *et al.* (2012) findings that demonstrated that the rs2989727 do not influence the *FCN1* expression in monocytes. Interestingly, children carrying the T/T genotype (rs1071583 of *FCN1*) presented an earlier age of diagnosis compared with genotypes C/C or C/T in a recessive model. The rs1071583 is a synonymous SNP at exon 9, that changes CAA to CAG, and the codon CAA has lower codon usage related to CAG (12.3 versus 34.2 per thousand of transcription) http://www.kazusa.or.jp/codon/, consequently, individuals carrying the genotype T/T (i.e. the codon CAA) could produce smaller quantity of ficolin 1. This finding suggests that *FCN1* is not directly implicated in triggering T1D onset but it could be involved in enhancing its consequences by creating a favourable environment for chronic conditions

in T1D patients. After the initial autoimmune response, the recruitment of leucocytes that produce a smaller quantity of ficolin 1 could diminish the apoptotic clearance and anticipating the autoimmune profile. However, further functional studies should be performed to clarify this hypothesis.

Conclusion

This is the first report to study the genetic role of *FCN1* and *FCN2* in northeastern Brazilian children and adolescents with T1D. *FCN1* rs1071583 SNP was correlated with earlier age of T1D diagnosis. In addition, the SNP combination rs2989727 and rs1071583 was involved with T1D protection. We are aware of the limitations of our study, related basically to the low number of individuals analysed as well as to the absence of functional validation (ELISA, Western, etc.) of the impact of *FCN1* and *FCN2* SNPs on the production and functionality of the proteins: thus both genetic replica and immunological functional studies should be done to increase the knowledge of *FCN* genes in the development of T1D.

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