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Reduced expression of *NLRP3* and *MEFV* in human ischemic heart tissue

Cecilia Hermansson [a], Annika Lundqvist [a], Carina Wasslavik [b], Lars Palmqvist [b], Anders Jeppsson [c], Lillemor Mattsson Hultén [a,*]

[a] Sahlgrenska Center for Cardiovascular and Metabolic Research, Wallenberg Laboratory, Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden
[b] Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
[c] Department of Cardiothoracic Surgery, Sahlgrenska University Hospital, Gothenburg, Sweden

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ABSTRACT

The innate immune system and, in particular, activation of the multi-protein complex known as the inflammasome complex are involved in ischemic injury in myocardial cells. The nucleotide-binding leucine-rich repeat-containing pyrin receptor 3 (NLRP3) inflammasome has been linked to inflammation and NLRP3 is especially important for increased inflammation in atherosclerosis, which may lead to myocardial infarction. Here we investigated how inflammasome molecules are affected in human ischemic heart tissue. Surprisingly the important member of the inflammasome complex, *NLRP3*, displayed markedly decreased levels in human ischemic heart tissue compared with non ischemic control heart tissue. However, subsequent gene analysis revealed mutations in *NLRP3* in human ischemic heart tissues but not in non-ischemic control tissue. Gene polymorphisms in the *NLRP3* inflammasome have been shown to be associated with increased IL-1β and IL-18 production and severe inflammation.

The autoinflammatory disorder familial Mediterranean fever (FMF) is associated with decreased expression of the Mediterranean fever gene (*MEFV*) and increased inflammation. We also observed reduced expression of *MEFV* in ischemic versus non-ischemic heart tissue. Further analyses showed a mutation in *MEFV* in human ischemic heart tissue but not in non-ischemic control tissue.

Our data show that defects in the inflammasome and associated proteins may be involved in promoting ischemic heart disease.

1. Introduction

The signaling pathway in ischemic injury in the heart and in myocardial infarction involves the innate immunity [1]. Inflammation is involved in all phases of atherosclerosis and several proinflammatory cytokines such as interleukin (IL)-1β, IL-18 and tumor necrosis factor (TNF)-α play a role in the pathophysiology of ischemic heart diseases [2]. We have previously shown high expression of proinflammatory markers in human ischemic heart tissue [3]. IL-1β has been shown to have a direct role in ischemic heart disease, and IL-1β levels are higher in atherosclerotic coronary arteries compared with normal coronary arteries [4]. In addition, elevated IL-18 levels have been associated with a less favorable prognosis in patients with acute coronary syndromes [5].

The production of IL-1β and IL-18 requires activation of caspase (CASP)-1 or IL-1β converting enzyme, which are activated by a complex of intracellular proteins known as the nucleotide-binding leucine-rich repeat-containing pyrin receptor 3 (*NLRP3*) inflammasome [6]. The *NLRP3* inflammasome activity has been linked to various inflammatory disorders including atherosclerosis, metabolic syndrome and type II diabetes [7]. *NLRP3*-deficient mice show decreased inflammation and reduced levels of IL-18 [8]. Recent experimental data show that inflammasome activation and increased IL-β production is important for myocardial ischemic injury [9]. However, little is known about the role of inflammasome in the pathophysiology of human ischemic heart disease. In this study, we investigate how the inflammasome and associated proteins are affected in the ischemic human heart.

2. Materials and methods

2.1. Human heart tissue

Endomyocardial tissue from the right atrium was obtained from 5 patients undergoing coronary bypass surgery (at the Sahlgrenska University Hospital, Gothenburg, Sweden). Samples were frozen immediately on dry ice and stored at −70 °C until analysis. The study protocol was approved by the Ethical Committee of the University of Gothenburg and all subjects gave written informed con-

* Corresponding author. Address: Wallenberg Laboratory, Sahlgrenska University Hospital, SE-413 45 Gothenburg, Sweden. Fax: +46 31 823762.
E-mail address: Lillemor.Mattsson@wlab.gu.se (L.M. Hultén).

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sent. For control samples, we purchased total RNA from right atrium from normal tissue from three human adults (Invitrogen and BioChain, CA, USA).

2.2. Quantitative PCR (Q-PCR)

RNA was isolated from human myocardial tissue with the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized with the RT2 First Strand Kit (C-03, SuperArray, SABiosciences). The RT reactions were performed with a Gene Amp PCR system 9700 (Applied Biosystems).

For gene expression analyses of inflammatory genes, the human Inflammasomes PCR Array (PAHS-097A, Qiagen) was used. All PCR amplification was performed for 40 cycles on an ABI PRISM 7700 sequence detection system (Applied Biosystems).

2.3. Mutation analysis

We analyzed human ischemic and non ischemic heart tissue and the coding regions of *NLRP3* and Mediterranean fever (*MEFV*) genes were amplified by PCR with specific primers. For *NLRP3* we used primers *NLRP-3* sence(s) TGGACCACATGGTTTCTTCC and *NLRP3*-antisence (as) TGGAGCGTTTCACACAACAC, for *MEFV* exon 2: *MEFV*-s AGCCTGAAGACTCCAGACCA and *MEFV*-as CCTTCTCTCTGCGTTTGCTC for *MEFV* exon 10: *MEFV*-s TTACTGGGAGGTGGAGGTTG and *MEFV*-as GTGTCCCAGGGCTGAAGATA were used. The amplification was performed using the HotStarTaq DNA Polymerase (Qiagen) in PCR buffer containing 2.5 mM $MgCl_2$ (Qiagen), and dNTP 200 μM (Roche) using a PTC-200 thermal cycler (BioRad). Amplification bands was observed in a 2% agarose gel and sequencing reactions were made in all samples. Sequencing was generated using ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The amplicons were analyzed using the ABI PRISM 3100 (Applied Biosystems) automated DNA sequencer. Each template was sequenced in both directions. The results of the analysis were edited using the ABI PRISM SeqScape v.2.6 software (Applied Biosystems).

2.4. Statistics

Data are plotted as mean and SEM unless stated otherwise. Differences between groups were determined with Student's *t* test using GraphPad Prism version 5.01, GraphPad Software, San Diego, California USA (www.graphpad.com). *P* values <0.05 (two-sided) were considered statistically significant.

3. Results

We compared expression levels of inflammasome genes in ischemic versus non-ischemic human heart tissue, and observed reduced levels of *NLRP3*, caspase 8 and FADD-like apoptosis regulator (*CFLAR*), pannexin 1 (*PANX1*), *MEFV*, and heat shock protein (*HSP*) 90AB1 in ischemic heart tissue (Fig. 1A–E). A trend towards significantly reduced levels of caspase recruitment domain family member 6 (*CARD6*) was also noted in ischemic heart tissue

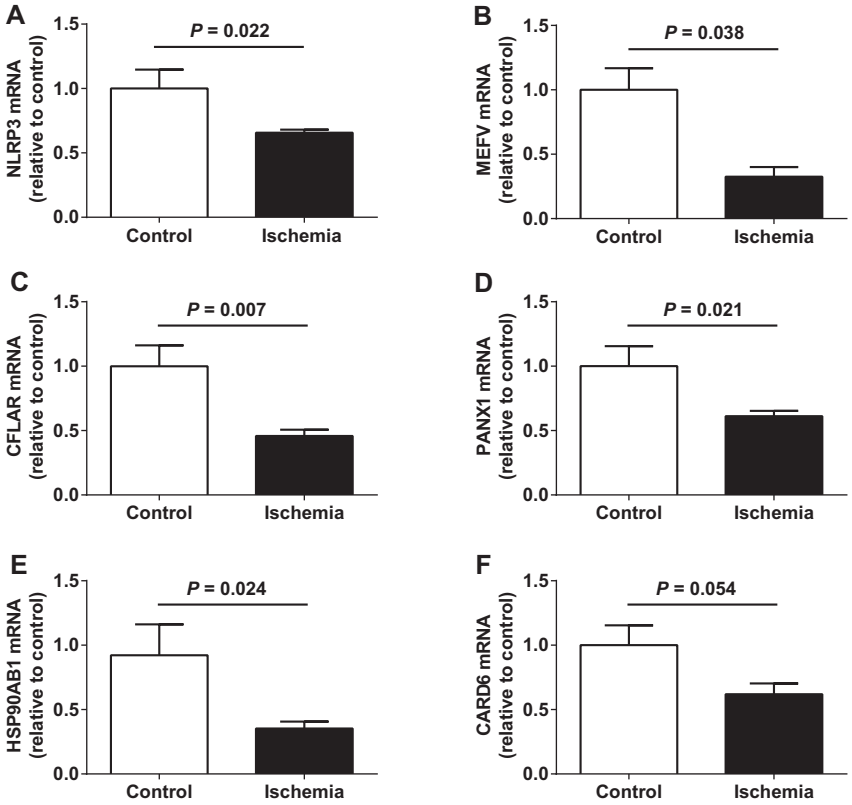

Fig. 1. Decreased expression of inflammasomes in human ischemic heart tissue. Total cellular RNA was extracted from human heart tissue and inflammasome mRNA and α-actin mRNA levels were measured by Q-PCR. (A) *NLRP3* mRNA expression in myocardial tissue from non-ischemic and in ischemic myocardium was normalized to α-actin. (B) *MEFV* expression is decreased in human ischemic heart tissue. (C) Decreased expression of FADD-like apoptosis regulator (*CFLAR*) mRNA in ischemic myocardial specimen relative to non ischemic control. (D) Decreased expression of Pannexin 1 (*PANX1*) mRNA in ischemic myocardial tissue compared with non ischemic control. (E) Heat shock protein 90AB1 is decreased in ischemic myocardium. (F) Expression of Caspase recruitment domain family, member 6 (*CARD6*) mRNA in myocardial tissue from non-ischemic and in ischemic myocardium. Data from Q-PCR showing the relative change in expression in myocardial tissue from non-ischemic (Control) ($n = 3$) and in ischemic myocardium (Ischemia) ($n = 5$). Data are expressed as mean ± SEM.

Table 1
Inflammasome components in human heart tissue.

Gene symbol	mRNA level (relative to control)	*P* value
AIM2	1.31 ± 0.78	0.75
CARD18	0.19 ± 0.04	0.47
CASP1	1.08 ± 0.11	0.74
CASP4	0.94 ± 0.21	0.82
CASP5	0.52 ± 0.11	0.09
CASP8	0.32 ± 0.05	0.13
HSP90AA1	0.83 ± 0.08	0.26
HSP90B1	0.89 ± 0.03	0.23
NLRC4	0.72 ± 0.10	0.11
NLRC5	0.59 ± 0.07	0.24
NLRP1	0.57 ± 0.06	0.28
NLRP4	1.65 ± 0.30	0.23
NLRP5	1.60 ± 0.29	0.22
NLRP6	0.61 ± 0.26	0.32
NLRP9	1.15 ± 0.15	0.59
NLRP12	0.93 ± 0.21	0.87
NLRX1	1.47 ± 0.28	0.20
PSTPIP1	0.54 ± 0.08	0.12
PYCARD	1.01 ± 0.09	0.92
PYDC1	0.48 ± 0.20	0.09

Data from Q-PCR showing the relative change in expression in ischemic versus non-ischemic human myocardium. Data are expressed as mean ± SEM.
AIM2, Absent in melanoma 2; *CARD18*, Caspase recruitment domain family, member 18; *CASP1*, Caspase 1 apoptosis-related cysteine peptidase; *HSP90A1*, heat shock protein 90 kDa alpha (cytosolic), class A member 1; *HSP90B1*, HSP90 class B member 1; NLRC, NACHT, leucin-rich repeat and pyrin domain (PYD) containing protein family CARD domain; *NLRP*, NLR family PYD domain; *NLRX1*, NLR family member X1; *PSTPIP1*, Proline-serine-threonine phosphatase interacting protein 1; *PYCARD*, PYD and CARD domain containing; *PYDC1*, PYD containing 1.

(Fig. 1F). We did not observe any significant changes in mRNA levels of other members of the inflammasome complex (Table 1).

We performed genetic analyses and sequencing of *NLRP3* in ischemic and non-ischemic heart tissue. We showed that two of the patients with ischemic heart tissue carried the gene polymorphism Q705 K in *NLRP3*: one individual was heterozygous (Q705 K,Q; Fig. 2A) and one was homozygous (Q705 K; Fig. 2B). None of the non-ischemic tissue samples showed this mutation (representative sequencing of the non-ischemic heart tissue is shown in Fig. 2C). In one specimen from a patient with ischemic heart disease, gel electrophoresis of *NLRP3* PCR products showed an unknown expression pattern predicted to be a splice variant of *NLRP3* (Fig. 2D). No similar *NLRP3* expression pattern was found in the non-ischemic control heart tissue.

We also performed genetic analysis and sequencing of *MEFV* in ischemic and non-ischemic heart tissue. We identified one novel homozygote (K671 M) mutation in a specimen from one patient with ischemic heart disease (Fig. 2E). No similar mutation in *MEFV* was found in the other ischemic or non-ischemic heart tissue samples (Fig. 2F).

4. Discussion

In this study, we investigated how the inflammasome is affected in the ischemic human heart, and observed decreased mRNA levels of *NLRP3*, *CFLAR*, *MEFV* and *PANX1* in ischemic tissue compared with non-ischemic controls.

Our observation of reduced mRNA levels of *NLRP3* in the ischemic heart was in contrast to our expectations since increased expression and activation of the NLRP3 inflammasome has been linked to inflammatory disorders [10]. However, gene polymorphisms in the *NLRP3* inflammasome in humans have been shown to be associated with increased IL-1β and IL-18 production and severe inflammation [11,12] in diseases such as familial periodic fever syndromes and Muckle-Wells syndrome, and a connection between elevated levels of IL-1β and mutations in *NLRP3* has been reported [13]. We performed genetic and sequencing analysis of *NLRP3* in human heart tissue and identified one patient with ischemic heart disease who was homozygous one who was heterozygous for the Q705 K mutation in *NLRP3*. The prevalence of the Q705 K mutation has been found to be common in a Swedish population with an allele frequency of 6.5% [14]. We also identified an

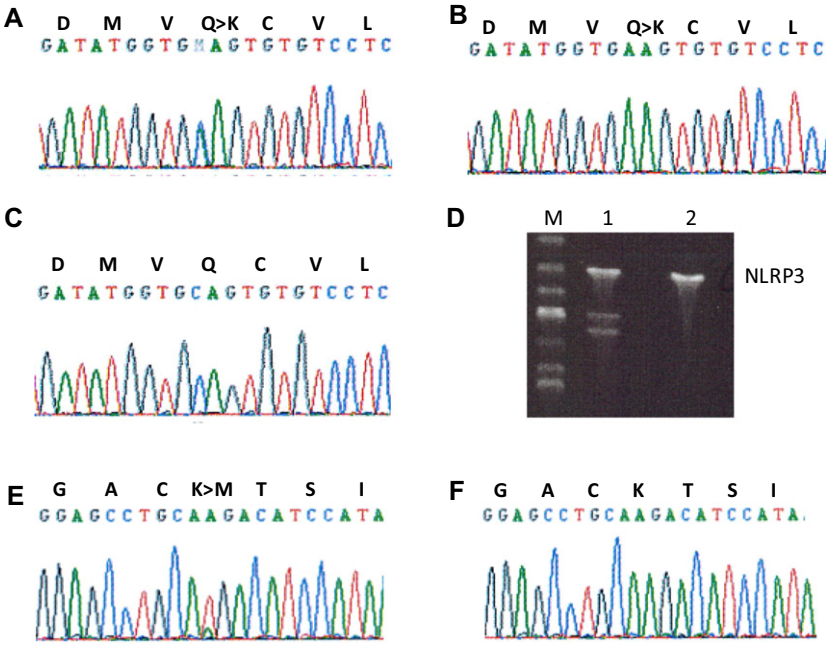

Fig. 2. DNA sequence of mutations in patients with ischemic heart disease. (A) DNA sequence of the *NLRP3* heterozygous mutation Q705 K, Q in exon 3 in a patient with ischemic heart disease. (B) DNA sequence of the *NLRP3* homozygous mutation Q705 K in a patient with ischemic heart disease. (C) The corresponding DNA sequence in a control individual. (D) Gel electrophoresis of *NLRP3* PCR products in ischemic heart tissue and control. Agarose gel showing *NLRP3* expression, lane M; molecular weight marker, lane 1 patient; lane 2 control. (E) DNA sequence of the *MEFV* homozygous mutation K617 M in exon 10 in a patient with ischemic heart disease and (F) the corresponding DNA sequence in a control individual.

unknown expression pattern of *NLRP3* predicted to be a splice variant in one additional patient. The Q705 K mutation is described as a gain of function alteration, and is associated with increased levels of IL-1β, IL-18 [11,12]. This is consistent with our earlier data showing increased levels of inflammatory markers in human ischemic versus non-ischemic- heart tissue [3]. The *NLRP3* gene is potentially an interesting target for further studies of atherosclerosis and ischemic heart disease.

The pyrin domain in *NLRP3* is highly homologous to the pyrin domain encoded by the Mediterranean fever (*MEFV*) gene and mutations in both these genes are associated with inflammatory diseases [15,16]. We observed reduced mRNA levels of *MEFV* in ischemic versus non-ischemic heart tissue. This result is consistent with the established association between low levels of *MEFV* mRNA and inflammation in patients with the autoinflammatory disorder familial Mediterranean fever (FMF) [17]. FMF patients with mutations in *MEFV* have been shown to express low levels of *MEFV* mRNA [18]. We performed sequencing of *MEFV* and identified one patient with ischemic heart disease homozygous for the K671 M mutation. Reduced levels of *MEFV* mRNA and a defect in *MEFV* may contribute to increased inflammation.

In addition, we observed decreased expression of *HSP90AB1* in ischemic versus non-ischemic human heart tissue. This may potentially indicate a decreased defense mechanism against ischemic injury of myocardial cells since HSP90 has been shown to protect cardiomyocytes from ischemic cell injury [19]. We also observed significant lower levels of *PANX1* in ischemic versus non-ischemic human heart tissue. NLRP3 functions downstream of PANX1 to regulate caspase activation in response to bacterial components, and PANX1 are suggested to be the missing link between bacterial stimuli and activation of the NLRP3 inflammasome [20]. We therefore propose that reduced *PANX1* expression is further evidence of a defective defense mechanism and activation of the innate immune response in ischemic hearts.

In conclusion, our work identifies decreased expression of *NLRP3* and *MEFV* mRNA in human ischemic heart tissue compared with non-ischemic myocardium. Furthermore, we demonstrate mutations in these genes in ischemic heart tissue but not in non ischemic controls. Low expression of *NLRP3* and *MEFV* may have a role in a defective immune response leading to increased inflammation and subsequent atherosclerosis and ischemic heart disease.

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References

[1] J.W. Dong, J.G. Vallejo, H.P. Tzeng, J.A. Thomas, D.L. Mann, Innate immunity mediates myocardial preconditioning through Toll-like receptor 2 and TIRAP-dependent signaling pathways, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H1079–H1087.

[2] P.M. Ridker, T. Thuren, A. Zalewski, P. Libby, Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), Am. Heart J. 162 (2011) 597–605.

[3] L.U. Magnusson, A. Lundqvist, J. Asp, J. Synnergren, C.T. Johansson, L. Palmqvist, A. Jeppsson, L.M. Hulten, High expression of arachidonate 15-lipoxygenase and proinflammatory markers in human ischemic heart tissue, Biochem. Biophys. Res. Commun. 424 (2012) 327–330.

[4] J. Galea, J. Armstrong, P. Gadsdon, H. Holden, S.E. Francis, C.M. Holt, Interleukin-1 beta in coronary arteries of patients with ischemic heart disease, Arterioscler Thromb. Vasc. Biol. 16 (1996) 1000–1006.

[5] M. Hartford, O. Wiklund, L.M. Hulten, A. Persson, T. Karlsson, J. Herlitz, J. Hulthe, K. Caidahl, Interleukin-18 as a predictor of future events in patients with acute coronary syndromes, Arterioscler Thromb. Vasc. Biol. 30 (2010) 2039–2046.

[6] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Mol. Cell 10 (2002) 417–426.

[7] T. Strowig, J. Henao-Mejia, E. Elinav, R. Flavell, Inflammasomes in health and disease, Nature 481 (2012) 278–286.

[8] P. Duewell, H. Kono, K.J. Rayner, C.M. Sirois, G. Vladimer, F.G. Bauernfeind, G.S. Abela, L. Franchi, G. Nunez, M. Schnurr, T. Espevik, E. Lien, K.A. Fitzgerald, K.L. Rock, K.J. Moore, S.D. Wright, V. Hornung, E. Latz, NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals, Nature 464 (2010) 1357–1361.

[9] M. Kawaguchi, M. Takahashi, T. Hata, Y. Kashima, F. Usui, H. Morimoto, A. Izawa, Y. Takahashi, J. Masumoto, J. Koyama, M. Hongo, T. Noda, J. Nakayama, J. Sagara, S. Taniguchi, U. Ikeda, Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury, Circulation 123 (2011) 594–604.

[10] F.G. Bauernfeind, G. Horvath, A. Stutz, E.S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B.G. Monks, K.A. Fitzgerald, V. Hornung, E. Latz, Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression, J. Immunol. 183 (2009) 787–791.

[11] D. Verma, M. Lerm, R. Blomgran Julinder, P. Eriksson, P. Soderkvist, E. Sarndahl, Gene polymorphisms in the NALP3 inflammasome are associated with interleukin-1 production and severe inflammation: relation to common inflammatory diseases?,, Arthritis Rheum. 58 (2008) 888–894.

[12] D. Verma, E. Sarndahl, H. Andersson, P. Eriksson, M. Fredrikson, J.I. Jonsson, M. Lerm, P. Soderkvist, The Q705K polymorphism in NLRP3 is a gain-of-function alteration leading to excessive interleukin-1beta and IL-18 production, PLoS ONE 7 (2012) e34977.

[13] H.M. Hoffman, J.L. Mueller, D.H. Broide, A.A. Wanderer, R.D. Kolodner, Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome, Nat. Genet. 29 (2001) 301–305.

[14] R. Blomgran, V. Patcha Brodin, D. Verma, I. Bergstrom, P. Soderkvist, C. Sjowall, P. Eriksson, M. Lerm, O. Stendahl, E. Sarndahl, Common genetic variations in the NALP3 inflammasome are associated with delayed apoptosis of human neutrophils, PLoS ONE 7 (2012) e31326.

[15] S. Grandemange, I. Aksentijevich, I. Jeru, A. Gul, I. Touitou, The regulation of MEFV expression and its role in health and familial Mediterranean fever, Genes Immun. 12 (2011) 497–503.

[16] J.A. Harton, M.W. Linhoff, J. Zhang, J.P. Ting, Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains, J. Immunol. 169 (2002) 4088–4093.

[17] D. Ustek, C.G. Ekmekci, F. Selcukbiricik, A. Cakiris, B. Oku, B. Vural, H. Yanar, K. Taviloglu, U. Ozbek, A. Gul, Association between reduced levels of MEFV messenger RNA in peripheral blood leukocytes and acute inflammation, Arthritis Rheum. 56 (2007) 345–350.

[18] C. Notarnicola, M.N. Didelot, I. Kone-Paut, F. Seguret, J. Demaille, I. Touitou, Reduced MEFV messenger RNA expression in patients with familial Mediterranean fever, Arthritis Rheum. 46 (2002) 2785–2793.

[19] K. Wu, W. Xu, Q. You, R. Guo, J. Feng, C. Zhang, W. Wu, Increased expression of heat shock protein 90 under chemical hypoxic conditions protects cardiomyocytes against injury induced by serum and glucose deprivation, Int. J. Mol. Med. (2012).

[20] T.D. Kanneganti, M. Lamkanfi, Y.G. Kim, G. Chen, J.H. Park, L. Franchi, P. Vandenabeele, G. Nunez, Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling, Immunity 26 (2007) 433–443.