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Vztah solubilních faktorů imunitního systému k fenotypu idiopatických zánětlivých myopatií

Relation of Soluble Factors of Immune System to Fenotype of Idiopathic Inflammatory Myopathies

Disertační práce

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Abstrakt

Úvod: Idiopatické zánětlivé myopatie (myositidy, IIM), představují heterogenní skupinu vzácných autoimunitních systémových onemocnění, společně charakterizovaných zejména svalovou slabostí, postihující převážně proximální skupiny příčně pruhovaného svalstva. Heterogenita myositid je více než v klinickém obraze vyjádřena v jejich patogenetických mechanismech a odráží se v imunofenotypové odpovědi u jednotlivých podtypů.

Cíle práce: Cílem této práce bylo popsat asociace a vliv solubilních faktorů imunitního systému, přítomných v sérech pacientů s IIM, s fenotypovými charakteristikami myositid a jejich podtypů, ověřit případnou expresi těchto molekul v zánětlivě změněné svalové tkáni pacientů a zhodnotit jejich význam v patogenezi analýzou jejich působení na imunitní a svalové buňky *in vitro*.

Výsledky: Popsali jsme prevalenci a charakteristiku kloubního postižení u pacientů s myositidou a jeho významnou asociaci s anti-Jo-1 autoprotilátkou. Dále jsme potvrdili vztah anti-HMGCR protilátky k imunitně zprostředkované nekrotizující myopatii, její těsný vztah k předchozí léčbě statiny a recentní nárůst incidence. Prokázali jsme překvapivou negativní asociaci hladin IFNα s aktivitou svalového postižení na magnetické rezonanci, avšak popisujeme korelaci klinické aktivity onemocnění s aktivací dráhy interferonu typu I u pacientů s dermatomyositidou. Dále jsme prokázali korelaci hladin resistinu a klinické aktivity a hladin visfatinu s klinickou aktivitou u anti-Jo-1 pozitivních pacientů. Resistin i visfatin jsou zvýšeně exprimované ve svalové tkáni pacientů s IIM. Navíc prokazujeme odlišnou specifickou expresi některých miRNA v sérech pacientů s PM a DM. Séra pacientů s IIM jsou schopna aktivace dráhy interferonu typu I *in vitro* a tato aktivace je zprostředkována především IFNα. Taktéž prokazujeme schopnost resistinu indukovat expresi prozánětlivých cytokinů (IL-1β, IL-6, MCP-1) v mononukleárních buňkách.

Závěr: Naše výsledky ukazují na vztah jednotlivých molekul imunitního systému k jednotlivým podtypům či fenotypovým projevům IIM a demonstrují jejich význam v patogenezi tohoto onemocnění.

Klíčová slova: idiopatické zánětlivé myopatie, autoprotilátky, cytokiny

Summary

Introduction: Idiopathic inflammatory myopathies (myositis, IIM) are heterogeneous group of rare autoimmune systemic diseases, characterized particularly by proximal skeletal muscle weakness. Heretogeneity of myositis is based on different pathogenetic mechanisms which may be reflected by variable imunophenotypic response in individual subtypes.

Objectives: The aim of this work was to explore the associations and influence of soluble factors of immune system in patient's sera on phenotypic characteristics and subtypes of IIM, to describe their expression in inflammed muscle tissue and study their eventual role in pathogenesis by analysis of effect on immune and muscle cells *in vitro*.

Results: We have described prevalence and characteristics of joint involvement in myositis patients and its significant association with anti-Jo-1 autoantibody. Further we confirmed the relation of anti-HMGCR antibody to immune mediated necrotizing myopathy, its tight relation to statins and recent increase in incidence. We showed inverse association of IFNα serum levels with muscle activity detected on MRI. Clinical activity positively correlated with IFN type-I pathway activation in patients with dermatomyositis. We also show positive correlation of resistin levels and clinical activity and correlation of activity with visfatin serum levels in anti-Jo-1 positive patients. Both resistin and visfatin are up-regulated in muscle biopsies. Moreover, we showed differentially expressed characteristic miRNA in sera of patients with PM and DM. Sera of patients with IIM are capable to activate IFN-type I pathway *in vitro* and IFNα seems to be responsible for that. We also demonstrate the ability of resistin to induce expression of pro-inflammatory cytokines (IL-1β, IL-l, MCP-1) in mononuclear cells.

Conclusions: Our results show the relation of particular molecules of immune system to individual subtypes of IIM and their phenotypic manifestations and suggest the role of soluble mediators in pathogenesis of idiopathic inflammatory myopathies.

Key words: idiopathic inflammatory myopathies, autoantibodies, cytokines

Poděkování:

Tato práce by nikdy nemohla vzniknout bez podpory celé řady lidí, kterých si velmi vážím a kterým touto cestou patří veliké poděkování.

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Obsah:

1. Úvod – idiopatické zánětlivé myopatie

1.1. Definice

Idiopatické zánětlivé myopatie (idiopathic inflammatory myopathies, IIM; též myositidyV laboratorním nálezu jsou přítomny zvýšené hodnoty sérových hladin svalových enzymů a proteinů (alaninaminotransferáza, aspartátaminotransferáza, kreatinkináza, laktát dehydrogenáza, myoglobin) (Dalakas, M.C., 1991, Dalakas, M.C. and Hohlfeld, R., 2003). Pro autoimunitní charakter onemocnění svědčí přítomnost zánětlivého infiltrátu ve svalech, přítomnost autoprotilátek, asociace s některými HLA alelami a odpověď na imunosupresivní léčbu.

1.2. Epidemiologie

Roční incidence myositid je odhadována na 2-10 na milion obyvatel, roční prevalence se pak pohybuje okolo 20/100 000 obyvatel (Dalakas, M.C. and Hohlfeld, R., 2003). V současnosti je trend k jejich nárůstu, pravděpodobně způsobeného spíše zlepšenou diagnostikou. Idiopatické zánětlivé myopatie se mohou vyskytnout v kterémkoli věku s vrcholy v dětství a v dospělosti. Celkový poměr incidence u žen a mužů je asi 2,5:1, tento poměr je nižší (téměř 1:1) u juvenilních forem a u myositid asociovaných s malignitami, naopak stoupá až k 10:1 v případě koexistence s jiným systémovým autoimunitním onemocněním pojiva. Idiopatické zánětlivé myopatie se poměrně často vyskytují v rámci překryvných syndromů s dalšími systémovými onemocněními, např. se systémovou sklerodermií (SSc), smíšeným onemocněním pojiva (MCTD), systémovým lupus erytematodes (SLE), Sjögrenovým syndromem (SjS) nebo revmatoidní artritidou (RA) (Alarcon-Segovia, D., 1994, Troyanov, Y. et al., 2005, Vancsa, A. et al., 2010).

1.3. Klasifikace

Hlavními zástupci skupiny idiopatických zánětlivých myopatií jsou polymyositida (PM), dermatomyositida (DM), juvenilní dermatomyositida (JDM), myositida s inklusními tělísky (IBM), imunitně zprostředkovaná nekrotizující myopatie (immune mediated necrotizing myopathy, IMNM), popřípadě myositida asociovaná s maligním onemocněním (cancer associated myostits, CAM). V průběhu posledních několika dekád bylo různými autory navrženo a publikováno několik klasifikačních kriterií (Bohan, A. and Peter, J.B., 1975, Bohan, A. and Peter, J.B., 1975, Dalakas, M.C., 1991, Dalakas, M.C. and Hohlfeld, R., 2003, Griggs, R.C. et al., 1995, Hoogendijk, J.E. et al., 2004, Mammen, A.L. et al., 2011, Troyanov, Y. et al., 2005). Původní Bohanova a Peterova kritéria, přes své četné nedostatky (např. absence autoprotilátek nebo histologických změn typických pro IBM či IMNM) zůstávají dodnes nejpoužívanějšími. Jednotlivé jednotky se mezi sebou liší v klinickém obraze, v charakteru zánětlivé infiltrace, resp. bioptického nálezu svalu a přítomností nebo typem autoprotilátek.

- *Polymyositida*

Polymyositida postihuje častěji dospělé než děti, projeví se jako progredující slabost proximálních svalových skupin. Pro svou podobnost s ostatními kategoriemi IIM je často určována jako diagnosa *per exclusionem*; dále nebývá přítomna vyrážka kromě typického postižení radiálních stran prstů rukou (tzv. „ruce mechanika“). V histologickém obraze převažuje přítomnost endomysiálního $CD8^+$ lymfocytárních infiltrátu, který až invaduje do non-nekrotických svaolových vláken a zvýšené expresi MHC molekul I třídy na svalových vláknech. Časté, i v závislosti na přítomnosti autoprotilátek, bývá postižení plic charakteru intersticiální plicní fibrózy (Colafrancesco, S. et al., 2015, Dalakas, M.C., 2015, Hengstman, G.J. et al., 2008).

- *Dermatomyositida*

Dermatomyositida se může vyskytnout jak v dospělém, tak i v dětském věku. Svalová slabost je manifestována podobně jako u ostatních druhů IIM, navíc však bývá přítomna typická kožní vyrážka, která obvykle bývá fotosenzitivní: periorbitální heliotropní raš, erytém ve výstřihu, na ramenou a dorzální části krku, dále na kůži nad lokty a koleny, na bocích a laterálních částí stehen a konečně typickým příznakem bývají tzv. Gottronovy skvrny až papuly nad drobnými klouby rukou. Nezřídka bývá, zejména u juvenilních forem, přítomna kalcinóza. Mikroskopicky bývá přítomen spíše $CD4^+$ lymfocytární infiltrát v perivaskulární, eventuálně perifascikulární lokalizaci, perifascikulární atrofie svalových vláken a dále depozita komplemetnu (membránu atakující komplex) na stěně malých cév, snížená hustota kapilár a případně overexprese molekul MHC-I (Colafrancesco, S. et al., 2015, Dalakas, M.C. and Hohlfeld, R., 2003, Hoogendijk, J.E. et al., 2004).

- *Myositida s inkluzními tělísky*

Myositida s inkluzními tělísky postihuje nejčastěji osoby od pátého decenia výše; na rozdíl od předešlých dvou typů IIM je častější u mužů. Svalová slabost zde častěji bývá asymetrická a typickým obrazem je výrazné postižení čtyřhlavého stehenního svalu, především pak m. rectus femoris a svalů předloktí, což je doprovázeno manifestovanou atrofií. Celkový průběh bývá u této jednotky spíše subakutní; charakteristická je i výrazná refrakterita k terapii. Histologicky nalézáme lemované (rimmed) vakuoly, bazofilní granulární inkluze, nepravidelná červená a cytochrom-oxidáza negativní svalová vlákna cytoplasmatické inkluze obsahující Tau protein uvnitř svalových vláken a též mononukleární buňky invadující do non-nekrotickcých vláken (Colafrancesco, S. et al., 2015, Dalakas, M.C., 2015, Griggs, R.C. et al., 1995, Hoogendijk, J.E. et al., 2004).

- *Imunitně zprostředkovaná nekrotizující myopatie*

Imunitně zprostředkovaná nekrotizující myopatie je poměrně recentně popsaná jednotka opět charakterizovaná proximální svalovou slabostí s akutním, někdy až dramatickým průběhem charakteru rhabdomyolýzy s extrémními hodnotami sérových hladin svalových enzymů. V histologickém obraze dominuje nález nekrotických svalových vláken bez přítomnosti zánětlivého infiltrátu. Pravidelně bývají přítomny autoprotilátky anti-SRP nebo anti-HMGCR u IMNM vyvolané užíváním statinů (Mammen, A.L. et al., 2011, Quinn, C. et al., 2015).

- *Myositida asociovaná s maligním onemocněním*

Myositida jako paranoeplastický projev se obvykle manifestuje jako dermatomyositida, eventuálně jako nespecifická myositida. Nejčastějšími nádorovými onemocněními bývají karcinomy ovarií a prsu u žen a karcinom plic u mužů a dále karcinomy pankreatu, žaludku a kolorektální karcinom (Hill, C.L. et al., 2001).

- *Myositida jako součást překryvného syndromu*

Myositida může být součástí klinického obrazu dalších systémových autoimunitních onemocnění, zejména systémového lupus erytematodes (SLE), smíšeného onemocnění pojiva (MCTD), Sjögrenova syndromu a systémové sklerodermie; vyjímkou není ani překryvný syndrom s revmatoidní artritidou. Pro tyto syndromy je charakteristická přítomnost autprotilátek anti-PM-Scl 75 a 100, anti-Ku a anti-U1RNP (Ghirardello, A. et al., 2014).

- *Antisyntetázový syndrom*

Společný výskyt příznaků myositidy se systémovými projevy zánětu (horečky) a postižením dalších orgánových soustav (intersticiální plicní fibróza, neerozivní artritida, Raynaudův fenomén, prsty mechanika, kožní vyrážky) a přítomnost pro myositidy specifických autoprotilátek, konkrétně tzv. antisyntetázových protilátek (viz oddíl 1.5.3.1.

Autoprotilátky) tvoří dohromady antisysntetázový syndrom. V jeho rámci mohou být jeho jednotlivé součásti více či méně vyjádřeny (Mahler, M. et al., 2014).

- *Juvenilní dermatomyositida*

Myositida u dětí má řadu společných klinických znaků s obrazem nacházeným u dospělých pacientů (proximální svalová slabost, chronické zánětlivé postižení svalů a typické kožní projevy). Častěji než u dospělých je se vyskytuje kalcinóza. Nečastějšími autoprotilátkami, nacházenými u juvenilní formy jsou anti-TIF1, anti-NXP2 a anti-MDA5 (Rider, L.G. and Nistala, K., 2016).

1.4. Etiologie

V etiologii idiopatických zánětlivých myopatií se pravděpodobně uplatňují jak faktory genetické, tak i vlivy vnějšího prostředí, jejich podíl, popřípadě vzájemné interakce však nejsou dosud objasněny.

Z genetických faktorů byla identifikována jako nejvýznamnější asociace s molekulami hlavního histokompatibilního systému (HLA) DRB1*0301 a DQA1*0501 (Arnett, F.C. et al., 1996, Shamim, E.A. et al., 2000), jsou popsány silné asociace haplotypů HLA systému s přítomností některých, pro myositidy specifických, autoprotilátek (Chinoy, H. et al., 2009, Chinoy, H. et al., 2007). V celogenomové asociační studii (Genom-wide association study, GWAS) byly identifikovány signifikantní asociace tří jednonukleotidových polymorfismů (single nucleotid polymorphism, SNP) s dermatomyositidou, konkrétně v genech pro PLCL1, BLK a CCL21, u nichž byla již dříve popsána asociace s autoimunitními onemocněními (Miller, F.W. et al., 2013). Mimo to byly popsány genetické asociace s geny pro tumor nekrotizující faktor alfa (TNF-α) a antagonistu receptoru pro interleukin-1 (IL-1ra) (Hassan, A.B. et al., 2004, Pachman, L.M. et al., 2000, Rider, L.G. et al., 2000).

Z faktorů vnějšího prostředí je určitý vliv přisuzován ultrafialovému záření: Mi-2 (jaderná helikáza) je též upregulována v keratinocytech exponovaných UV záření (Burd, C.J. et al.,

2008) a podíl pacientů s dermatomyositidou (s kožním postižením) a přítomnost anti-Mi-2 autoprotilátek koreluje se stoupající expozicí UV záření v severojižním směru (Love, L.A. et al., 2009, Okada, S. et al., 2003). Autoimunitně podmíněnou nekrotizující myopatii jsou schopny vyvolat léky ze skupiny statinů, včetně indukce autoprotilátek proti hydroxy-3-methyl-glutaryl-koenzym-A-reduktáze (Christopher-Stine, L. et al., 2010, Mammen, A.L. et al., 2011); mimo to byly popsány případy polymyositidy po léčbě interferonem (IFN-α) (Kalkner, K.M. et al., 1998) a TNF-α blokujícími preparáty (Brunasso, A.M. et al., 2010, Ishikawa, Y. et al., 2011, Musial, J. et al., 2003). Kouření představuje u myositických pacientů s HLA DRB1*03 pozitivitou rizikový faktor pro vznik autoprotilátek anti-Jo-1, lze se domnívat, že k tomu dochází ještě před klinickou manifestací choroby (Chinoy, H. et al., 2012). Velká část T-lymfocytů v periferní krvi a ve svalové tkáni u pacientů s IIM má $CD28^{null}$ fenotyp, zastoupení těchto lymfocytů bylo signifikantně vyšší u myositických pacientů se seropozitivitou proti cytomegaloviru, což může naznačovat účast některých virových infekcí v etiologii IIM (Fasth, A.E. et al., 2009). Samostatnou kapitolu pak představuje asociace idiopatických zánětlivých myopatií s nádorovým onemocněním (CAM), nejčastěji s nádory ovarií, plic, pankreatu, žaludku, kolorektálního karcinomu a non-Hodgkinského lymfomu (Hill, C.L. et al., 2001, Sigurgeirsson, B. et al., 1992).

1.5. Patogeneze

Patogeneze autoimunitních myopatií není v současné době zcela jasná; předpokládá se kombinace imunitních a neimunitních mechanismů ve vývoji chronického zánětu v kosterním svalstvu a hlavních klinických symptomů – svalové slabosti a únavnosti:

- přímý, zejména cytotoxický efekt infiltrujících leukocytů, zejména T-lymfocytů a makrofágů na svalové buňky,

- nepřímý efekt molekul imunitního systémů (autoprotilátek, molekul MHC, cytokinů, chemokinů a dalších) a

- alterace metabolismu, zejména prostřednictvím poškození mikrocirkulace (Hochberg, M.C., 2011).

1.5.1. Buněčná imunopatologie

Zánětlivá celulární infiltrace kosterního svalu se vyskytuje ve dvou hlavních vzorcích. V prvním případě se jedná o predominantně endomysiální lokalizaci, sestávající se zejména z $CD8^+$ a $CD4^+$ T-lymfocytů, makrofágů a dendritických buněk, které obkružují a někdy i invadují do ne-nekrotických svalových vláken (Dalakas, M.C., 1991, Page, G. et al., 2004, Plotz, P.H. et al., 1995). Tento vzorec je nejčastěji pozorován u polymyositidy a myositid s inkluzními tělísky. Druhý typ pak představuje převážně $CD4^+$ T-buňky, makrofágy, dendritické buňky a B-lymfocyty v perivaskulární a perimysiální lokalizaci a je pozorován zejména u dermatomyositidy (Dalakas, M.C., 1991, Page, G. et al., 2004, Plotz, P.H. et al., 1995). Rozdílné mikrofenotypy poukazují na odlišné patogenetické mechanismy uplatněné v jednotlivých subtypech myositid s predominantím T-lymfocyty zprostředkovaným poškozením svalových vláken u polymyositid a myositid s inklusními tělísky a roli mikrovaskulárního poškození u dermatomyositid. Zcela odlišný případ pak představuje imunitně zprostředkovaná nekrotizující myopatie, kde zánětlivý infiltrát chybí, popřípadě jsou jen roztroušeně přítomné makrofágy (Vattemi, G. et al., 2014).

- *T-lymfocyty*

Přesná úloha T-lymfocytů v patogenezi myositid není dosud zcela objasněná. V myositickém svalu je nacházena široká řada subtypů T-lymfocytů: $CD4^+$ (helper) lymfocyty, kterým mohou být prezentovány antigeny prostřednictvím MHC molekul exprimovaných na svalových vláknech, nicméně přesvědčivé důkazy o interakci s T-cell receptorem (TCR) zatím chybí. $CD8^+$ lymfocyty, produkující cytotoxické molekuly perforin-1 a granzym B, mohou působit myotoxicky, obdobně ale dosud nebyla u myositických svalových vláken popsána přítomnost potřebných kostimulačních faktorů ke spuštění tohoto

mechanismu (Behrens, L. et al., 1998). Určitou úlohu mohou hrát $CD28^{null}$ T-lymfocyty (postrádající aktivační molekulu CD28) (Fasth, A.E. et al., 2009, Pandya, J.M. et al., 2010), přítomné v zánětlivém milieu: představují chronicky stimulované, klonálně omezené efektorové buňky, které nahrazují kostimulaci pomocí CD86 antigenem jinými mechanismy (Fasth, A.E. et al., 2010), mají cytotoxické účinky podobné NK buňkám, jsou prozánětlivé, rezistentní k apoptóze a mohou přispívat k chronicitě a rezistenci, pozorované u myositid. V *in vitro* ko-kulturách vykazovaly $CD28^{null}$ (jak $CD4^{+}$ tak $CD8^{+}$) lymfocyty vysokou míru myotoxicity vůči autologním myocytům (Pandya, J. et al., 2014).

T-lymfocyty mohou přispívat k patogenezi myositid nejen přímým cytotoxickým působením, ale například i posouváním imunitní rovnováhy od supresivní k prozánětlivé: Th17 T-lymfocyty, produkující interleukin IL-17 (IL-17 mRNA je zvýšené exprimována v biopsiích myositických pacientů) (Kondo, M. et al., 2009), mohou tímto ovlivňovat migraci, diferenciaci a vyzrávání zánětlivých buněk. IL-17 v kombinaci s IL-1β vede ke zvýšení produkce IL-6, MHC-I, CCL20 a nukleárního faktoru NF-κB v myoblastech (Chevrel, G. et al., 2003), což může přispívat k poškození svalu a k chronickému zánětu (Tournadre, A. et al., 2009).

- *B-lymfocyty*

Pro význam B-lymfocytů a plasmatických buněk v patogenezi myositid svědčí řada nepřímých důkazů. Jsou přítomny v zánětlivém infiltrátu, prodělávají klonální expanzi, somatické mutace a isotypový přesmyk, což ukazuje na antigenem indukovanou B-buněčnou odpověď, byť konkrétní antigeny nebyly dosud identifikovány (Bradshaw, E.M. et al., 2007). U pacientů s pozitivitou anti-Jo-1 autoprotilátek a s dermatomyositidou je pozorována zvýšená hladina faktoru aktivujícího B-lymfocyty (BAFF) (Krystufkova, O. et al., 2009, Peng, Q.L. et al., 2012). Dalším faktem, podporujícím význam B-lymfocytů v patogenezi IIM je řada případů uspokojivé klinické odpovědi na terapii B-lymfocyty blokující monoklonální

protilátkou (rituximabem) (Couderc, M. et al., 2011, Limaye, V. et al., 2012, Oddis, C.V. et al., 2013).

- *Dendritické buňky a makrofágy*

Nezralé i zralé formy dendritických buněk, stejně jako plasmocytoidní dendritické buňky a infiltrující makrofágy jsou často nalézány ve svalových biopsiích u myositid a v kůži u dermatomyositis (McNiff, J.M. and Kaplan, D.H., 2008). Přepokládá se jejich úloha při prezentování antigenů (Page, G. et al., 2004) a produkci prozánětlivých cytokinů a chemokinů (Civatte, M. et al., 2005, Confalonieri, P. et al., 2000, De Paepe, B. and De Bleecker, J.L., 2005, Rostasy, K.M. et al., 2004), zejména pak IFN-β (Greenberg, S.A. et al., 2005).

1.5.2. Molekuly HLA (MHC) I a II třídy

In vitro kultivované myoblasty konstitutivně exprimují molekuly HLA I třídy (HLA-A, -B a –C) (Hardiman, O. et al., 1993) a tato exprese je zvyšována prozánětlivými cytokiny (IFN-γ, TNF-α, IL-1α, IL-1β) a chemokinem MIP-1α a naopak snižována působením TGF-β (Marino, M. et al., 2003, Michaelis, D. et al., 1993). Interferon-γ navíc indukuje i expresi HLA II třídy (HLA-DR, -DP a –DQ) (Mantegazza, R. et al., 1991). Za normálních okolností nejsou in vivo molekuly HLA povrchu svalových buněk exprimovány, významný nález z tohoto hlediska představuje častá exprese MHC I v myositickém svalu (Emslie-Smith, A.M. et al., 1989, Karpati, G. et al., 1988). Tato exprese MHC I antigenů, obvykle difúzní, je pozorována u většiny myositických pacientů jak na regenerujících, tak i na jinak histopatologicky normálních vláknech. MHC II třídy jsou exprimovány méně konzistentně. Molekuly MHC I i II třídy mohou být v myositickém svalu exprimovány i bez přítomnosti zánětlivé infiltrace, popřípadě jí předcházejí (Englund, P. et al., 2001).

Mimo MHC I a II jsou svalové buňky schopny exprimovat i HLA-G in vivo u myositid a in vitro po stimulaci (Wiendl, H. et al., 2000). HLA-G je „non-klasická“ molekula MHC I,

strukturálně příbuzná klasické MHC-I. Jejich význam není zcela objasněn, podobně jako HLA I váží CD8 a antigenní peptidy, mohou tedy teoreticky působit jako antigen-presentující struktury (Diehl, M. et al., 1996), nicméně HLA-G byly popsány jako molekuly zprostředkovávající imunotoleranci (Carosella, E.D. et al., 2001), například chrání myoblasty před aloreaktivní lýzou inhibicí NK-buněk, CD4+- a CD8+-lymfocytů in vitro (Wiendl, H. et al., 2003).

1.5.3. Solubilní faktory imunitního systému s významem u myositid

1.5.3.1. Autoprotilátky

Sérové autoprotilátky jsou nalézány u většiny pacientů s autoimunitními myopatiemi; pozitivita antinukleárních nebo anticytoplasmatických protilátek se vyskytuje mezi 55-80% nemocných (Targoff, I.N., 2002). Identifikace autoprotilátek přispívá ke klasifikaci jednotlivých podtypů myositid, asociaci s klinickými syndromy a specifickým orgánovým postižením a v predikci prognózy základního onemocnění (Betteridge, Z. and McHugh, N., 2015, Ghirardello, A. et al., 2014, Gunawardena, H. et al., 2009, Troyanov, Y. et al., 2005).

Protilátky, které mohou být přítomny i u jiných autoimunitních onemocnění, byť zde nemusí být přítomny známky myositidy, jsou označovány jako protilátky asociované s myositidou (myositis-associated autoantibodies, MAA), například protilátky proti ribonukleoproteinu (anti-RNP), anti-PM-Scl a anti-Ku u překryvných syndromů se systémovou sklerodermií, nebo anti-Ro52 a Ro60 (Targoff, I.N., 2002).

Zástupci druhé skupiny autoprotilátek jsou nacházeny výhradně u myositid a jsou proto nazývány protilátkami specifickými pro myositidu (myositis-specific autoantibodies, MSA). Do této skupiny jsou řazeny tzv. antisyntetázové protilátky (anti-Jo-1, -PL-7, -PL-12, -EJ, -OJ, -KS, -Zo, -Ha), anti-SRP, protilátky proti jaderné helikáze (anti-Mi-2), anti-TIF1γ, anti-CADM-140 proti melanom diferenciačně-asociovamému proteinu 5 (anti-MDA5), protilátka

proti nukleárnímu matrixovému proteinu 2 (anti-NXP2) a anti-cNA1 (proti cytosolické endonukleáze 1A) (Betteridge, Z. et al., 2007, Betteridge, Z. and McHugh, N., 2015, Cruellas, M.G. et al., 2013, Targoff, I.N., 2002). Specifickou protilátkou je pak anti-HMGCR, s antigenem 3-hydroxy-3-methyl-glutaryl-CoA-reduktázou, vyskytující se u pacientů se statiny indukovanou imunitně zprostředkovanou nekrotizující myopatií (Christopher-Stine, L. et al., 2010, Mammen, A.L. et al., 2011).

Patogenetický význam autoprotilátek u myositid není dosud jasný, a to ani v otázce, proč dochází k jejich produkci, ani zda mají přímý (etio)patogenetický účinek v onemocnění. Žádný z cílových autoantigenů dosud nebyl identifikován jako tkáňově specifický pro příčně pruhovanou svalovinu. Hladina autoprotilátek ve většině případů nekoreluje s klinickou aktivitou nemoci, byla však pozorována mírná závislost mezi množstvím protilátek anti-Jo-1 a hladinami sérové kreatinkinázy (sCK) a aktivitou svalového i orgánového onemocnění (Stone, K.B. et al., 2007) a obdobně hladin sCK a anti-SRP a anti-HMGCR protilátek (Aggarwal, R. et al., 2015, Benveniste, O. et al., 2011, Tansley, S. et al., 2012). Ačkoli nebyly doposud podány jasné důkazy o přímé účasti autoprotilátek v patogenezi myositid, existuje řada nepřímých indicií, že tomu tak je. Například fenotyp pacientů s antisyntetázovými autoprotilátkami se liší na míře postižení svalů (myositida jako taková) a plic (intersticiální fibróza) v závislosti na přítomnosti konkrétní antisyntetázové protilátky: od výrazného svalového postižení s eventuální plicní nemocí u anti-Jo-1 k téměř čisté plicní fibróze s pouze vzácně přítomným onemocněním plicní tkáně u anti-PL-12 (Marie, I. et al., 2012). Obdobně se (etio)patogenetická úloha anti-HMGCR autoprotilátek nabízí u statiny indukované nekrotizující myopatie, kdy dochází k masivnímu poškození svalových vláken, až rhabdomyolýze v podstatě bez přítomnosti zánětlivého infiltrátu, a kdy je cílový antigen zvýšeně exprimován díky předchozímu blokování statiny, konkrétní práce, potvrzující přímý vliv těchto protilátek však zatím chybějí (Mammen, A.L. et al., 2011).

- *Antisyntetázové protilátky*

Antisyntetázové protilátky jsou namířeny proti tRNA syntetázám, cytoplasmatickým enzymům vážícím specifickou aminokyselinu na příslušnou tRNA a formující tím aminoacyl-tRNA. V současnosti je známo 8 antisyntetázových autoprotilátek, z nichž nejčastější je anti-Jo-1 (proti histidyl-tRNA syntetáze), vyskytující se u 9-24% pacientů s IIM., dále pak následují anti-PL12 (alanyl), anti-PL7 (threonyl), anti-EJ (glycyl), anti-OJ (isoleucyl), anti-KS (asparaginyl), anti-Zo (phenylalaninyl) a anti-Ha (tyrosyl), které jsou zastoupeny po přibližně 5% a celkem se vyskytují u 6-12% pacientů s myositidami (Betteridge, Z. and McHugh, N., 2015, Lega, J.C. et al., 2014). Pozitivita pro antisyntetázovou protilátku podmiňuje přítomnost antisyntetázového syndromu; konkrétní autoprotilátky pak jsou asociovány s rozdílnou manifestací jednotlivých orgánových postižení: fenotyp pacientů se liší v míře postižení svalů (myositida jako taková) a plic (intersticiální fibróza) od výrazného svalového postižení s eventuální plicní nemocí u anti-Jo-1, k téměř čisté plicní fibróze s pouze vzácně přítomným onemocněním svalové tkáně u anti-PL-12 (Marie, I. et al., 2012). Obdobně, Raynaudův fenomén je častěji pozorován u anti-PL12 a anti-PL7 pozitivních jedinců, dermatomyositická vyrážka je více asociovaná s přítomností anti-EJ, anti-PL12 a anti-PL7 (Hamaguchi, Y. et al., 2013). To, spolu s pozorovanou závislostí mezi množstvím protilátek anti-Jo-1 a hladinami sérové kreatinkinázy (sCK) a aktivitou svalového i orgánového onemocnění (Stone, K.B. et al., 2007) nepřímo svědčí pro patogenetické působení těchto protilátek.

- *Anti-Mi-2*

Autoprotilátky anti-Mi-2, mířené proti jaderné helikáze (Wang, H.B. and Zhang, Y., 2001) jsou specifickým markerem pro dermatomyositidu (Targoff, I.N. and Reichlin, M., 1985), poměrně časté jsou u juvenilní dermatomyostidy a jsou signifikantně asociované s dermatomyositickým spektrem kožních příznaků (Gottronovy papuly, heliotropní raš, V-příznak, šálový příznak) (Ghirardello, A. et al., 2005, Targoff, I.N. and Reichlin, M., 1985).

Pozitivita pro anti-Mi-2 představuje poměrně dobrý prognostický faktor, zahrnující menší míru svalového postižení, nižší riziko intersticiálního plicnho postižení a dobrou odpověď na imunosupresi (Betteridge, Z. and McHugh, N., 2015, Hengstman, G.J. et al., 2006, Petri, M.H. et al., 2013).

- *Anti-SAE*

Autoprotilátky anti-SAE (anti-small ubiquitin-like modifier activating enzyme) (Betteridge, Z.E. et al., 2009) se vyskytují se přibližně u 6-8% dospělých kavkazoidních pacientů s myositidou (Bodoki, L. et al., 2014). Anti-SAE jsou opět asociovány s kožním postižením, v tomto případě předcházejícím svalové příznaky a též korelují s přítomností dysfagie (Betteridge, Z. and McHugh, N., 2015, Betteridge, Z.E. et al., 2009, Fujimoto, M. et al., 2013).

-*Anti-TIF1*

Anti-TIF1 jsou autoprotilátky proti rodině transkripčního intermediárního faktoru 1 (TIF1), tvořené proteiny TIF1-γ (155 kDa), TIF1-α (140 kDa) a TIF1-β (122 kDa), z nichž největší význam mají anti-TIF1-γ (Fiorentino, D.F. et al., 2015, Fujimoto, M. et al., 2012, Kaji, K. et al., 2007, Targoff, I.N. et al., 2006, Targoff, I.N. et al., 2006). Anti-TIF1-γ pozitivita je u myositid asociována jednak s kožním postižením a jednak (u dospělých pacientů) s nádory asociovanou myositidou, a to velmi silně (senzitivita 78%, specificita 89%, positivní a negativní prediktivní hodnota 58% a 95%) (Trallero-Araguas, E. et al., 2012). Tato asociace však nebyla pozorována u dětí a mladých dospělých (Gunawardena, H. et al., 2008).

- *Anti-NXP2*

Autoprotilátky anti-NXP2 mají za cíl nukleární matrixový protein 2 a vyskytují se asi u čtvrtiny pacientů s juvenilní dermatomyositidou (Gunawardena, H. et al., 2009) a jsou asociovány s kalcinózou, svalovými kontrakturami, atrofií a celkově sníženým výkonostním

stavem (Betteridge, Z. et al., 2012, Espada, G. et al., 2009). Byla též popsána jejich přítomnost u dospělých pacientů (Ceribelli, A. et al., 2012, Ichimura, Y. et al., 2012), u kterých, a především u mužů, s věkem stoupá při pozitivitě anti-NXP2 riziko přítomnosti maligního onemocnění (Fiorentino, D.F. et al., 2013). Obdobně jako anti-TIF1-γ, jsou anti-NXP2 autoprotilátky asociované s rozdílným fenotypem onemocnění v závislosti na věku pacientů.

- *Anti-MDA5*

Anti-MDA5 (anti-melanoma differentiation-associated protein 5) se frekventně , přibližně v jedné pětině případů, vyskytují u východoasijských pacientů, u kterých je jejich přítomnost asociována s rychle progredující intersticiální plicní fibrózou a velice špatnou prognózou (Nakashima, R. et al., 2010). Přítomnost anti-MDA5 byla popsána i v evropské populaci myositiků, u kterých je též asociována s plicním postižením, avšak nejedná se o rychle progredující formu. V kavkazské populaci je též anti-MDA5 pozitivita spojena s výraznějším kožním postižením (Fiorentino, D. et al., 2011).

- *Anti-SRP*

Signal recognition particle (SRP), cytoplasmatický RNP komplex podílející se na rozpoznání a transportu nově syntetizovaných proteinů do endoplazmatického retikula je cílovým antigenem pro anti-SRP autoprotilátky (Muro, Y. et al., 2012, Reeves, W.H. et al., 1986). Jejich přítomnost je významně asociována s nekrotizující myopatií, s obvykle těžkým průběhem, rychlou progresí svalové slabosti, dysfagií, obvykle bez nebo s pouze mírným kožním postižením (Kao, A.H. et al., 2004, Targoff, I.N. et al., 1990). Titr anti-SRP pozitivně koreluje se sérovými hladinami kreatinkinázy a jeho změny odrážejí změny ve svalové síle (Aggarwal, R. et al., 2015, Benveniste, O. et al., 2011). Anti-SRP pozitivní pacienti bývají refrakternější na léčbu, ale přežívání pacientů s pozitivními nebo negativními antisyntetázovými autoprotilátkami je srovnatelné (Kao, A.H. et al., 2004).

- *Anti-HMGCR*

Antigenem pro anti-HMGCR autoprotilátky je 3-hydroxy-3-methylglutaryl-koenzym A reduktáza, klíčový enzym v syntéze cholesterolu (Christopher-Stine, L. et al., 2010, Mammen, A.L. et al., 2011). Anti-HMGCR pozitivita je asociována s imunitně podmíněnou nekrotizující myopatií, s obvykle těžkým průběhem, někdy až charakteru rhabdomyolýzy; obdobně jako u anti-SRP hladiny protilátek odrážejí klinickou aktivitu (Tansley, S. et al., 2012). Přítomnost anti-HMGCR autoprotilátek je velmi významně ascociována s předchozím užívání statinů, a to až v 92% případů u pacientů starších 50 let (Mammen, A.L. et al., 2011), nicméně jsou popisovány případy i u statin-naivních pacientů (Allenbach, Y. et al., 2014, Watanabe, Y. et al., 2015). V posledních letech je pozorován značný nárůst počtu pacientů s anti-HMGCR pozitivitou (Klein, M. et al., 2015).

- *Anti-cN1A*

Anti-cN1A je recentně objevená autoprotilátka proti cytosolickéu 5'nukleotidáze 1A (cN1A), enzymu hydrolyzujícího adenosin monofosfát (Pluk, H. et al., 2013, Salajegheh, M. et al., 2011), která se vyskytuje u zhruba jedné třetiny pacientů s myositidou s inkluzními tělísky (IBM) (Herbert, M.K. et al., 2016, Larman, H.B. et al., 2013); u tohoto podtypu do té doby nebyly autoprotilátky specifické pro IIM nalézány (Rojana-Udomsart, A. et al., 2012). Nicméně podle recentní studie byly tyto autoprotilátky identifikovány též u pacientů se Sjögrenovým syndromem (36%) a systémovým lupus erytematodes (20%); z tohoto hledisky by pak měly být řazeny mezi autoprotilátky s myositidami asociovanými (Herbert, M.K. et al., 2016).

1.5.3.2. Cytokiny a chemokiny

Cytokiny, proteiny o relativně nízké molekulové hmotnosti, působí jako solubilní chemičtí poslové mezi imunitními i somatickými buňkami. Podílejí se na integrované signální síti regulující jak vrozenou, antigen-nespecifickou imunitou, tak i v adaptivní imunitní odpovědi.

- Rodina interferonu

Řada indicií poukazuje na to, že interferony typu I (IFN-α, -β, -ω, -ε, -κ) hrají významnou úlohu v patogenezi idiopatických zánětlivých myopatií: sérové hladiny IFN-α jsou vyšší u dermatomyositických pacientů oproti zdravým kontrolám a inverzně korelují s délkou neléčeného onemocnění (Niewold, T.B. et al., 2009), proti očekávání ale jeho sérové hladiny nekorelují s aktivitou onemocnění.(Krol, P. et al., 2011) IFN typu I jsou silnými induktory exprese MHC molekul I a II třídy (Tournadre, A. et al., 2012), v periferních leukocytech i svalové tkáni myositických pacientů byly nalezeny zvýšené exprese genů indukovatelných interferonem typu I (tzv. IFN I signature) (Walsh, R.J. et al., 2007). Obdobně byla prokázána i zvýšená exprese proteinů indukovaných IFN typu I: protein rezistence proti myxoviru A (MxA) v kožních (Wenzel, J. et al., 2005) a svalových (Greenberg, S.A. et al., 2005) biopsiích pacientů s DM, kde byl specificky zvýšen v místech aktivního zánětu a atrofizace svalových vláken (Salajegheh, M. et al., 2010). Imunokomplexy obsahující antisyntetázové protilátky (např. anti-Jo-1) mohou jako endogenní stimulační faktor indukovat produkci IFN-α v plasmocytoidních dendritických buňkách (Eloranta, M.L. et al., 2009). Roli IFN typu I v patogenezi myositid podporují i případy polymyositid a dermatomyositid indukovaných terapeutickým podáváním IFN-α a -β u jiných autoimunitních nebo infekčních onemocnění (Aouba, A. et al., 2011, Dietrich, L.L. et al., 2000, Kalkner, K.M. et al., 1998, Somani, A.K. et al., 2008, Stubgen, J.P., 2009, Venezia, G. et al., 2005). Zdroje IFN typu I u myositid nebyly přesvědčivě demonstrovány, za významné producenty jsou považovány plasmocytoidní dendritické buňky, přítomné v zánětlivých infiltrátech myositických svalů (Eloranta, M.L. et al., 2007, Greenberg, S.A. et al., 2005). Navíc nezralé prekurzory svalových buněk ($CD56^{+}$) jsou po stimulaci toll-like receptoru 3 a IFN-γ schopny produkce IFN-β (Tournadre, A. et al., 2012).

Data o úloze IFN-γ, řazeného do skupiny interferonů typu II, nejsou jednoznačná; jednotlivé studie nekonsistentně uvádějí nebo nepotvrzují zvýšenou expresi nebo přítomnost IFN-γ či jeho mRNA v leukocytech a svalových biopsiích zejména u myositidy s inklusními tělísky a dermatomyisitidy (Baird, G.S. and Montine, T.J., 2008, Dastmalchi, M. et al., 2008, Lundberg, I. et al., 1997, Schmidt, J. et al., 2008).

- *Rodina interleukinu-1 (IL-1)*

Exprese IL-1α a IL-1β, které spolu s IL-18 vytvářejí rodinu interleukinu-1, je jeden z nejvíce konzistentních nálezů cytokinů ve svalové tkáni u PM a DM (Dorph, C. et al., 2006, Lepidi, H. et al., 1998, Tews, D.S. and Goebel, H.H., 1996). IL-1 se mimo jiné podílí na indukci konverze regulačních T-lymfocytů (T_{regs}) na Th-17 (Miossec, P. and Kolls, J.K., 2012). U pacientů, kteří odpovídají na léčbu glukokortikoidy, dochází k poklesu těchto cytokinů (Lundberg, I. et al., 2000); léčba antagonistou IL-1 (anakinra) má pozitivní výsledky u refrakterních myositid (Furlan, A. et al., 2008, Zong, M. et al., 2014).

- *Rodina tumor nekrotizujícího faktoru α (TNF-α)*

TNF-α a lymfotoxiny jsou strukturně příbuzné cytokiny s podobnými cytotoxickými účinky. Zatímco TNF-α může být produkován celou škálou buněk, lymfotoxiny pocházejí převážně z aktivovaných NK-buněk. Cytokiny TNF-α rodiny prostřednictvím indukce adhezních molekul a ostatních cytokinů a chemokinů regulují vývoj lymfoidních orgánů a zánětu jako takového (De Paepe, B. et al., 2009). TNF-α je významným regulátorem chronického zánětu asociovaného s myositidami, výrazně a signifikantně zvýšené exprese mRNA TNF-α byly nalezeny v infiltrátech u pacientů s PM, DM i IBM (Schmidt, J. et al., 2008). Paradoxně, studie účinnosti léčby blokátory TNF-α u pacientů s myositidami měly spíše rozporuplné než úspěšné výsledky (Dastmalchi, M. et al., 2008, Hengstman, G.J. et al., 2008), byly dokonce popsány i případy indukce myositidy u pacientů léčených anti-TNFα preparáty (Brunasso, A.M. et al., 2010, Ishikawa, Y. et al., 2011, Urata, Y. et al., 2006).

- *Transforming growht factor β (TGF-β)*

TGF-β byl tradičně považován za protizánětlivý cytokin, nicméně tento cytokin má pro- i protizánětlivé vlastnosti a to v závislosti na konkrétní situaci. Zvýšená exprese TGF-β mRNA byla pozorována ve svalech pacientů s DM a IBM a po léčbě intravenózními imunoglobuliny došlo u DM pacientů, na rozdíl od IBM, k poklesu této exprese. Navíc tato zvýšená exprese byla u těchto pacientů (DM) asociována s kožní fibrózou, což naznačuje význam TGF-β v patogenezi DM (Moran, E.M. and Mastaglia, F.L., 2014).

- *Chemokiny*

Chemokiny, též chemotaktické cytokiny, se podílí na regulaci migrace leukocytů z krve do místa zánětu. Na základě aminokyselin obklopujících konzervovaná cysteinová rezidua jsou klasifikovány do skupin, většina z nich pak náleží do dvou skupin α- a β-chemokinů.

V zánětlivě změněné svalové tkáni u IIM byla pozorována zvýšená exprese jak α-chemokinů (CXCL9 a CXCL10), tak β-chemokinů (CCL2, CCL3, CCL4, CCL19 a CCL21) (Baird, G.S. and Montine, T.J., 2008, De Paepe, B. et al., 2007, Marino, M. et al., 2008, Schmidt, J. et al., 2008).

β-chemokiny (CCL) kromě známých účinků na aktivaci a atrakci leukocytů se podílí na regeneraci svalových vláken; ta v buněčných kulturách proliferují po stimulaci CCL2, CCL3 a CCL4 (Yahiaoui, L. et al., 2008).

1.5.4. Neimuitní mechanismy

Vliv neimunitních mechanismů se v poslední době stává jedním z aspektů pozornosti rostoucího zájmu výzkumu patogeneze idiopatických zánětlivých myopatií. Tyto mechanismy zahrnují především hypoxii, stres endoplasmatického retikula (ER stres) a autofagii. Jednotlivé mechanismy spolu úzce souvisejí a mohou se navzájem ovlivňovat.

- *Hypoxie*

Pro hypoxii, jako jeden z možných patogenetických mechanismů, uplatňujících se u myositid, může svědčit řada pozorování: svalová slabost bez přítomnosti zánětlivého infiltrátu či ztráta kapilár ve svalech (Emslie-Smith, A.M. and Engel, A.G., 1990, Estruch, R. et al., 1992) a zvýšená exprese hypoxií inducibilního faktoru 1-α (HIF-1-α), včetně následků jeho působení (Dastmalchi, M. et al., 2008, Probst-Cousin, S. et al., 2010). Hypoxie může způsobovat svalovou slabost různými způsoby, jako například ovlivněním energetického metabolismu (snížení hladin ATP a fosfokreatininu), indukcí produkce prozánětlivých cytokinů (IL-1α a IL-1β). Tyto mechanismy jsou přítomny i u idiopatických zánětlivých myopatií (Hamada, T. et al., 2008, Park, J.H. et al., 1995).

- *Stres endoplazmatického retikula*

Stres endoplazmatického retikula nastává za situace, kdy se v této buněčné organele hromadí nedostatečně nebo vůbec složené („folded“) proteiny, což vede k reakcím, označovaným jako „unfolded protein response“ (UPR) a „ER overload response“ (cestou aktivace NF-κB) a následné apoptóze buňky. Ve svalových biopsiích pacientů s myositidami byla pozorována upregulace metabolitů drah UPR a NF-κB (Nagaraju, K. et al., 2005).

- *Autofagie*

Za fyziologických podmínek slouží autofagie jako způsob odstraňování nechtěných nebo poškozených proteinů uvnitř buňky jejich přesunem do lysosomů a tam následnou degradací. V případě narušení tohoto mechanismu může dojít k autofagické buněčné smrti. Podíl autofagie se předpokládá zejména u IBM (Nagaraju, K. et al., 2000), kde byla pozorována četná mrtvá svalová vlákna i přes vysokou expresi antiapoptotických molekul.

- *Poruchy metabolismu*

Porucha metabolismu hraje pravděpodobně roli v patogenezi statiny indukované IMNM. Myši s knockoutovaným genem pro svalovou HMGCR obdobné znaky (elevace sCK,

poškození svalových vláken až počínající nekrotické změny) jako pacienti s IMNM (Osaki, Y. et al., 2015). Toto postižení bylo reverzibilní po perorálním podávání mevalonové kyseliny, produktu HMGCR, což svědčí pro to, že postižení charakteristické pro IMNM může být způsobeno postižením metabolické dráhy cholesterolu. V současné době však nejsou údaje o tom, zda anti-HMGCR autprotilátky, přítomné u tohoto typu IIM jsou namířeny specificky proti svalovému isoenzymu, či mají inhibiční vliv na jeho aktivitu.

2. Hypotéza a cíle práce

Hypotéza této disertační práce vycházela z předpokladu, že sérové solubilní faktory, jako jsou prozánětlivé cytokiny a chemokiny, autoprotilátky atd., produkované organismem pacientů nemocných IIM mohou ovlivňovat chování svalových buněk vyvoláním morfologických změn, změn v expresi molekul MHC-I, expresi autoantigenů, změn produkce cytokinů, chemokinů či adhezivních a kostimulačních molekul v jejich kvalitě i kvantitě, aktivace procesů vedoucích k apoptóze nebo nekróze atd., které následně vedou ke klinickým projevům IIM. Zamýšleli jsme pokusit se objasnit případný vliv těcho solubilních faktorů vyvoláním uvedených změn jejich působením na normální svalové buňky v tkáňových kulturách, vytvořením simulovaného prostředí IIM a analýzou produktů zánětlivé tkáně. V průběhu experimentů jsme často naráželi na technické a metodologické obtíže, zejména při kultivaci svalových buněk, proto jsme naši hypotézu rozšířili o klinickou úroveň a rozšířili jsme práci o analýzu fenotypových projevů u IIM a jejich podtypů ve vztahu ke konkrétním faktorům imunitního systému. Předpokládáme tedy, že tyto solubilní faktory imunitního systému mají vliv na konkrétní fenotyp onemocnění na molekulární, buněčné, i klinické úrovni. Dále předpokládáme, že heterogenita onemocnění ze skupiny IIM má svůj korelát v odlišném složení molekul imunitního systému.

Cílem této disertační práce je popsat asociace solubilních faktorů s jednotlivými subtypy IIM a manifestacemi klinických symptomů a pokusit se objasnit případný vliv těchto faktorů na patogenezi onemocnění jejich působením na kultivované svalové a imunitní buňky v tkáňové kultuře:

1. Analyzovat klinické fenotypy a symptomy v kohortě pacientů s idiopatickými zánětlivými myopatiemi a tyto asociovat s přítomností solubilních faktorů (cytokiny, chemokiny, autoprotilátky) v sérech pacientů.
2. Ověřit případnou expresi těchto faktorů a její význam ve svalové tkáni pacientů s IIM
3. Zhodnotit vliv solubilních sérových faktorů na svalové buňky a leukocyty periferní krve *in vitro*.

3. Vlastní výsledky výzkumné práce

3.1. Artritida u idiopatických zánětlivých myopatií: klinické charakteristiky a asociace s autoprotilátkami

Úvod: Artritida, respektive kloubní postižení jako takové, je obecně akceptována jako jeden ze symptomů v klinickém obraze idiopatických zánětlivých myopatií (idiopathic inflammatory myopathies, IIM), nicméně přes obecné povědomí časté prevalence kloubních příznaků nebyla dosud kloubní manifestace u IIM systematicky popsána.

Cíle práce: Tato práce sledovala prevalenci, distribuci, klinickou manifestaci a vztah artritidy k časovému průběhu základního onemocnění u kohorty pacientů s IIM, a dále její vztah k přítomnosti autoprotilátkek, a genetickému pozadí (HLA).

Metody: Od 106 pacientů s idiopatickými zánětlivými myopatiemi jsme získali anamnézu kloubního postižení, klinické charakteristiky IIM; u všech pacientů byl zjištěn aktuální kloubní status prostřednictvím klinického vyšetření, osobního rozhovoru a čerpáním ze zdravotnické dokumentace, u 47 nemocných jsme měli k dispozici rentgenový snímek drobných kloubů ruky nebo nohy. U 71 a 73 pacientů jsme stanovili High-resolution genotypizaci v lokusech HLA-DRB1 a HLA-DQB1.

Výsledky: Artritida se kdykoli během průběhu základního onemocnění objevila u 56 (53%) pacientů: u 39 (37%) byla přítomna na začátku svalového onemocnění, včetně 23 případů (22%), kdy svalovým příznakům předcházela (Tabulka 1). V době klinického vyšetření byl u 31 (29%) pacientů přítomen alespoň 1 oteklý kloub. Nejčastěji postižené kloubní oblasti byly zápěstí, metakarpofalangeální a proximální interfalangeální klouby. Myositida, kdykoli během průběhu onemocnění, relabovala u 31 pacientů a artritida byla jedním z projevů

relapsu u 15 pacientů (48,8%), nejčastěji se vyskytla současně s ostatními symptomy. Přítomnost artritidy na začátku onemocnění však nebyla prediktivní pro přítomnost artritidy při relapsu myositidy a stejně tak přítomnost artritidy v počátku onemocnění nebyla prediktivní pro to, zda případný relaps myostidy bude nebo nebude doprovázen artritidou. Z dostupných rentgenových snímků (47 pacientů) pouze u 2 pacientů jsme identifikovali kloubní eroze: jeden pacient trpěl překryvným syndromem s revmatoidní artritidou a druhý byl pozitivně testován na anti-Jo-1 autoprotilátku. 27 z 29 pacientů s pozitivitou anti-Jo-1 mělo artritidu kdykoli v průběhu IIM; tato prevalence je signifikantně vyšší v porovnání anti-Jo-1 negativními pacienty ($p<0,0001$). Nenalezli jsme žádnou asociaci artritidy s jednotlivými HLA alelami.

Závěr: Artritida je častým příznamkem idiopatických zánětlivých myopatií. Je často přítomna na začátku onemocnění a dokonce může předcházet objevení se svalových symptomů. Nejčastěji se projeví jako neerozivní polyartritida postihující zvláště zápěstí, ramena a drobné klouby rukou a připomínající tak distribuci kloubního postižení u revmatoidní artritidy. Tato práce potvrzuje silnou asociaci artritidy s přítomností anti-Jo-1 autoprotilátek, asociaci s jinými autprotilátkami nebo s jednotlivými HLA alelami jsme nepotvrdili.

Tabulka 1. Artritida u jednotlivých podtypů IIM

Diagnóza IIM	Artritida kdykoli	Artritida na začátku onemocnění	Současná artritida (≥1 oteklý koub)
PM (46)	27 (59%)	19 (41%)	17 (40%)
DM (40)	22 (55%)	15 (38%)	11 (28%)
CAM (8)	2 (25%)	1 (13%)	0 (0%)
IMNM (11)	4 (36%)	1 (13%)	2 (18%)
IBM (1)	1 (100%)	1 (100%)	1 (100%)
Celkem (106)	56 (53%)	39 (37%)	31 (29%)

PM – polymyositida; DM – dermatomyositida; CAM – myositida asociovaná s nádorem; IMNM – imunitně zprostředkovaná nekrotizující myositida; IBM – myositida s inkluzními tělísky.
Artritida za začátku onemocnění – dle anamnestických dat. Současná artritida – artritida přítomná v čase vyšetření. Artritida kdykoli – kombinace anamnestických dat a klinického vyšetření.

Tuto práci komentovali autoři Jearn a Think-You (Jearn, L.H. and Kim, T.Y., 2015) s použitím vlastních dat. V souboru 23 anti-Jo-1 pozitivních pacientů s PM/DM pozorovali kloubní postižení u 13 pacientů (56,5%), ale nepozorovali signifikantní asociaci artritidy a přítomností anti-Jo-1 ($p>0,05$), naproti tomu popsali signifikantní vztah s přítomností plicní nemoci (20/23, 87,0%, $p<0,05$). Při srovnání skupin anti-Jo-1 pozitivních a negativních pacientů měla pozitivní skupiny signifikantně vyšší výskyt extramuskulárních projevů (artritida a intersticiální plicní nemoc) u anti-Jo-1 pozitivních (22/23, 95,7%, $p=0,007$). Autoři uvádějí, že anti-Jo-1 pozitivita je mnohem spíše asociovaná s plicním než s kloubním postižením.

V naší odpovědi jsme se opírali jednak o to, že naše výsledky prevalence kloubního positžení jsou sice vyšší než předchozí zjištění (93% oproti pracem s prevalencí přesahující 50%). Hlavní příčinu však spatřujeme v jiném metodologickém přístupu: v naší kohortě jsme po artritidě aktivně pátrali pomocí klinického vyšetření nebo se opírali o validní a věrohodná anamnestická data. Klinické vyšetření je doposud považováno za zlatý standard v detekci artritidy, zatímco autoři Jearn a Think-You použili rentgenové snímky. Co se týče plicního postižení, k jeho vztahu k pozitivitě antisyntetázových autoprotilátek, resp. anti-Jo-1 existuje řada důkazů a průkaz tohoto faktu nebyl cílem naší práce.

Práce byla publikována v časopise The Journal of Rheumatology a její kompletní znění a znění naší odpovědi jsou přiloženy v anglickém jazyce (Příloha 1A a 1B).

3.2. Stoupající incidence imunitně zprostředkované nekrotizující myopatie: zkušenosti z jednoho centra

Úvod: Imunitně zprostředkovaná nekrotizující myopatie (immune-mediated necrotizing myopathy, IMNM) je recentně popsaná forma idiopatických zánětlivých myopatií (idiopathic inflammatory myopathies, IIM), charakterizovaná progresivní, často těžkou svalovou slabostí, výraznými elevacemi svalových enzymů v séru a v histologickém obraze nekrotickým postižením, až rhabdomyolýzou, kosterní svaloviny bez přítomnosti zánětlivého infiltrátu. Charakteristická bývá přitomnost některých specifických autoprotilátek (anti-SRP, anti-HMGCR).

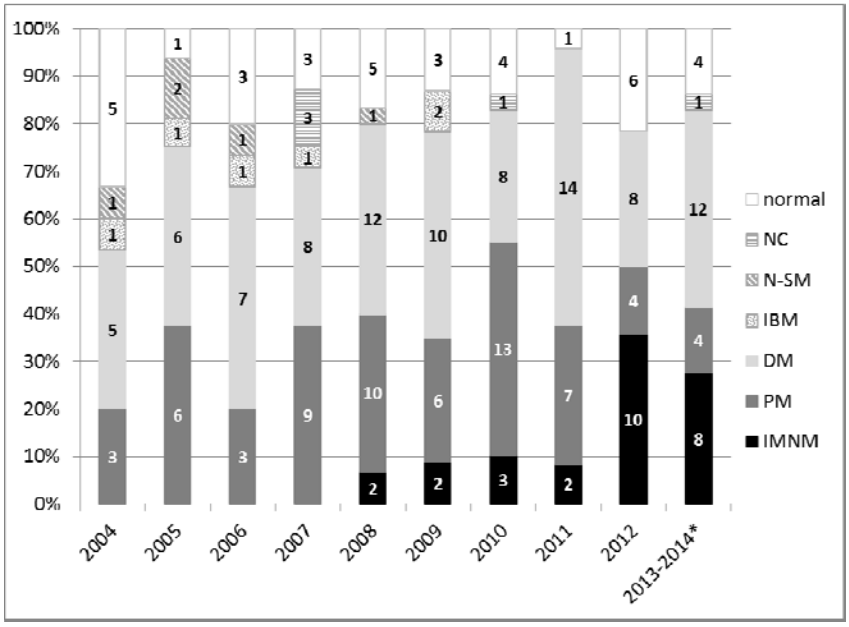
Cíle práce: Tato práce se věnovala současnému trendu nárůstu incidence IMNM, analyzovala autoprotilátkové profily pacientů a sledovala vliv statinů jako možných vyvolávajících činitelů.

Metody: Retrospektivně jsme reevaluovali výsledky svalových biopsií, klinických a laboratorních dat včetně autoprotilátkových profilů u všech pacientů s idiopatickou zánětlivou myopatií diagnostikovanou mezi lednem 2004 a červnem 2014 v Revmatologickém ústavu. 217 dostupných sér od těchto pacientů jsme testovali metodou ELISA na přítomnost autoprotilátek proti 3-hydroxy-3-methylglutaryl koenzym A reduktáze (anti-HMGCR). U pacientů s IMNM nebo pozitivitou anti-HMGCR jsme ověřili farmakologickou anamnézu užívání statinů.

Výsledky: Z 357 pacientů bioptovaných ve sledovaném období jich 233 splnilo kritéria pro některou z idiopatických zánětlivých myopatií, včetně 27 (11,6%) IMNM. Nikdo z nich nebyl diagnostikován mezi lety 2004 až 2007, v období 2008-2011 se vyskytovaly 2-3 nové případy ročně; mezi lety 2012-2014 došlo k významnému nárůstu incidence na 18 případů ($p<0,0001$)

(Obrázek 1). 13 z 27 pacientů (48%) mělo pozitivní farmakoanamnézu užívání statinů, u 11 z nich (58%) byly přítomny anti-HMGCR autoprotilátky. U 4 (14,8%) pacientů jsme zjistili přítomnost anti-SRP autoprotilátky, 3 (11,1%) byli anti-Jo-1 pozitivní, z ostatních autprotilátek asociovaných s IIM jsme idnetifikovali pozitivitu pro ANA (6x), anti-Ro (2x), anti-La (1x) a anti-Ku (1x). U 2 pacientů jsme neprokázali přítomnost žádné známé autoprotilátky. Neidentifikovali jsme žádného pacienta bez předchozího užívání statinů, který by byl anti-HMGCR pozitivní. Kromě anti-HMGCR protilátek a anamnézy užívání statinů jsme neprokázali asociaci IMNM s žádným demografickým, klinickým, laboratorním nebo jiným environmentálním faktorem.

Obrázek 1. Procentuální zastoupení výsledků biopsí v jednotlivých letech



* Data od ledna 2013 do června 2014. Čísla v boxech představují absolutní počty. IMNM – imunitně zprostředkovná nekrotizující myopatie; PM – polymyositida; DM – dermatomyositida; IBM – myositida s inklusními tělísky; NC – neklasifikovatelná; N-SM – non specific myositis.

Závěr: Naše data ukazují významný nárůst incidence imunitně zprostředkované nekrotizující myopatie v posledních 2 letech. IMNM je převážně spojená s pozitivitou anti-HMGCR autoprotilátek a je asociována s předchozím užíváním statinů.

Práce byla publikována v časopise Rheumatology a její kompletní znění je přiloženo v anglickém jazyce (Příloha 2).

3.3. Zvýšené hodnoty visfatinu jsou asociované s vyšší aktivitou nemoci u anti-Jo-1 pozitivních pacientů s myositidou

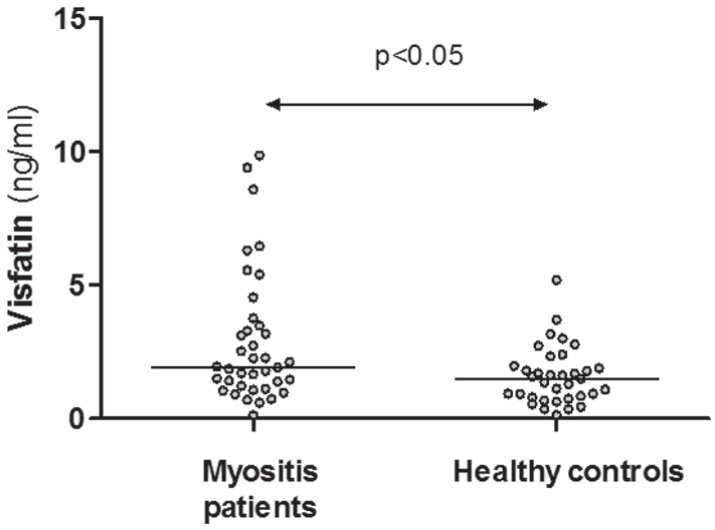
Úvod: Visfatin, též pre-B cell colony-enhancing factor (PBEF) je ubikvitně exprimován ve všech tkáních, je řazen mezi adipokiny a je upregulován v tukové tkáni. Mimo své účinky na energetický metabolismus jsou významné jeho imunomodulační a prozánětlivé vlastnosti: zesiluje aktivaci leukocytů, syntézu adhezivních molekul a produkci prozánětlivých cytokinů, např. IL-1, TNF-α a IL-6 v monocytech. Jeho produkce je zvýšena v lymfocytech a stimuluje jejich proliferaci během polyklonální imunitní odpovědi; dále se účastní vyzrávání B-lymfocytů. Zvýšené hladiny visfatinu byly popsány u řady autoimunitních zánětlivých onemocnění, jako systémový lupus nebo revmatoidní artritida.

Cíle práce: Cílem práce bylo stanovit sérové hladiny visfatinu u myositických anti-Jo-1 pozitivních pacientů se zdravými kontrolami a zhodnotit jejich možný vztah s anti-Jo-1 autoprotilátkovou pozitivitou, aktivitou onemocnění a sérovými hladinami faktoru aktivujícím B-lymfocyty (BAFF), cytokinu zásadnímu pro vyzrávání a přežívání B-lymfocytů, u kterého je známa asociace s myosidou a anti-Jo-1 autoprotilátkami. Dále jsme chtěli analyzovat exprese visfatinu ve svalových biopsiích pacientů s myositidou a kontrolních pacientů s nezánětlivým svalovým onemocněním (myasthenia gravis).

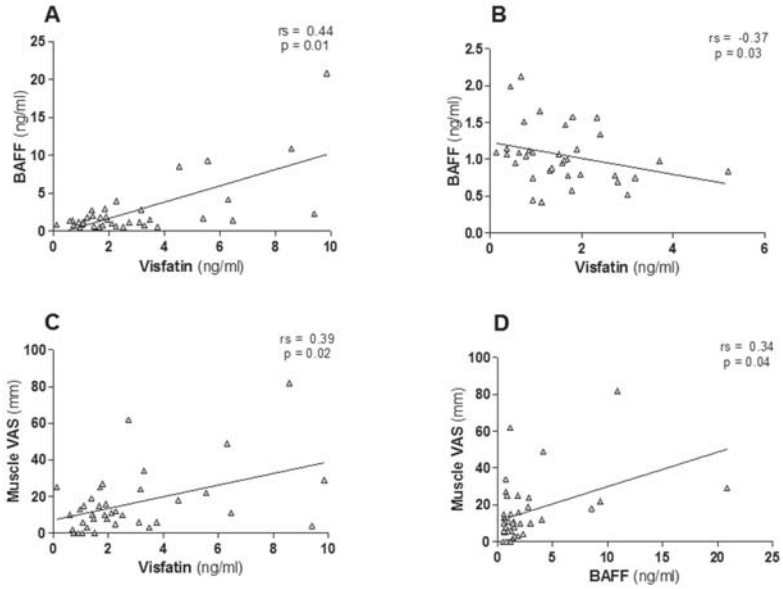
Metody: Změřili jsme sérové hladiny visfatinu a BAFF u 38 anti-Jo-1 pozitivních pacientů s myositidou a u 35 zdravých kontrol. Aktivitu onemocnění jsme hodnotili jednak prostřednictvím skóre MYOACT za použití visuální analogové škály a dále pomocí sérových hladin svalových enzymů a dalších markerů (kreatinkináza [CK], laktát dehydrogenáza [LDH], myoglobin [Mb], C-reaktivní protien [CRP]). Expresi visfatinu ve svalové tkáni jsme stanovili imunohistochemicky (n=10) a porovnali jsme ji s kontrolními svalovými vzorky pacientů s nezánětlivým svalovým onemocněním (myasthenia gravis, n=5).

Výsledky: Sérové hladiny visfatinu a BAFF byly signifikantně vyšší u pacientů s myositidou než u zdravých kontrol (visfatin: 1,94 [0,13–9,86] vs. 1,51 [0,14–5,20] ng/ml; $p<0,05$) (Obrázek 2) a byly asociovány s klinickou aktivitou svalového postižení hodnocenou pomocí VAS (visfatin rs=0,39, p=0,02; BAFF rs=0,34, p=0,04). Sérové hladiny BAFF pozitivně korelovaly s koncentracemi svalových enzymů (Mb rs=0,57, p=0,002, CK rs=0,51, p=0,001) a anti-Jo-1 protilátek (rs=0,85, p=0,001); tento vztah jsme však neprokázali u visfatinu mimo korelace s LDH (rs=0,28, p=0,02). Dále jsme pozorovali pozitivní korelaci mezi sérovými hladinami BAFF a visfatinu (rs=0,44, p=0,01), naopak u zdravých jedinců byla tato korealce negativní (rs=-0,37, p=0,03). Neprokázali jsme rozdíly mezi hladinami visfatinu na základě stáří nebo pohlaví pacientů, době trvání nemoci nebo na délce léčby glukokortikoidy. U pacientů s myositidou byla, na rozdíl od kontrolních pacientů s myasthenií gravis, přítomna zvýšená exprese visfatinu ve svalové tkáni v endomysiálních a perimysiálních infiltrátech.

Obrázek 2. Sérové hladiny visfatinu u pacientů a zdravých kontrol



Obrázek 3. Korelace hladin sérového visfatinu a BAFF



Korelace mezi sérovými hladinami visfatinu a BAFF u myositických pacientů (A) a u zdravých kontrol (B). Sérové hladiny visfatinu i BAFF u myositických pacientů korelují s aktivitou svalového postižení (C, D). Statistika je vyjádřena pomocí Spearmanových korelačních koeficientů s hodnotami *p* a liniemi lineárních regresí.

Souhrn: Zvýšená exprese visfatinu ve svalové tkáni u pacienů s myositidou a asociace mezi jeho zvýšenými sérovými hladinami a aktivitou onemocnění u anti-Jo-1 pozitivních pacientů podporuje roli visfatinu na účasti v patogenezi myositid.

Práce byla publikována v časopise Clinical and Experimental Rheumatology a její kompletní znění je přiloženo v anglickém jazyce (Příloha 3).

3.4. Resistin u idiopatických zánětlivých myopatií

Úvod: Resistin, též adipocyty sekretovaný faktor (ADSF) je členem adipokinové rodiny. Původně byl popsán u myší jako faktor indukující insulinovou rezistenci a je asociován s řadou metabolických onemocnění, ale též s nádory a zánětlivými či imunitně zprostředkovanými chorobami. V mononukleárních buňkách periferní krve je upregulován prostřednictvím mediátorů zánětu a indukuje expresi prozánětlivých cytokinů, jako IL-6, IL-8,

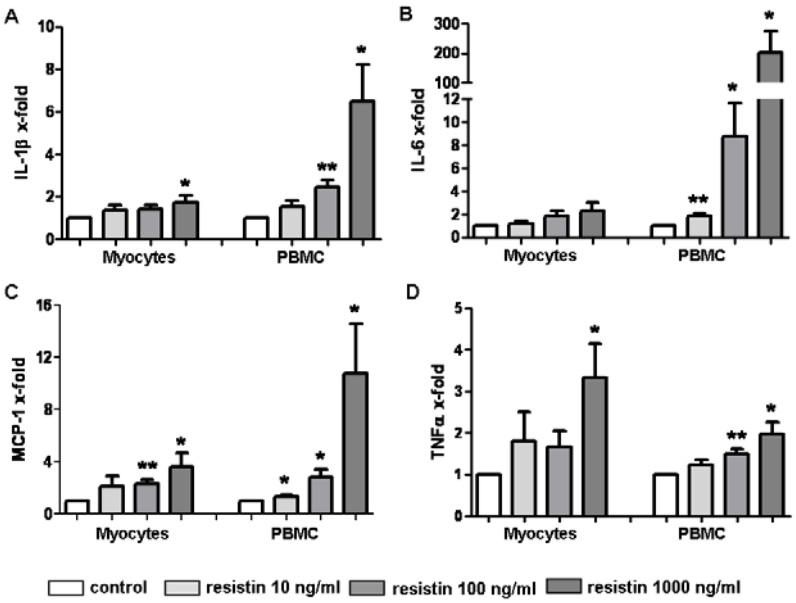
MCP-1 a TNF-α, angiogenních faktorů a extracelulárních metaloproteináz, z čehož lze usuzovat na jeho zapojení do patogeneze řady imunitně podmíněných onemocnění.

Cíle práce: Cílem studie bylo stanovit a porovnat sérové hladiny a lokální expresi resistinu u pacientů s idiopatickými zánětlivými myopatiemi (IIM) se zdravými kontrolami a dále objasnit vztah mezi hladinami resistinu, zánětem a aktivitou onemocnění.

Metody: Sérové hladiny resistinu jsme stanovili u 42 pacientů s IIM a u 27 zdravých kontrol, sledovali jsme expresi resistinu ve svalové tkáni pacientů a kontrol. Vyhodnotili jsme asociaci mezi hladinami resistinu, úrovní zánětu, celkovou aktivitou onemocnění a svalovou silou. *In vitro* jsme na genové a proteinové úrovni sledovali vliv stimulace monoukleárních buněk periferní krve a myocytů resistinem na expresi prozánětlivých cytokinů.

Výsledky: U pacientů s IIM jsme nalezli signifikantně vyšší hladiny sérového resistinu než u kontrol (8,53±6,84 vs. 4,54±1,08 ng/ml, p <0,0001) a tyto korelovaly s CRP (r=0,328, p=0,044) a s vizuální analogovou škálou hodnocení aktivity myositidy (MYOACT) (r=0,382, p=0,026). U anti-Jo-1 pozitivních pacientů jsme pozorovali silnější asociaci hladin resistinu s CRP (r=0,717, p=0,037), tak s hodnotami MYOACT (r=0,798, p=0,0007) a také jsme pozorovali trend ke korelaci s hladinami myoglobinu. U pacientů s dermatomyositidou signifikantně korelovaly sérové hladiny resistinu s MYOACT (r=0,667, p=0,001), kreatinkinázou (r=0,739, p=0,001) a hladinami myoglobinu (r=0,791, p=0,0003) a vykazovaly trend ke korelaci s CRP. Exprese resistinu ve svalové tkáni byla signifikantně vyšší u pacientů s IIM v porovnání s kontrolami. V mononukleárních buňkách indukovala stimulace resistinem expresi interleukinů (IL)-1β a IL-6 a chemokinu MCP-1. Tento efekt jsme nepozorovali u myocytů (Obrázek 4).

Obrázek 4. Efekt resistinu na zánětlivou odpověď u myocytů a PBMC *in vitro*.



Exprese mRNA IL-1α (A), IL-6 (B), MCP-1 (C) a TNF-α (D) po stimulaci lidským resistinem (10, 100, 1000 ng/ml) po šesti hodinách v myocytech a mononukleárních buňkách periferní krve (PBMC). Data jsou prezentována jako násobky změny ve srovnání s nestimulovanými kontrolami (bílý sloupec).
* $p<0,05$, ** $p<0,01$

Souhrn: Výsledky této studie poukazují na to, že vyšší hladiny resistinu jsou idiopatických zánětlivých myopatií asociované se zánětem, vyšším indexem celkové aktivity onemocnění a svalového poškození u pacientů s přítomností anti-Jo-1 autoprotilátek a u pacientů s dermatomyositidou. Upregulace resistinu ve svalové tkáni a resistinem indukovaná sekrece prozánětlivých cytokinů v mononukleárních buňkách poukazuje na potenciální roli resistinu v patogenezi IIM.

Práce byla publikována v časopise Arthritis Research & Therapy a její kompletní znění je přiloženo v anglickém jazyce (Příloha 4).

3.5. Sérové hladiny interferonu α u pacientů s dermatomyositidou/ polymyositidou nekorelují s aktivitou onemocnění

Úvod: Interferony typu I představují skupinu prozánětlivých cytokinů (IFN-α, -β, -ε, -κ, -τ, -δ, -ζ, -ω a –ν). U pacientů s idiopatickými zánětlivými myopatiemi (idiopathic inflammatory myopathy, IIM) byla popsána zvýšená exprese skupin genů indukovatelných IFNα (tzv. type-I-interferon signature) a u interferonů typu I je předpokládána účast na na prozánětlivých procesech vedoucch k manifestaci svalového onemocnění u pacientů s IIM.

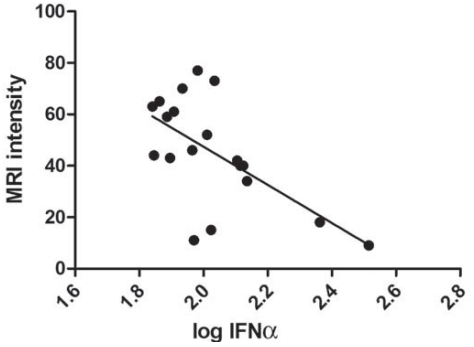
Cíle práce: Cílem práce bylo vyšetřit sérové hladiny solubilního INFα a porovnat je s klinickými a laboratorními charakteristikami a s aktivitou onemocnění u pacientů s IIM.

Metody: Sérové hladiny IFNα jsme stanovili u 24 pacientů s dermatomyositidou (DM) a 19 s polymyositidou (PM) metodou mikročásticové eseje), pacienti byli vybráni z kohorty 81 PM/DM pacientů s preferencí anti-Jo-1 pozitivních a těch, kteří měli provedenou magnetickou rezonanci (MRI) stehenních svalů. Klinická a laboratorní data a údaje o léčbě glukokortikoidy jsme získali ze zdravotnické dokumentace, aktivita onemocnění byla hodnocena jednak pomocí skóre MYOACT, založeném na visuální analogové škále a dále dle nálezu na MRI. Kontrolní skupinu představovalo 25 zdravých jedinců a dále 6 pacientů s akutní virovou infekcí.

Výsledky: Sérové hladiny IFNα byly signifikantně nižší u pacientů s IIM než u zdravých i pozitivních kontrol (p=<0,01) (Obrázek 5); nepozorovali jsme závislost na denní dávce glukokortikoidů. Pacienti s pozitivitou anti-Jo-1 autoprotilátek měli hladiny signifikantně vyšší oproti negativním pacientům (n=26, medián 117,8pg/ml oproti n=19, medián 93,4pg/ml; p=0,05). Neprokázali jsme rozdíl mezi pacienty s PM a DM. U pacientů s IIM sérové hladiny

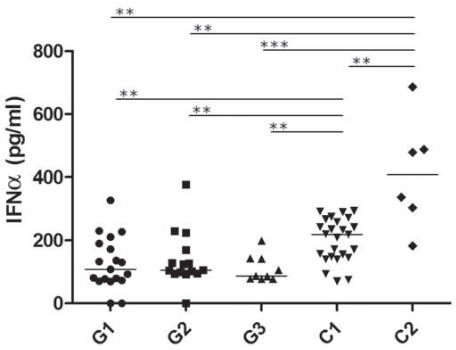
IFNα signifikantně negativně korelovaly s edémem svalů, vyjádřeném jako parametr intenzity signálu na MRI.

Obrázek 5. Vztah sérových hladin IFN-α a aktivity na MRI u pacientů s IIM.



Spearmanova korelace: sr= -0,58, p=0,0095

Obrázek 6. Sérové hladiny IFN-α v závislosti na léčbě.



G1 – pacienti před léčbou; G2 – léčení ≤20mg prednisonu denně; G3 léčení ≥20mg prednisounu denně; C1- zdravé kontroly; C2 kontrolní pacienti s akutní virovou infekcí. **p<0,01, ***p<0,001

Závěr: Interferon α je prozánětlivý cytokin s předpokládanou úlohou v patogenezi IIM, což potvrzuje nález vyšších hladin u pacentů s anti-Jo-1 autoprotilátkami, nicméně naše výsledky překvapivě ukazují nižší sérové hodnoty u pacientů v porovnání se zdravými kontrolami a neodrážejí tak dříve popsaný vyšší obsah IFNα ve svalové tkáni. Tento nález není důsledkem léčby glukokortikoidy, neboť jsme nepozorovali rozdíly v závislosti na jejich užívání (Obrázek 6). Sérový IFNα také nepředstavuje marker aktivity onemocnění, neboť jeho koncentrace negativně koreluje s nálezem na MRI. Možným vysvětlením může být předpoklad, že lokálně produkovaný IFNα je spotřebován v místě zánětu a není uvolňován do systémové cirkulace, eventuálně že signatura interferonů typu I, která je charakteristická pro pacienty s DM a PM, je způsobena jiným zástupcem než IFNα.

Práce byla publikována v časopise Annals of Rheumatic Diseases a její kompletní znění je přiloženo v anglickém jazyce (Příloha 5).

3.6. Autoprotilátkové specificity a aktivace dráhy interferonu typu I u idiopatických zánětlivých myopatií.

Úvod: U řady autoimunitních nemocí byla popsána aktivace signální dráhy interferonu typu I a existují i indicie, že je tomu tak i u myositid, zvláště u dermatomyositidy, nicméně mechanismus této aktivace není dosud znám. IFN I typu je u systémového lupus erytematodes (SLE) zvýšeně produkován periferními dendritickými buňkami. Séra pacientů s myositidami (pozitivní na autoprotilátky anti-Jo-1 nebo anti-Ro52/60) jsou schopny indukovat produkci IFN v dendritických buňkách. Tyto autoprotilátky by mohly představovat endogenní induktory produkce IFN.

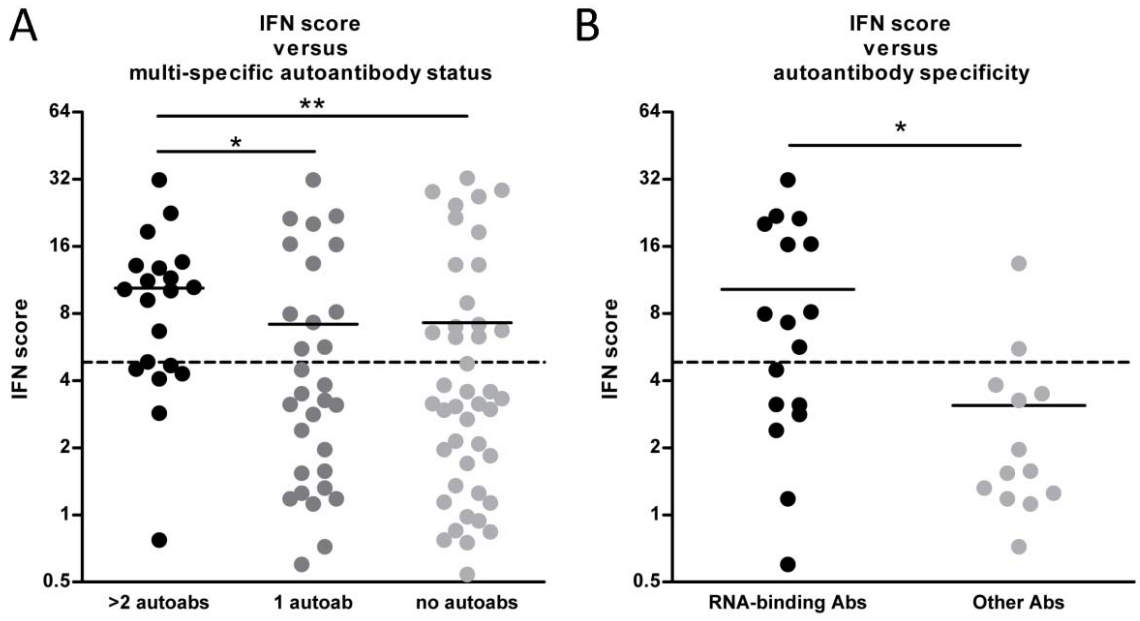
Cíle práce: Cílem této práce bylo ověřit, zda autoprotilátkový profil u myositických pacientů, respektive přítomnost protilátek proti RNA-vázajícím proteinům (anti-Jo-1, anti-Ro52, anti U1RNP), je asociován signaturou aktivace interferonu typu I (type-I-interferon signature) a tedy i potenciálně indukovat produkci IFN.

Metody: V této prospektivní studii jsme porovnávali séra 92 myositických pacientů se séry pacientů se SLE a zdravých kontrol. Analyzovali jsme autoprotilátkový status pomocí imunoesejů a imunoprecipitace. Aktivita IFN typu I jsme stanovovali v plné krvi za použití přímé analýzy genové exprese inducibilních genů, z jejich průměrné exprese pak bylo stanoveno IFN skóre a pacienti byli dle cut-off hodnoty rozděleni na skupiny s vysokým a s nízkým skórem. IFN typ-I inducibilní aktivitu séra jsme zjišťovali stimulací buněk periferní krve od zdravých kontrol. Blokovací experimenty jsme prováděli prostřednictvím neutralizačních protilátek proti interferonovému receptoru a proti interferonu α.

Výsledky: IFN aktivita byla signifikantně vyšší u pacientů s myositidou než u zdravých kontrol ($p=0,0007$). Pacienti s pozitivitou autoprotilátek proti RNA-vázajícím proteinům měli

vyšší IFN skóre než pacienti pro tyto protilátky negativní (p=0,011); toto skóre bylo také asociované s autoprotilátkovou multispecificitou (p=0,038) (Obrázek 7). Vyšší IFN skóre jsme nalezli u pacientů s dermatomyositidou a myositidou s inklusními tělísky v porovnání s polymyositidou (p=0,04 a p=0,04). V porovnání s pacienty s nízkým IFN skóre vykazovala séra pacientů s vyšším IFN skórem signifikantně vyšší schopnost aktivace exprese IFN typ-I indukovatelných genů v dendritických buňkách; u těchto sér byla tato schopnost významně inhibovatelná přidáním jak protilátek proti IFN receptoru (p=0,016), tak proti IFN-α (p=0,0095). IFN skóre pouze mírně korelovalo s aktivitou onemocnění hodnocenou lékařem a to pouze u pacientů s dermatomyositidou (r=0,3778, p=0,03). Nepozorovali jsme žádnou asociaci IFN signatury v séru s HLA-DRB1 genotypem.

Obrázek 7. IFN skóre ve vztahu k autoprotilátkové aktivitě.



Exprese 8 zájmových genů v závislosti na počtu pozivních autoprotilátek (A) a na přítomnosti RNA-vázajících autoprotilátek (B). *p<0,05; **p<0,001.

Závěr: V této práci prokazujeme asociaci mezi signaturou IFN typu I a pozitivitou autoprotilátek proti RNA-vázajícím proteinům a to nejen u dermatomyositid, ale i u dalších

podtypů idiopatických zánětlivých myopatií. Tato asociace je významnější u pacientů s autoprotilátkovou multispecificitou. Prokazujeme však pouze mírnou asociaci aktivace dráhy IFN typu I s aktivitou onemocnění a to pouze u pacientů s dermatomyositidou.

Práce byla přijata k publikaci v časopise Scandinavian Journal of Immunology a její kompletní znění je přiloženo v anglickém jazyce (Příloha 6).

3.7. Mikroaray-analýza cirkulující mikro RNA v sérech pacientů s polymyositidou a dermatomyositidou vykazuje odlišný expresní profil a je asociován s aktivitou onemocnění

Úvod: Mikro RNA (miRNA) jsou malé nekódující RNA molekuly, které ovlivněním stability a translační účinnosti postranskripčně regulující genovou expresi. mi-RNA expresní profily jsou tkánově a buněčně specifické a podléhají přísně kontrole. Odlišné expresní vzorce jsou zavzaty v patogenezi široké řady chorob včetně systémovým autoimunitních onemocnění. Byly popsány změny exprese určitých miRNA u některých svalových dystrofií a u pacientů s chronickou obstrukční plicní nemocí, kteří mívají redukovanou svalovou hmotu. Dosud nebyly provedeny studie popisující celkové expresní profily u pacientů s idiopatickými zánětlivými myopatiem (idiopathic inflammatory myopahties, IIM).

Cíle práce: Cílem práce bylo vyšetření cirkulujících mikro RNA (miRNA) asociovaných s IIM a dále určení exprese kandidátních molekul ve vztahu ke klinické aktivitě myositid.

Metody: Ze sér 28 pacientů s IIM, 16 zdravých kontrol a 16 kontrolních pacientů se systémovým lupus erytematodes (SLE) jsme izolovali celkovou RNA, obsahující též miRNA. Exprese miRNA jsme určili pomocí miRNA microaraye. Celkovou aktivitu onemocnění jsme hodnotili pomocí visuální analogové škály.

Výsledky: Identifikovali jsme 23 signifikantně odlišně exprimovaných miRNA. 6 miRNA mělo odlišnou expresi ve srovnání se zdravými kontrolami. Oproti zdravým kontrolám jsme u dermatomyositidy (DM) identifikovali 3 a u polymyositidy (PM) pak 6 odlišně exprimovaných miRNA. U pacientů s vysokou aktivitou nemoci jsme pozorovali vyšší expresi 3 miRNA oproti pacientům s nízkou aktivitou. Dále jsme zaznamenali signifikantně rozdílnou expresi 26 miRNA u pacientů se SLE ve srovnání s pacienty s IIM, PM a DM.

Závěr: Tato práce představuje první studii, která komplexně popisuje úrovně exprese cirkulujících miRNA v sérech pacientů s IIM. Lze předpokládat, že některé z těchto rozdílně exprimovaných miRNA jsou zapojeny do etiologie IIM a mají potenciál sloužit jako molekulární markery pro rozvoj IIM a monitoraci aktivity onemocnění.

Práce byla publikována v časopise Clinical Experimental Rheumatology a její kompletní znění je přiloženo v anglickém jazyce (Příloha 7).

4. Diskuze

Tato práce byla zaměřena na studium vztahu jednotlivých cirkulujících solubilních molekul imunitního systému, zejména autoprotilátek, prozánětlivých cytokinů a regulačních faktorů, k fenotypovým znakům idiopatických zánětlivých myopatií, expresi těchto molekul přímo ve svalové tkáni a na zhodnocení možného vlivu na buňky periferní krve i samotné svalové buňky a tím jejich významu v patogenezi IIM.

U autoprotilátek asociovaných s myositidami nebo specifických pro myositidy jsme prokázali silnou asociaci autoprotilátek anti-Jo-1 s kloubním postižením, respektive artritidou u pacientů s IIM; toto postižení má charakteristickou distribuci drobných ručních kloubů. Artritida nebo kloubní postižení je poměrně často vídaným příznakem IIM, nicméně souhrnná data na tuto problematiku jsou poměrně chudá (Citera, G. et al., 1994). Artritida je relativně častým příznakem u pacientů s autoprotilátkami proti tRNA syntetázám (Katzap, E. et al., 2011, Meyer, O. et al., 2009, Queiro-Silva, R. et al., 2001, Targoff, I.N., 1994). Naše výsledky ale ukazují výrazně silnější asociaci, než bylo v předchozích studiích naznačeno (Martinez-Cordero, E. et al., 2001, Nakajima, A. et al., 2012). Tato studie je dle našich poznatků první prací systematicky analyzující kloubní postižení u pacientů s IIM.

Také jsme potvrdili vztah autoprotilátek anti-HMGCR k imunitně zprostředkované nekrotizující myopatii (Mammen, A.L. et al., 2011) a popsali výrazný vzestup incidence této formy IIM v posledních letech. Dle našich dat tento nárůst připadá na vrub statiny indukované IMNM, přičemž spotřeba statinů v ČR setrvale narůstá. Většina našich pacientů se statinovou myopatií užívala atorvastatin, což ovšem může souviset s tím, že tento preparát je i nejčastěji předepisovaným statinem v ČR (SÚKL 2014 [A], SÚKL 2014 [B]).

Identifikovali jsme odlišně exprimované sérové miRNA u pacientů s PM a DM. Odlišná exprese cirkulujících miRNA byla popsána u jiných autoimunitních onemocnění (Qu, Z. et

al., 2014), zejména u SLE (Wang, G. et al., 2010). Většina z námi identifikovaných miRNA byla již studována ve vztahu k různým onemocněním, včetně nádorových, kardiovaskulárních atd., nicméně zatím žádná z nich nebyla spojována s autoimunitními chorobami. Vztah miRNA s IIM, a to převážně s DM, byl popsán u mi-R21 (Shimada, S. et al., 2013) a mi-R7 (Oshikawa, Y. et al., 2013), nicméně v našich výsledcích jsme tento vztah nepotvrdili.

Z prozánětlivých cytokinů jsme se zaměřili na skupinu interferonu typu I. IFN typu I je v řadě studií popsán jako významný faktor patogeneze onemocnění: IFN typu I jsou silnými induktory exprese MHC molekul I a II třídy (Tournadre, A. et al., 2012), v periferních leukocytech i svalové tkáni myositických pacientů byly nalezeny zvýšené exprese genů indukovatelných interferonem typu I (tzv. IFN I signature) (Walsh, R.J. et al., 2007). Obdobně byla prokázána i zvýšená exprese proteinů indukovaných IFN typu I: protein rezistence proti myxoviru A (MxA) v kožních (Wenzel, J. et al., 2005) a svalových (Greenberg, S.A. et al., 2005) biopsiích pacientů s DM, kde byl specificky zvýšen v místech aktivního zánětu a atrofizace svalových vláken (Salajegheh, M. et al., 2010). Překvapivým zjištěním je proto náš průkaz významné negativní korelace hladin IFNα a míry postižení svalů na magnetické rezonanci. Naproti tomu aktivita IFN-typu I, vyjádřená úrovní exprese IFN-typem-I indukovatelných genů korelovala s klinickou aktivitou onemocnění u pacientů s DM, avšak v kontrastu s tím jsme nepozorovali vztah klinické aktivity s hladinami IFNα s výjimkou trendu k asociaci s plicním postižením. Hladiny IFNα byly vyšší u anti-Jo-1 pozitivních pacientů a aktivita IFN-typu I byla též vyšší u pacientů s autoprotilátkovou multiplicitou, což je v souladu s tím, že imunokomplexy obsahující antisyntetázové protilátky (např. anti-Jo-1) mohou jako endogenní stimulační faktor indukovat produkci IFN-α v plasmocytoidních dendritických buňkách (Eloranta, M.L. et al., 2009).

Z dalších prozánětlivých cytokinů jsme prokázali asociaci klinické aktivity s hladinami resistinu, které byly u pacientů s DM i asociované s biochemickými ukazateli aktivity

onemocnění. Jakkoli byl resistin původně asociován s metabolickými onemocněními, byly jeho zvýšené hladiny zaznamenány u revmatoidní artritidy (Bokarewa, M. et al., 2005, Migita, K. et al., 2006). Vzhledem k tomu, že jsme neprokázali vztah resistinu a svalové slabosti, je možné, že vyšší hladiny mohou spíše nespecificky odrážet celkovou aktivitu nemoci, včetně extramuskulárního postižení, než funkční postižení u IIM. Obdobně jako v případě resistinu, je naše práce první, která popisuje pozitivní korelaci visfatinu s klinickou aktivitou u pacientů s anti-Jo-1 pozitivitou. V dřívějších studiích byl též prokázán jeho vztah s řadou onemocnění (kardiovaskulární, metabolická, zánětlivá, nádorová) (Nowell, M. et al., 2012) a jeho hladiny taktéž korelují s aktivitou a progresí revmatoidní artritidy a ankylozující spondylartritidy (Brentano, F. et al., 2007, Rho, Y.H. et al., 2009). Poněkud překvapivě se však neliší od zdravých kontrol u systémového lupusu a sklerodermie (Masui, Y. et al., 2013, Ozgen, M. et al., 2011, Vadacca, M. et al., 2009). Ve svalové tkáni pacientů s IIM jsme v porovnání se zdravými kontrolami detekovali zvýšené hladiny exprese resistinu a visfatinu.

Dle experimentálních výsledků naší studie mohou molekuly imunitního systému, cirkulující v sérech pacientů s IIM, hrát roli v patogenezi tohoto onemocnění. Prokázali jsme, že séra pacientů s IIM jsou schopna aktivovat dráhu interferonu typu I v buňkách periferní krve, za tuto aktivaci zodpovídá IFN-typu I, konkrétně především IFNα. V naší studii jsme též prokázali schopnost resistinu indukovat expresi dalších prozánětlivých cytokinů – interleukinů IL-1β a IL-6 a monocytárního chemoatraktivního proteinu MCP-1 v mononukleárních buňkách periferní krve; tento efekt jsme však neprokázali na myocytech.

5. Závěr

Tato práce byla zaměřena na studium vztahu jednotlivých cirkulujících solubilních molekul imunitního systému, zejména autoprotilátek, prozánětlivých cytokinů a regulačních faktorů, k fenotypovým znakům idiopatických zánětlivých myopatií, expresi těchto molekul přímo ve svalové tkáni a na zhodnocení možného vlivu na buňky periferní krve i samotné svalové buňky a tím jejich významu v patogenezi IIM.

Prokázali jsme silnou asociaci autoprotilátek anti-Jo-1 s kloubním postižením popsali jsme charakteristickou distribuci na drobných ručních kloubech. Tato studie je dle našich poznatků první prací systematicky analyzující kloubní postižení u pacientů s IIM. Dále jsme potvrdili vztah autoprotilátek anti-HMGCR k imunitně zprostředkované nekrotizující myopatii a výrazný vzestup incidence této formy IIM v posledních letech. Dle našich dat tento nárůst připadá na vrub statiny indukované IMNM. Mimo to jsme identifikovali odlišně exprimované sérové miRNA u pacientů s PM a DM.

Z prozánětlivých cytokinů jsme se zaměřili zejména na skupinu interferonu typu I. Překvapivě jsme pozorovali významnou negativní korelaci hladin IFN-α s mírou postižení svalů na magnetické rezonanci. Exprese IFN-typem-I indukovatelných genů korelovala s klinickou aktivitou onemocnění u pacientů s DM, avšak v kontrastu s tím jsme nepozorovali vztah klinické aktivity s hladinami IFNα. Sérové hladiny IFNα pak byly vyšší u pacientů s anti-Jo-1 autoprotilátkami a u pacientů s autoprotilátkovou multiplicitou jsme pozorovali zvýšenou aktivitu IFN-typu I. Z dalších prozánětlivých cytokinů jsme prokázali asociaci klinické aktivity s hladinami resistinu, které byly u pacientů s DM i asociované s biochemickými ukazateli aktivity onemocnění. Obdobně hladiny visfatinu pozitivně korelují s klinickou aktivitou u pacientů s anti-Jo-1 pozitivitou. Jak visfatin tak resistin se též zvýšeně exprimují ve svalové tkáni pacientů s IIM. Dále jsme prokázali schopnost resistinu indukovat

expresi dalších prozánětlivých cytokinů – interleukinů IL-1β a IL-6 a monocytárního chemoatraktivnícho proteinu MCP-1 v mononukleárních buňkách periferní krve; tento efekt se nám však nepodařilo prokázat na myocytech

Uvedené výsledky prokazují vztah jednotlivých molekul imunitního systému ke konkrétním podtypům či fenotypovým projevům idiopatických zánětlivých myopatií a demonstrují význam solubilních faktorů imunitního systému v patogenezi tohoto onemocnění.

Heterogenita myositid je více než v klinickém obraze vyjádřena v jejich patogenetických mechanismech a odráží se v imunofenotypové odpovědi u jednotlivých podtypů, což může být s výhodou použito v diagnostice a v budoucnu i v terapii těchto onemocnění.

6. Vlastní publikace autora

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Práce byla oceněna Cenou České revmatologické společnosti JEP za nejlepší publikaci za rok 2013 v kategorii do 35 let.

7. Seznam použitých zkratek

ADSF – adipocyty sekretovaný faktor

ATP – adenosintrifostfát

BAFF – B-cell activating factor

CAM – myositida asociovaná s nádorem, (cancer associated myositis)

CD – cluster of differentiation

CK, sCK – kreatinkináza, sérová kreatinkináza

cN1A – cytosolická 5'nukleotidáza 1A

CRP – C-reaktivní protein

DM – dermatomyositida

ELISA – enzyme-linked immunosorbent assay

ER – endoplazmatické retikulum

GWAS – genom-wide association study

HLA – human leukocyte antigen

HMGCR – 3-hydroxy-3-methylglutaryl koentym A reduktáza

IBM – myositida s inkluzními tělísky (inclusion body myositis)

IFN – interferon

IIM – idiopatické zánětlivé myopatie, (idiopathic inflammatory myopathies)

IL – interleukin

IMNM – imunitně zprostředkovaná nekrotizující myopatie, (immune mediated necrotizing myopathy)

JDM – juventilní dermatomyositida

LDH – laktát dehydrogenáza

MAA – autoprotilátky asociované s myositidou, (myositis-associated antibodies)

Mb – myoglobin

MCP-1 – monocyte chemoattractant protein-1

MCTD – smíšené onemocnění pojiva, (mixed cennective tissue diseae)

MDA5 – melanoma differentiation-associated protein 5

MHC – hlavní histokompatibliní komplex

miRNA – mikro ribonukleová kyselina

MRI – magnetická rezonance

mRNA – mediátorová ribonukleová kyselina

MSA – autoprotilátky specifické pro myositidu, (myositis-specific antibodies)

MxA – protein rezistence proti myxoviru A

MYOACT – myositis disease activity assessment visual analogue scale

N-SM – non specific myositis

NC – non classifiable

NF-κB – nukleární receptor NF kappa B

NXP2 – nukleární matrixový protein 2

PBEF – pre-B-cel colony-enhancing factor

PM – polymyositida

RA – revmatoidní artritida

SAE – small ubiquitin-like modifier activating enzyme

SjS – Sjögrenův syndrom

SLE – systémový lupus erytematodes

SRP – signal recognition particle

SSc – systémová sklerodermie

TCR – T-lymfocytární receptor, (T-cell receptor)

TGF-β – transforming growth factor β

TIF1 – transkripční intermediární faktor 1

TNF-α – tumor nekrotizující faktor alfa

tRNA – transferová ribonukleová kyselina

UPR – unfolded protein response

UV – ultrafialové záření

VAS – vizuální analogová škála

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9. Přílohy

Součástí přílohy jsou plná znění publikací, které jsou podkladem disertační práce.

Seznam příloh:

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Příloha 1

Arthritis in Idiopathic Inflammatory Myopathy: Clinical Features and Autoantibody Associations

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ABSTRACT. ***Objective.*** To determine the prevalence, distribution, and clinical manifestations of arthritis in a cohort of patients with idiopathic inflammatory myopathies (IIM). Associations with autoantibody status and HLA genetic background were also explored.

Methods. Consecutive patients with IIM treated in a single center were included in this cross-sectional study (n = 106). History of arthritis, 68-joint and 66-joint tender and swollen joint index, clinical features of IIM, and autoantibody profiles were obtained by clinical examination, personal interview, and review of patient records. High-resolution genotyping in HLA-DRB1 and HLA-DQB1 loci was performed in 71 and 73 patients, respectively.

Results. A combination of patients' medical history and cross-sectional physical examination revealed that arthritis at any time during the disease course had occurred in 56 patients (53%). It was present at the beginning of the disease in 39 patients (37%) including 23 cases (22%) with arthritis preceding the onset of muscle weakness. On physical examination, 29% of patients had at least 1 swollen joint. The most frequently affected areas were wrists, and metacarpophalangeal and proximal interphalangeal joints. Twenty-seven out of the 29 anti-Jo1-positive patients had arthritis at any time during the course of their illness; this prevalence was significantly higher compared to patients without the anti-Jo1 autoantibody ($p < 0.0001$). No association of arthritis with individual HLA alleles was found.

Conclusion. Our data suggest that arthritis is a common feature of myositis. It is frequently present at the onset of disease and it may even precede muscular manifestations of IIM. The most common presentation is a symmetrical, nonerosive polyarthritis affecting particularly the wrists, shoulders, and small joints of the hands. We have confirmed a strong association of arthritis with the presence of the anti-Jo1 antibody. (First Release May 1 2014; J Rheumatol 2014;41:1133–9; doi:10.3899/jrheum.131223)

Key Indexing Terms:
IDIOPATHIC INFLAMMATORY MYOPATHIES ARTHRITIS AUTOANTIBODIES

Idiopathic inflammatory myopathies (IIM) represent a group of systemic autoimmune disorders characterized by a nonsuppurative inflammation of skeletal muscles as the major manifestation. Distinct subgroups of IIM with variable clinical and laboratory manifestations are recognized, such as polymyositis (PM), dermatomyositis (DM), juvenile dermatomyositis, cancer-associated myositis, immune-mediated necrotizing myopathy, and inclusion body myositis (IBM).

Arthritis is commonly seen in patients with IIM; however, comprehensive data on its presentation and on manifestations in individual myositis subgroups are scarce[1]. Arthritis is particularly frequent in patients with autoantibodies directed against tRNA synthetases as part of the antisynthetase syndrome[2,3,4,5,6], but it is not limited to this subgroup[7,8]. Arthritis and/or arthralgias were reported in 33% of patients with IIM in a large multicenter Japanese cohort used for a formulation of new classification criteria, in which arthritis was included[9]. Other available information originates from case reports[10,11,12,13] or small cohorts selectively defined by the presence of a specific autoantibody or antisynthetase syndrome[5,14,15,16]. Arthritis in patients with IIM is considered less severe and less destructive when compared to the joint involvement in rheumatoid arthritis (RA), but the few available reports provide conflicting results on this aspect[5,9,10,11,12,13,14,15,16]. The degree of reported joint involvement in myositis varies from nonerosive arthritis[12] and subluxing arthropathy[5] to erosive and destructive arthritis[10,13,14].

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The aim of our study was to provide comprehensive data regarding joint involvement in unselected patients with IIM from a single center. Specifically, we determined the prevalence of arthritis in patients with IIM, its relation to the course of the muscle disease, characteristics of arthritis such as distribution and extent, as well as its association with autoantibody profiles and HLA allelic polymorphisms.

MATERIALS AND METHODS

Patients and controls. All patients with IIM fulfilling diagnostic criteria seen both at the outpatient and inpatient departments of the Institute of Rheumatology between January and September 2012 were recruited into the study. The diagnosis of PM and DM was based on the criteria of Bohan and Peter[17,18], necrotizing myopathy and amyopathic DM were diagnosed using the European Neuromuscular Centre Workshop (ENMC) criteria[19], and the diagnosis of IBM was established using the Griggs criteria[20]. All patients had a muscle biopsy performed during the course of their disease; however, the full description of the findings required for classification according to the ENMC criteria was available only for the 76 biopsies performed after 2004. The control group for the genetic substudy consisted of 179 healthy subjects. The study was approved by the ethics committee at the Institute of Rheumatology and all patients gave informed consent.

Clinical data. Basic demographic and clinical data including the history of IIM onset, disease course, smoking history, and presence of lung involvement (defined as a presence of active alveolitis or fibrotic changes on radiograph or high-resolution computed tomography scan, and/or DLCO < 70%) were obtained from all patients. Information regarding presence or absence of arthritis in the past and/or at the current time with respect to the onset, localization, and symmetry as well as other features of joint involvement such as presence of joint deformities ("floppy thumb") was obtained during personal interviews with patients and/or from medical records. Activity of arthritis and the degree of joint involvement were assessed by both patients and physicians using visual analog scales (VAS). A semiquantitative scale was used to evaluate the severity of joint involvement as a proportion of total morbidity. History of arthritis was established if the patient during personal interview reported having at least 1 painful and swollen joint in the past or if the presence of inflammatory arthritis detected by an experienced rheumatologist was recorded in the medical records. Current arthritis was defined as a presence of at least 1 swollen joint on physical examination using the 68/66-joint count. Radiographs of the joints of hands and/or feet were available from 47 patients.

Autoantibodies. Autoantibody profiles of patients with IIM were determined during routine diagnostic examination using indirect immunofluorescence to screen for antinuclear antibodies (ANA) and anti-dsDNA (Immuno Concepts), line immunoassay (Imtec Human), and Western blot-myositis (Euroimmun) for detection of individual autoantibodies directed against Jo-1, Mi-2, Ku, PM-Scl, PM-Scl75, PM-Scl100, PL-7, PL-12, EJ, OJ, SRP, Ro, Ro52, La, Scl-70, and U1-RNP antigens. In-house-made 35S radioimmunoprecipitation[21] was used to confirm the results and to detect autoantibodies not identified using commercial assays [against: transcriptional intermediary factor 1-γ (TIF1-γ), MDA5, NXP2, Zo, EIF, RNAP I (RNA polymerase antibodies), RNAP II, and RNAP III]. Rheumatoid factors (RF) were detected using a particle-agglutination assay (Fujirebio Inc.), and an ELISA test for anticyclic citrullinated peptide (anti-CCP; TestLine Clinical Diagnostics) was used to detect anticitrullinated protein antibodies (ACPA).

HLA typing. Allelic polymorphism of HLA-DRB1 and HLA-DQB1 genes was analyzed by DNA-based typing using commercial sets (OneLambda) according to manufacturer's instructions.

Statistics. Demographics, clinical characteristics, and results are presented as descriptive statistics. The continuous not-normally distributed variables were analyzed by Mann-Whitney test; categorical data were analyzed by Fisher's exact test having p values estimated by Monte Carlo simulations (n = 10,000), and Kaplan-Meier estimator was used for calculation of survival analysis of arthritis. The significance of differences in allele and gene frequencies was evaluated by Fisher's exact test. We used GraphPad Prism 5 (GraphPad Software), QuickCalcs online calculator (graph-pad.com), and R (r-project.org) for statistical analyses. P values < 0.05 were considered statistically significant.

RESULTS

Demographic and clinical data of patients. In total, 106 patients with IIM were included in the study. Basic demographic and clinical characteristics are summarized in Table 1.

Prevalence and characteristics of joint involvement. Combining patients' medical history and cross-sectional physical examination revealed that arthritis at any time during the disease course had occurred in 56 patients (52.8%; Table 2). Thirty-nine patients (36.8%) had arthritis at disease onset.

Thirty-one patients (29.2%) presented with at least 1 swollen joint at the time of cross-sectional evaluation. Nine additional patients had only joint tenderness, with no swelling. We did not find any difference in the prevalence of arthritis among individual IIM subgroups.

Table 1. Demographic and basic clinical data.

Patients, n	106
Sex; male/female	32/74
Age, yrs	
Mean ± SD	55.6 ± 14.1
Median (95% CI)	59 (52.8–58.3)
Diagnosis[a]	PM = 46[†] (43.4%)
	Definite/probable/possible 26/11/9
	DM = 40[§‡] (37.7%)
	Definite/probable/possible 35/3/2
	CAM = 8 (7.5%)
	IMNM = 11 (7.5%)
	IBM = 1 (0.9%)
Disease duration, yrs[b]	
Whole group	6.1 ± 6.3 (4.4, 4.9–7.3)
Arthritis patients	6.8 ± 5.7 (5.0, 5.3–8.3)[*]
Nonarthritis patients	5.3 ± 6.8 (3.0, 3.3–7.2)[*]
Lung involvement	37 (34.9%)
Ever smokers	40 (37.7%)

[a] Muscle biopsy evaluable according to the ENMC criteria was available in 76 patients: 25 patients satisfied biopsy criteria for PM, 28 for DM, 11 for IMNM, 1 for IBM; 9 biopsies did not have typical changes, and 2 were nonclassifiable, with significant pathologies, but not consistent with a single diagnostic category[19]. [†] Including 5 patients with overlap syndromes: 3 systemic sclerosis (SSc), 1 Sjögren syndrome (SS), and 1 rheumatoid arthritis. [§] Including 1 patient with clinically amyopathic dermatomyositis. [‡] Including 3 patients with overlap syndromes: 2 SSc and 1 SS. [b] Shown as mean ± SD (median, 95% CI). [*] A significant difference was found between disease duration in arthritis and nonarthritis patients (p = 0.04). ENMC: European Neuromuscular Centre Workshop; PM: polymyositis; DM: dermatomyositis; CAM: cancer-associated myositis; IMNM: immune-mediated necrotizing myopathy; IBM: inclusion body myositis.

Table 2. Arthritis in myositis subtypes. Data are n (%).

Diagnosis	Arthritis at Any Time*	Arthritis at Disease Onset**	Current Arthritis# (≥ 1 swollen joint)
PM (46)	27 (59)	19 (41)	17 (40)
DM (40)	22 (55)	15 (38)	11 (28)
CAM (8)	2 (25)	1 (13)	0 (0)
IMNM (11)	4 (36)	1 (13)	2 (18)
IBM (1)	1 (100)	1 (100)	1 (100)
Total (106)	56 (53)	39 (37)	31 (29)

*Combination of patient history and clinical examination; ** Based on patient history; # Arthritis present at the time of evaluation. PM: polymyositis; DM: dermatomyositis; CAM: cancer associated myositis; IMNM: immune mediated necrotizing myopathy; IBM: inclusion body myositis.

Probability of arthritis development. Patients with arthritis had significantly longer disease duration than those without arthritis (p = 0.04; Table 1). Patients who did not have arthritis at disease onset have a 65.6% (95% CI 57.1–75.2) overall probability of its future development and this probability gradually decreases to 33.9% (95% CI 23.6–48.7) after 10 arthritis-free years (Figure 1). Thus, the probability of having arthritis increases with the disease duration up to 66.1% (95% CI 51.3–76.4) after 10 years.

Arthritis at disease onset. Out of the 39 patients who had arthritis at the onset of IIM, joint symptoms preceded muscle weakness in 23 patients (59%) and occurred simultaneously in 16 (41%). Arthritis most commonly manifested as symmetrical polyarthritis in 33 cases (84.6%); oligoarthritis (involvement of 2–4 joints) and monoarthritis occurred in 5 (12.8%) and in 1 (2.6%) patient, respectively.

Current arthritis at the time of evaluation. Out of the 31 patients (29.2%) presenting with at least 1 swollen joint at the cross-sectional evaluation, 5, 14, and 12 patients had 1, 2–4, and more than 4 swollen joints, respectively. One patient had a newly diagnosed swollen joint at this examination for the first time.

Mean affected/tender/swollen joint counts in patients with arthritis were 8.3 ± 9.1, 8.4 ± 9.4, and 5.3 ± 5.4, respectively. Metacarpophalangeal (MCP) and proximal interphalangeal joints of the fingers and thumbs, wrists, and shoulders were the most frequently involved joints (Table 3).

Other forms of joint involvement. Deforming arthropathy was present in 15 patients (14.2%). Extreme lateral instability of the first interphalangeal joint ("floppy thumb") occurred in 5 patients (4.7%); 4 of them had anti-Jo1-positive PM, and 1 had anti-Mi-2-positive DM. Deformity in the first MCP was present in 3 patients (2.8%). All other deformities affected separate individual joints. Five patients (4.7%) had more than 1 joint deformity.

Radiographic characteristics of joint involvement. Radiographs of peripheral joints were available in 47 patients. Forty-six and 37 patients had radiographs of the hands and feet, respectively. Out of the 15 patients with clinically apparent deforming arthropathy, radiographs were available in 9 (60%). Joint erosions were present in 2 patients:

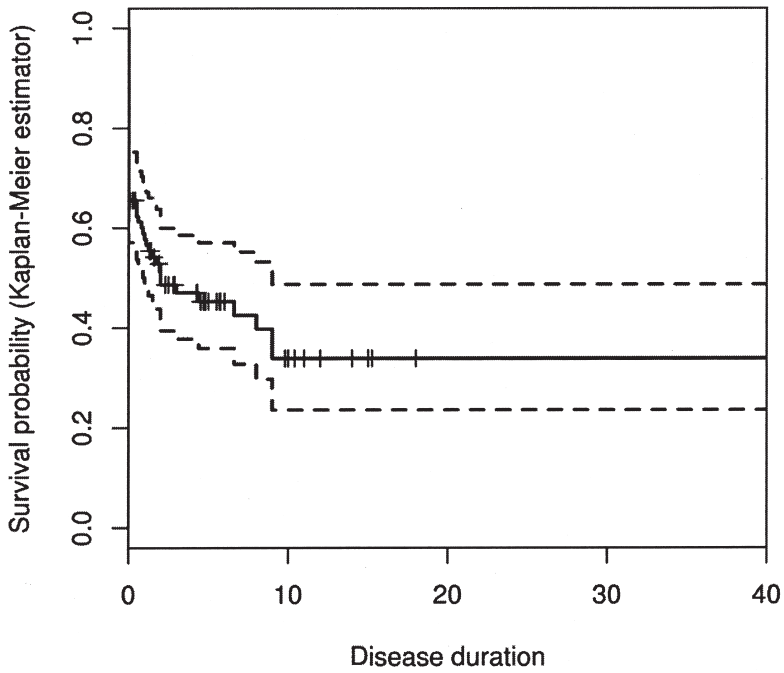

Figure 1. Probability of survival without arthritis. Probability of future development of arthritis in patients with arthritis-free survival (solid line) with 95% CI (dashed lines). Crosses (+) indicate censoring.

Table 3. Distribution of arthritis at the time of examination.

Joint	No. Patients with Affected Joints (%)		
	Tender	Swollen	Tender and/or Swollen
Temporomandibular	7 (6.6)	1 (0.9)	7 (6.6)
Sternoclavicular	7 (6.6)	2 (1.9)	7 (6.6)
Acromioclavicular	13 (12.3)	0	13 (12.3)
Shoulder	22 (20.8)	0	22 (20.8)
Elbow	14 (13.2)	5 (4.7)	14 (13.2)
Wrist	20 (18.9)	11 (10.4)	23 (21.7)
Metacarpophalangeal I-V	18 (16.9)	12 (11.3)	22 (20.8)
Proximal interphalangeal I-V	20 (18.9)	20 (18.9)	22 (20.8)
Distal interphalangeal II-V	6 (5.7)	5 (4.7)	7 (6.6)
Hip	4 (3.8)	—	4 (3.8)
Knee	9 (8.5)	3 (2.8)	10 (9.4)
Ankle	12 (11.3)	6 (5.7)	13 (12.3)
Tarsal joint	11 (10.4)	3 (2.8)	13 (12.3)
Metatarsophalangeal I-V	11 (10.4)	1 (0.9)	11 (10.4)
Interphalangeal I-V	2 (1.9)	0	2 (1.9)
Patients with at least 1 joint affected	45 (42.5)	31 (29.2)	52 (49)

destructive arthritis of carpal joints in a patient with an overlap of PM with RA with a positivity of RF and anti-CCP autoantibodies; and destructive arthritis of the second and third metatarsophalangeal joints in 1 patient who was anti-Jo1-positive for DM.

Clinical aspects of IIM with arthritis. Myositis relapsed in 31 patients (29.2%) at any time during the course of the illness. Arthritis was a feature of the relapse in 15 patients (48.4%) and occurred most frequently concurrently with the myositis flare (8 cases; 53.3%) or shortly before or after the relapse of other myositis symptoms in 4 and 2 patients, respectively; temporal pattern was not specified in 1 patient. The most common manifestation of arthritis during IIM relapses was polyarthritis or oligoarthritis in 7 and 6 patients, respectively (46.7 and 40%); detailed data on the number of affected joints were not available in 2 individuals.

Arthritis was present at disease onset in 13 out of 31 patients (41.9%) who later relapsed and in 29 out of 75 (38.7%) who did not. Thus, the presence of arthritis at the early phase of the disease is not predictive of future myositis relapses ($p = 0.8$). Similarly, the presence of arthritis at disease onset does not predict whether the relapse will be associated with arthritis ($p = 0.16$).

Clinical relevance of arthritis. Arthritis activity and joint damage was assessed using a VAS by both the patient and the evaluating physician. The mean arthritis activity and joint damage scores were relatively low in the whole group owing to a significant proportion of unaffected individuals. However, in patients with joint involvement, both mean activity of arthritis and joint damage were considered to be moderate (Table 4). Joint disease activity was rated higher by patients than by physicians ($p = 0.01$). When joint disease was considered as a proportion of total morbidity on a semiquantitative scale, 66 patients (62.3%) felt that arthritis did not play any role in the disease burden. Contribution of arthritis to the overall morbidity was reported to be small by 24 patients (22.6%), medium by 9 (8.5%), and large by 7 (6.6%).

Table 4. Arthritis activity and damage. Data shown as mean ± SD (n).

	All Patients	Patients with VAS > 0
MD activity	7.5 ± 15.8	19.4 ± 20.5 (41)
MD damage	6.2 ± 15.6	21.2 ± 22.8 (31)
Pt activity	14.0 ± 21.5	26.5 ± 23.4 (56)

MD/Pt: Physician's/patient's assessment of activity/damage on visual analog scale (VAS; 100 mm).

Autoantibodies. Myositis-specific and myositis-associated autoantibodies were tested in all patients and most patients were also evaluated for the presence of additional autoantibodies associated with other rheumatic diseases with frequent joint involvement (i.e., RA, systemic lupus erythematosus, Sjögren syndrome, and systemic sclerosis). Autoantibodies were found in 87 patients (82.1%). Not surprisingly, a strong association of anti-Jo1 antibodies with arthritis was confirmed, with 27 out of 29 anti-Jo1-positive patients (93%) having arthritis at some point during the course of IIM. The incidence of arthritis among anti-Jo1-positive patients was significantly higher compared to the anti-Jo1-negative subjects ($p < 0.0001$). No significant association between arthritis and autoantibody positivity could be found for ANA (positive in 42.6% of tested patients), RF (10.2%), anti-Ro52 (32.7%), anti-Ro (11.5%), anti-PM-Scl (12.3%), anti-Mi-2 (6.7%), and anti-TIF-1γ (8.2%). Other autoantibodies were found in very low frequencies and could not be statistically evaluated. Seven

of 9 RF-positive patients and both patients with ACPA had arthritis. ACPA were positive at relatively high levels (patient No. 2: 239 U/l, patient No. 99: 114 U/l). Only patient No. 2 fulfilled the 1987 American College of Rheumatology (ACR) classification criteria for RA and was classified with an overlap syndrome.

Arthritis and HLA status. Allelic polymorphism of HLA-DRB1 and -DQB1 genes was analyzed in 71 and 73 patients and 179 and 175 healthy controls, respectively. Patients had higher frequencies of HLA-DRB1*03 (56%) and HLA-DQB1*02 (70%) alleles in comparison with the control group (25% and 42%; $p < 0.0001$), but no evidence of an association between arthritis and these or any other HLA-DRB1 or -DQB1 alleles was observed (from $p = 0.2205$ to $p = 1.0000$).

DISCUSSION

We present patient history as well as cross-sectional data regarding joint involvement in a cohort of 106 consecutive patients with IIM seen between January and September 2012 in a single center. To our knowledge, our study represents the largest comprehensive overview of arthritis in an unselected cohort of such patients. The results show that arthritis is a common feature of myositis, affecting more than half of patients overall. Individual subtype of IIM most likely does not play a role in prevalence of arthritis. In the majority of patients with arthritis it manifests already at the onset of IIM, preceding symptoms of muscle weakness in half and appearing simultaneously in another one-third. Survival analysis shows that the highest probability of future development of arthritis is at the beginning of the muscle disease, gradually decreasing for up to 10 years of disease duration, with a significant residual risk even after that time (Figure 1). This is supported by the fact that patients with arthritis had longer mean disease durations. We may hypothesize that joint tenderness, without swelling, found in 9 patients at cross-sectional examination might represent the first sign of newly developing arthritis in these patients.

Arthritis is also a common feature of disease relapses, being present in about half of relapsing patients, most frequently occurring at the same time as the muscle symptoms. However, arthritis at the myositis onset is not predictive of the presence of arthritis during myositis relapses or of any future relapses of IIM.

The most frequently involved joints are the shoulders and small joints of the hands — wrists, metacarpophalangeal and proximal interphalangeal joints (each affecting about one-fifth of the patients), followed by elbows, ankles, and tarsal and acromioclavicular joints. The involvement of hand joints mimicking the distribution of involvement in RA together with the fact that arthritis often precedes the onset of muscle weakness may contribute to an occasional misdiagnosis of IIM as RA[22].

We have combined data obtained both in retrospective and cross-sectional fashion; therefore we were not able to use a uniform definition of inflammatory arthritis. For the purpose of retrospective analysis, arthritis was defined as either an inflammatory arthritis diagnosed by a rheumatologist in the past and documented in the medical records or as a presence of both joint swelling and pain reported by the patient during the interview. Every attempt was made to confirm the inflammatory nature of the joint involvement and to rule out symptoms that could have been caused by osteoarthritis. For this reason a more stringent definition of arthritis requiring a simultaneous presence of both joint swelling and pain was applied. We are aware that, despite our efforts, retrospectively collected data might have caused arthritis overestimation. However, relying on medical records only would miss many arthritis cases, because especially mild and transitory forms of arthritis could have gone unreported.

Almost 30% of patients with IIM in our cohort had clinically apparent arthritis, defined as a presence of at least 1 swollen joint, at the time of cross-sectional evaluation. Polyarthritis was the most frequent manifestation with a mean of 5 affected joints. This suggests that, when specifically looked for, arthritis is a frequent manifestation of IIM.

In our cohort, a strong association of arthritis with the presence of the anti-Jo1 autoantibody was confirmed. The prevalence of arthritis among anti-Jo1-positive patients was over 93%, which is more than was reported in some other studies[14,23]. The distribution of affected joints in our anti-Jo1-positive patients was similar to previous reports and confirms a close relationship of joint disease with anti-Jo1 antibodies. We could not demonstrate an association of arthritis with the presence of any other autoantibody, presumably because of the low numbers of patients in other autoantibody subgroups. Indeed, an association of arthritis with 2 other antisynthetase antibodies, anti-PL-7[16] and anti-PL-12[24], has been recently described. In those studies, unlike in our cohort, patients were included on the basis of autoantibody positivity rather than the diagnosis of IIM, therefore introducing a selection bias. Seven of 9 RF-positive patients and both patients with ACPA had arthritis. Only 1 of those patients fulfilled the 1987 ACR classification criteria for RA. These findings are in contrast with reports[25,26] describing a relatively frequent overlap of IIM with RA, but are consistent with the original study of Bohan, *et al*, who observed this combination more rarely in 2.3% of patients[27], and also with other reports[28]. It may be clinically difficult to distinguish between arthritis as a manifestation of myositis and as a main clinical feature in RA, but the rare presence of typical deformities and radiographic erosions in those with available radiographs argues against the frequent existence of the overlap syndrome.

Association of HLA-DRB1 or HLA-DQB1 polymorphism with arthritis was not found. As expected, we

observed a higher frequency of HLA-DRB1*03 in our cohort. This allele has been associated with anti-Jo1 antibodies[29], but it may be present also in patients with some other autoantibodies or without them. The absence of the association of HLA-DRB1*03 and arthritis in our patients suggests that the presence of anti-Jo1 rather than HLA-DRB1*03 is the contributing factor.

Arthritis and joint involvement do not seem to significantly contribute to the overall disease burden in most patients with IIM. The activity of arthritis was generally considered to be low to moderate by both patients and physicians. Most patients assessed the contribution of arthritis to the overall morbidity to be none or only mild. It gives an impression that many patients perceive arthritis as less bothersome than other manifestations of myositis. However, in some patients arthritis contributes significantly to the overall morbidity. Seven patients judged the contribution of arthritis to be of large significance and in 2 of them the total morbidity was driven mostly by joint involvement. Moreover, nearly half of the IIM relapses were associated with arthritis. Therefore, in some patients arthritis may be the main complaint and the choice of drug therapy should reflect this. However, because it is not known what treatment is best for arthritis in myositis, the same drugs used to treat other IIM manifestations are usually prescribed.

Arthritis in IIM is rarely deforming or erosive. In our cohort, deforming arthropathy was present in 15% of patients and radiographic erosions were detected in only 2 patients (1 overlap syndrome with RA and 1 with anti-Jo1-positive DM); both patients had clinically apparent deformities. These findings may be limited because not all of our patients had radiographs performed. However, because the radiographs were indicated based on the presence and severity of joint involvement, it is likely that most, if not all, patients with radiographic changes were identified. Five of our patients had so-called floppy thumb deformity[30]; 4 of them (80%) were positive for anti-Jo1 autoantibody, thus confirming a previous report on this type of subluxing arthropathy[14].

We have documented that arthritis is a common, although usually not severe, feature of IIM. It is often present at the beginning of the disease, even preceding the onset of muscle weakness in a substantial proportion of patients. Distribution of the most frequently involved joints is similar to that seen in RA. In our group, arthritis was mostly not deforming, although we found some previously described characteristic deformities in some patients.

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Dr. Vencovský, *et al* reply

To the Editor:

We thank Drs. Jearn and Kim for their comments[1] on our report regarding arthritis in idiopathic inflammatory myopathies (IIM)[2]. They point to a very high incidence of arthritis among our patients with anti-Jo1–positive myositis, and use their cohort of patients to show that there is a more intimate association with pulmonary involvement rather than arthritis in anti-Jo1–positive patients.

We reported 93% prevalence of arthritis in anti-Jo1–positive patients with IIM. The association between arthritis and the presence of anti-Jo1 antibodies is well accepted and has been described in many reports with variable frequencies, usually exceeding 50%[3,4,5,6,7,8,9,10]. Several landmark papers show a frequency that is remarkably similar to our data. Love, *et al*[3] reported 94% prevalence of arthritis in a group of antisynthetase antibody-positive patients, three-fourths of which had anti-Jo1. Marguerie, *et al*[4] found arthritis/arthralgias in 95% of patients with anti-Jo1 antibodies. Yoshida, *et al*[5] reported frequency of arthritis to be 100% among a group of anti-Jo1–positive patients, with significantly lower prevalence of 48% in patients with anti-Jo1–negative myositis. Interestingly, in Yoshida, *et al*'s paper, autoantibodies were detected by double immunodiffusion (DID), the same method used by Jearn and Kim in the study described in their commentary[1] on our publication.

Therefore, we see our observation as not considerably different from previous data. The main reason for the high prevalence of arthritis in our patients is the methodological approach used. We have actively searched for swelling and tenderness on physical examination of 66/68 joints or used a credible history of arthritis reported by the patient or treating rheumatologist, as explained in our paper. We point out that physical examination is still considered to be the standard method of joint involvement evaluation in arthritis, as illustrated in the current classification guidelines for rheumatoid arthritis. The main intention of our report was to describe various aspects of arthritis in patients with myositis, primarily because we frequently encounter this symptom in our patients and were not able to find a comprehensive evaluation of arthritis in a sufficiently sized cohort of patients with IIM in the literature. There is a large body of evidence that anti-Jo1, as well as other antisynthetase antibodies, are strongly associated with pulmonary disease in IIM, and numerous papers describe the association in various levels of detail. Therefore, we reported only on the basic data regarding other features of IIM, including lung involvement, and concentrated on the lesser-known aspects pertaining to joint involvement. Indeed, we were able to establish that 53% of patients had arthritis at any point during the disease course, even preceding the onset of myositis in some cases.

Jearn and Kim[1] analyzed 2 groups of patients with and without anti-Jo1 antibodies and were unable to find a statistical difference in arthritis presence. They suggest that ethnic background or anti-Jo1 antibody detection techniques might be responsible for the differences. The difference in methods used to detect arthritis is much more likely to explain the conflicting results. The use of radiographs to screen for joint involvement in IIM is clearly much less sensitive than physical examination and therefore may not be a reliable way to assess the prevalence of arthritis.

DID is probably less sensitive than the enzyme immunoassays and radioactive immunoprecipitation that we used for anti-Jo1 detection. However, that fact does not explain the different results because there was a significant difference in the prevalence of arthritis between groups defined by antibody presence identified by DID in Yoshida, *et al*[5]. We agree that other differences in the patient groups and their genetic background might have played some role.

Arthritis should be recognized as a frequent extramuscular manifestation in patients with IIM, which may pose a diagnostic dilemma at the early stages of the disease. Because of the strong association of arthritis with anti-Jo1 antibodies, their detection may be particularly helpful in establishing the correct diagnosis.

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Příloha 2
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Concise report

Increasing incidence of immune-mediated necrotizing myopathy: single-centre experience

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Abstract

Objectives. Immune-mediated necrotizing myopathy (IMNM) is characterized by the predominant presence of necrotic muscle fibres in muscle biopsy and variable response to immunosuppressive treatment. The aims of this study were to analyse the temporal trend of IMNM incidence in our centre over the past 10 years and to explore the role of statins as possible causative agents.

Methods. A retrospective evaluation of muscle biopsy results, clinical and laboratory data, including antibody associations of all patients with idiopathic inflammatory myopathy newly diagnosed between 2004 and June 2014, was performed. Available sera were tested for the presence of anti-3-hydroxy-3-methylglutaryl coenzyme A reductase (anti-HMGCR) autoantibodies.

Results. Of 357 biopsied patients, 233 fulfilled criteria for inflammatory/immune-mediated myopathy, including 27 (11.6%) classified as IMNM. There were no patients with IMNM diagnosed between 2004 and 2007; subsequently, two to three cases of IMNM per year were seen during the period 2008–11, with a substantial increase to 18 cases (66.6% of all IMNM biopsies) in 2012–14. Thirteen of 27 patients (48%) had a history of statin use, 11 (85%) of whom had positive anti-HMGCR antibodies. There was no IMNM patient without a history of statin use who was anti-HMGCR antibody positive.

Conclusion. Our data show an increasing incidence of IMNM, which is mainly accounted for by anti-HMGCR-positive IMNM associated with the use of statins.

Key words: myositis, necrotizing myopathy, muscle biopsy, anti-3-hydroxy-3-methylglutaryl coenzyme A reductase autoantibodies.

CLINICAL SCIENCE

Rheumatology key messages

- A significant increase in necrotizing myopathy incidence has been observed in a single centre in recent years.
- Statin-induced necrotizing myopathies are mainly responsible for increased incidence of immune-mediated necrotizing myopathy.

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Introduction

Immune-mediated necrotizing myopathy (IMNM) is a relatively newly recognized category of idiopathic inflammatory myopathy (IIM). It is characterized by the predominant presence of necrotic muscle fibres with minimal or no inflammatory infiltrates in muscle biopsy and a variable degree of response to immunosuppressive treatment [1–3]. IMNM itself is a heterogeneous group; it is often associated with the presence of autoantibodies, for example, anti-SRP or anti-3-hydroxy-3-methylglutaryl

coenzyme A reductase (anti-HMGCR), each representing about 3–6% of IIMs [3, 4]. The diagnosis of IMNM seems to be becoming more frequent in our centre; therefore, we decided to carry out a retrospective analysis of the annual incidence of IMNM since 2004 and to compare it with the incidence of other forms of myositis. We have also investigated a possible contribution of statins as causative agent.

Patients and methods

Muscle biopsies, as well as clinical and laboratory data, of all patients who were evaluated at the Institute of Rheumatology between January 2004 and June 2014 for suspicion of IIM were retrospectively reviewed. This time period was selected because since 2004 all muscle biopsies have been performed in the same hospital and were read by a single expert pathologist (J.Z.) [5]. Patients who fulfilled Bohan and Peter myositis criteria [6, 7] or European Neuromuscular Centre (ENMC) [1] criteria for necrotizing myopathy or Griggs criteria for IBM [8] were included in the analysis as cases of inflammatory/immune-mediated myopathy. All patients signed an informed consent form, and the study was approved by the local ethics committee (Ethics committee of the Institute of Rheumatology, Prague, Czech Republic).

Muscle samples were obtained by open biopsy from the quadriceps muscle (mostly lateral vastus muscle) under local anaesthesia. Isopentane-frozen samples were examined using haematoxylin and eosin staining, a spectrum of histochemical and immunohistochemistry reactions and by electron microscopy [9]. Biopsy results were subcategorized, blinded to the clinical diagnosis, according to the report of the 119th ENMC workshop [1] as: IMNM, PM, DM, non-specific myositis and IBM. Biopsies with significant pathologies but not consistent with a single diagnostic category, were labelled as non-classifiable. Biopsies with no pathological changes or with mild non-specific abnormalities were classified as normal. Only good-quality muscle biopsies that provided a sufficient amount of tissue were considered for the study. Personal history, clinical data, laboratory results and data regarding environmental exposure were obtained from the patient database and/or hospital records.

Anti-HMGCR autoantibodies were measured by ELISA in 218 patients and in 62 healthy controls. Sera were tested without knowledge of clinical details. Briefly, 96-well plates were coated with 1.0 μl/ml HMGCR antigen (Sigma-Aldrich, St Louis, MO, USA) in PBS overnight at 4°C prior to being blocked in PBS–0.1% BSA–0.1% Tween at room temperature for 2 h. Serum samples were diluted 1/200 in PBS–0.1% Tween and were added to the plate in duplicate for 2 h at 4°C. Plates were washed three times in PBS–0.1% Tween prior to the addition of 1:30 000 goat anti-human IgG (Sigma-Aldrich) at 4°C for 30 min. Plates were washed three times in PBS–0.1% Tween and incubated in TMB Substrate Solution (Sigma-Aldrich) at 4°C for 10 min. Reactions were stopped with 1 M H_2SO_4, and plates were read at 450 nm. Negative cut-offs were calculated from the mean optical density + 3 S.D. of healthy controls. All positive samples were confirmed on at least two repeated ELISAs.

Autoantibody profiles of IIM patients were determined during routine diagnostic work-up using IIF to screen for ANA and anti-dsDNA (Immuno Concepts, Sacramento, CA, USA), line immuno-assay (Imtec Human, Wiesbaden, Germany) and myositis-westernblot (Euroimmun, Lübeck, Germany) for detection of individual autoantibodies directed against Jo-1, Mi-2, Ku, PM-Scl, PM-Scl75, PM-Scl100, PL-7, PL-12, EJ, OJ, SRP, Ro, Ro52, La, Scl-70 and U1-RNP antigens. In-house-made ^{35}S radioimmunoprecipitation [10] was used to confirm the results and to detect autoantibodies not captured using commercial assays (antibodies to TIF-1γ, MDA5, NXP2, Zo, EIF3, RNAP I, RNAP II and RNAP III). RFs were detected using a particle-agglutination assay (Fujirebio Inc., Tokyo, Japan), and an ELISA test for anti-CCP (Test-Line Clinical Diagnostics, Brno, Czech Republic) was used to detect antibodies against ACPA. Data on country-wide statin use were obtained from publicly available information at the Czech State Institute for Drug Control Web page [11, 12].

Demographics, clinical characteristics and results are presented as descriptive statistics. Categorical data were analysed by χ^2 test and Fisher's exact test. We used GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) for statistical analysis.

Results

Of the 357 patients who had muscle biopsy performed during the period 2004–14, 233 patients [171 (73.4%) females; mean age 55.45 (13.66) years] were diagnosed with inflammatory/immune-mediated myopathy. Muscle biopsy results evaluated according to the 119th ENMC workshop report [1] were classified as PM in 65 (27.9%), DM in 90 (38.6%), IMNM in 27 (11.6%), non-specific myositis in 5 (2.1%) and IBM in 6 (2.6%) cases. Five (2.1%) biopsies were non-classifiable and 35 (15.0%) were normal based on the above-mentioned criteria.

Eight patients had overlap syndromes: five with SLE, and three with SSc. Two patients fulfilled criteria for MCTD [13]. Twenty-seven patients were diagnosed with cancer-associated myositis, defined as occurrence of cancer within 3 years of IIM diagnosis, with breast and ovarian cancer being the most frequent tumours in seven (26%) and five cases (18%), respectively. Based on the clinical and histology ENMC criteria, IMNM was diagnosed in 27 patients, of whom one had an overlap with RA and one had skin melanoma.

Biopsy results, autoantibody profiles and selected clinical and environmental characteristics of the patients with necrotic biopsies are shown in Table 1. Apart from anti-HMGCR autoantibodies and previous use of statins, we have not found an association with any of the demographic, clinical, laboratory or environmental factors that have been analysed.

Incidence

Overall, 27 (11.6%) cases were histologically classified as necrotizing myopathy and diagnosed with IMNM based

Table 1 Characteristics of patients with necrotizing myopathy in the muscle biopsy

Gender/age, years	Biopsy date, month/year	Autoantibodies to	Statin use	Statin type	Duration of statin use, years	Muscle strength, %[a]	Myalgia	Maximal serum CK level, μkat/l[b]	Systemic and organ involvement
M/24	8/2008	SRP	N	—	—	62.5	N	37	—
M/61	9/2008	Ro, La	N	—	—	74.3	Y	75	Fever
M/31	11/2009	Jo-1	N	—	—	97.5	N	111	Fever, arthritis
F/62	11/2009	ANA	N	—	—	73.8	N	16	—
F/55	1/2010	Ro52	Y	S	3	56.3	Y	0.4	—
F/54[c]	5/2010	ANA	N	—	—	88.6	N	38	—
M/19	12/2010	Jo-1	N	—	—	88.8	Y	279	Fever, arthritis
M/55	4/2011	HMGCR	Y	A	UN	76.3	Y	211	—
F/67	10/2011	HMGCR	Y	S/A	3.75	73.8	N	172	—
F/64	2/2012	HMGCR	Y	A/R	6.5	66.3	Y	229	—
F/76	2/2012	HMGCR	Y	S/A	2	67.5	N	158	—
M/70	3/2012	HMGCR	Y	A	3	67.5	Y	157	—
F/43[d]	3/2012	CCP, ANA	N	—	—	67.5	N	138	Arthritis
F/67	7/2012	SRP	N	—	—	60	N	366	—
F/56	9/2012	SRP	N	—	—	67.5	N	103	—
F/67	10/2012	HMGCR	Y	A	3	91.3	N	112	—
F/57	10/2012	N	N	—	—	83.8	N	15	—
F/65	11/2012	HMGCR, CCP	Y	F	0.5	70	N	68	—
F/19	12/2012	N	N	—	—	56.3	Y	200	—
M/73	1/2013	HMGCR	Y	A	UN	63.8	N	80	-
F/72	2/2013	HMGCR	Y	A	1 year	62.5	N	24	-
F/77	7/2013	ANA	Y	A	UN	UN	N	82	-
F/67	9/2013	HMGCR	Y	A	4	UN	N	94	-
M/66	1/2014	HMGCR	Y	A	0.2	UN	Y	27	-
M/49	4/2014	ANA, Ro60, La	N	—	—	97.5	Y	128	Arthritis, RP
M/65	6/2014	SRP	N	—	—	70	N	139	-
M/28[e]	6/2014	ANA, Ku	N	—	—	85	N	70	-

[a]Muscle strength is shown as a percentage of maximal strength derived from the eight-muscle Manual Muscle Test [14]. [b]Normal range in our laboratory is 0.05–2.42 μkat/l. [c]Skin melanoma. [d]Overlap syndrome with RA. [e]Overlap syndrome with SLE. A: atorvastatin; F: fluvastatin; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; M/F: male/female; R: rosuvastatin; S: simvastatin; UN: unknown; Y/N: yes/no.

on the ENMC criteria. There were no necrotizing myopathies diagnosed between 2004 and 2007. Subsequently, two or three cases of IMNM per year were seen during the period 2008–11, with a substantial increase to 10 cases in 2012 (43.5% of all necrotizing myopathy biopsies and 35.8% of biopsies performed that year), which is significantly more than during the 2004–11 period [χ^2 (df1) = 54.124, $P < 0.0001$]. This trend was confirmed in the following 18 months (January 2013 to June 2014; Fig. 1): eight identified necrotizing myopathies also exceeds the incidence observed during the years 2004–11 [χ^2 (df1) = 30.268, $P < 0.0001$]. The rapid increase in the incidence of necrotizing myopathy in the recent 2.5 years (2012 to June 2014) represents a significant change compared with previous years [χ^2 (df1) = 82.460, $P < 0.0001$].

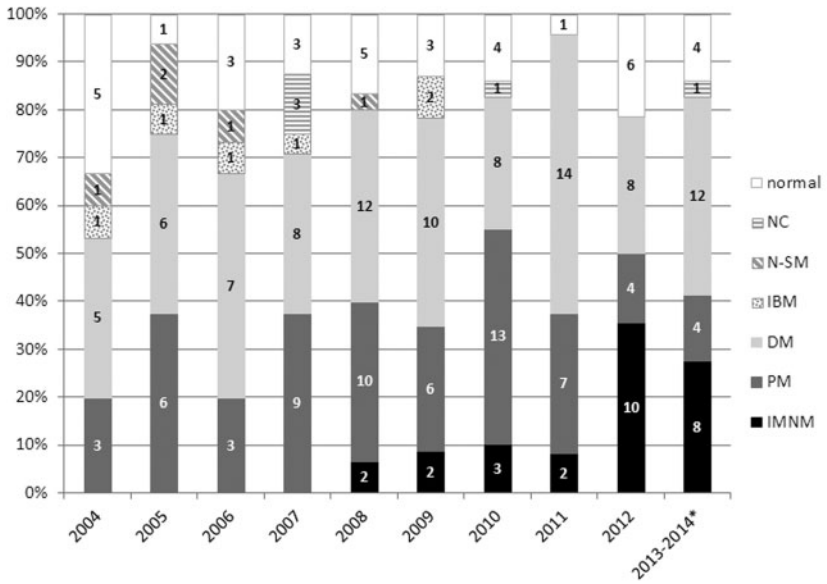
Most biopsies of patients with IMNM displayed prominent muscle fibre necrosis without any inflammatory infiltrates. Ten biopsies contained scarce lymphocytes, which stained positively for CD8 in six cases and for CD20 in one case, while in the remaining three cases lymphocytes could not be typed because they were not present on the slides used for immunostaining. There were no detectable differences in biopsy pattern with respect to the presence or absence of anti-HMGCR antibodies.

Autoantibodies

Of 217 serum samples available, anti-HMGCR autoantibodies were found in 15 (6.9%). Eleven of these 15 anti-HMGCR-positive patients had necrotic histology on biopsy, and 4 were classified as PM based on a classical finding of invasion of inflammatory cells into muscle fibres in three of them and inflammatory infiltration surrounding muscle fibres without invasion in one case. Sixteen patients with IIM who were not tested for anti-HMGCR antibodies neither had necrotic findings on biopsy nor used statins.

Four IMNM patients were positive for anti-SRP antibodies, two were anti-Jo-1 positive, two were anti-Ro/La positive, one was anti-Ku positive, one had isolated anti-Ro52 positivity and four were ANA positive with no identifiable specific autoantibody. Anti-CCP antibodies, considered highly specific for RA, were detected in two

FIG. 1 Percentage distribution of biopsy results in individual years



*Data from January 2013 to June 2014. Numbers in boxes indicate absolute numbers of biopsy results. IMNM: immune-mediated necrotizing myopathy; NC: non-classifiable; N-SM: non-specific myositis.

IMNM patients; one of them had an overlap with RA (patient 13). Anti-HMGCR autoantibodies overlapped with other autoantibodies in two patients only (anti-CCP and ANA). There were no differences in muscle strength, presence of myalgia or serum creatine kinase levels between anti-HMGCR-positive and -negative patients with IMNM.

Statin use

Of the total of 233 patients with IIM, 36 (15.5%) had a history of prior statin use (data were not available for two individuals). Among the 36 statin users, 15 (41.7%) developed anti-HMGCR antibodies and 13 (36.1%) presented with necrotizing myopathy in the biopsy. Eleven of the 13 patients with IMNM and a history of statin use were positive for anti-HMGCR antibodies. The two IMNM patients exposed to statins without anti-HMGCR antibodies were anti-Ro52 and ANA positive, respectively. All 15 anti-HMGCR-positive patients had been treated with statins in the past (100%), whereas only 21 out of the remaining 202 (10.4%) anti-HMGCR-negative IIM patients had a history of statin use ($P < 0.0001$). Thirteen of the 27 IMNM patients were statin users (48.1%), whereas only 23 among 204 (11.3%) non-IMNM patients had been exposed to statins ($P < 0.0001$).

There is a strong association in our cohort between statin use and the presence of anti-HMGCR-positive necrotizing myopathy, because 11 of 15 anti-HMGCR-positive patients who used statins developed IMNM. There were only four anti-HMGCR-positive patients with history of statin exposure who did not have necrotizing myopathy. These four patients showed a classical polymyositis pattern on biopsy.

These findings suggest a strong association of statin use both with the development of anti-HMGCR antibodies and with necrotizing myopathy.

Atorvastatin, simvastatin, fluvastatin and rosuvastatin were used by 22 (61%), 9 (25%), 2 (5.5%) and 2 (5.5%) patients, respectively. Four patients switched to different statins, and in five patients (13.8%) the specific statin used was not known. Individual drugs and details on their use are summarized in Table 1.

None of the IMNM patients was on statins at the time of biopsy [mean time between statin discontinuation and muscle biopsy was 16.2 (28.8) months; range 2 weeks to 8 years]. In the group of non-IMNM patients, five had biopsy performed while using statins, and in two individuals the exact date of statin discontinuation was not known. In all the other non-IMNM patients, the mean time between stopping statins and muscle biopsy was 7.5 (15.2) months (range 2 weeks to 5 years; not significant). Three patients were on a combination of statin and fibrate; two of them had a classical PM pattern in muscle biopsy with one of them being anti-HMGCR positive, while the third patient had normal biopsy findings and was anti-HMGCR negative. Patients using statins were older than non-users [64.5 (7.7) *vs* 53.8 (13.9) years; $P < 0.0001$ for whole group and 67.6 (6.6) *vs* 45.9 (18.1) years; $P = 0.0004$ for the IMNM cohort].

Discussion

We report a significantly increasing incidence of IMNM among patients evaluated for IIM in our centre over the past 10 years. No IMNM cases were seen in the years 2004–07. The first patients with IMNM started to appear during the 2008–11 period, with a sharp increase in 2012,

and this higher frequency has subsequently been maintained for 18 months. The referral pattern remained unchanged during the whole period of our study. We have evaluated all biopsies performed since 2004 in our centre, irrespective of the final diagnosis, and all biopsies were taken and processed in the same way at the same department and read by a single experienced pathologist using a pre-specified protocol for the recording of pathological findings. Therefore, we believe the number of IMNM cases is genuinely increasing over time and the results are not influenced by recent interest in necrotizing myopathies.

Based on our results, it seems that, for the most part, statin-induced IMNM is responsible for the increasing incidence. Ten out of 18 patients with IMNM seen in 2012–14 were treated with statins and all but one had anti-HMGCR antibodies, thus confirming the likely role of statins in the pathogenesis of the disease. In addition, two other patients seen in 2011 were treated with statins and were anti-HMGCR positive. Only two statin users among IMNM patients did not have the anti-HMGCR antibodies. Moreover, anti-HMGCR antibodies were not found in any statin non-user with either IMNM or another subtype of IIM. This strong association of anti-HMGCR antibodies and statin use among IMNM patients is remarkable and confirms previous results reported in patients from Johns Hopkins University [15].

More than 80% of patients with statin-associated IMNM from our cohort used atorvastatin. This finding probably reflects the fact that atorvastatin has been the most frequently prescribed statin in the Czech Republic since 2006 and is becoming increasingly popular (supplementary Fig. S1, available at *Rheumatology* Online) [11, 12]. However, the disease is not limited to atorvastatin users, and other statins are also implicated. The average duration of statin use prior to the onset of symptoms was 2.67 years (range 2 months to 6.5 years), in accordance with previous findings [16].

In summary, we describe an increased incidence of necrotizing myopathy in recent years. Almost half of the cases are anti-HMGCR-positive IMNM patients. Statins are most likely to be responsible given the striking association with the presence of anti-HMGCR positivity.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Příloha 3

Increased visfatin levels are associated with higher disease activity in anti-Jo-1-positive myositis patients

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Abstract

Objective

The aim of this study was to evaluate serum levels of visfatin in anti-Jo-1-positive myositis patients, its expression in muscle tissue and to investigate potential relationships between visfatin, B-cell activating factor of the TNF family (BAFF), disease activity and anti-Jo-1 autoantibody levels.

Methods

Serum levels of visfatin and BAFF were measured in 38 anti-Jo-1 positive myositis patients and 35 healthy subjects. Disease activity was evaluated by myositis disease activity assessment tool (MYOACT) using visual analogue scales (VAS) and by serum muscle enzymes. Visfatin expression was evaluated by immunohistochemistry in muscle tissue of myositis patients (n=10) and compared with non-inflammatory control muscle tissue samples from patients with myasthenia gravis (n=5).

Results

Serum visfatin and BAFF levels were significantly higher in myositis patients compared to healthy subjects and were associated with clinical muscle activity assessed by VAS. Only serum BAFF levels, but not visfatin levels, positively correlated with muscle enzyme concentrations and anti-Jo1 antibody levels. There was a positive correlation between visfatin and BAFF serum levels in myositis patients but a negative correlation was observed in healthy subjects. Visfatin expression was up-regulated in endomysial and perimysial inflammatory infiltrates of muscle tissue from myositis patients.

Conclusion

Up-regulation of visfatin in myositis muscle tissue and an association between increased visfatin levels and muscle disease activity evaluated by MYOACT in anti-Jo-1 positive myositis patients could support possible role of visfatin in the pathogenesis of myositis.

Key words

myositis, visfatin, BAFF, anti-Jo-1, muscle enzymes

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Introduction

The idiopathic inflammatory myopathies including polymyositis (PM) and dermatomyositis (DM) are characterised by muscle weakness and inflammatory infiltrates in skeletal muscle tissue (1). Other organs such as skin or lungs may be affected as well.

Autoantibodies, some of which are considered to be myositis-specific, are present in 60–80% of patients with myositis (2, 3). The most frequent myositis specific autoantibodies directed against histidyl-tRNA synthetase (anti-Jo-1) are present in approximately 10–30% of myositis patients (4) and are associated with a distinct clinical phenotype (5). Presence of these autoantibodies before the onset of clinical symptoms (6, 7), as well as the role of the target antigen in the breach of immune tolerance (8, 9), suggest a possible role of anti-Jo-1 in the pathogenesis of myositis. Furthermore, a correlation between serum levels of anti-Jo-1 autoantibodies and myositis activity was previously reported (10).

Visfatin, also known as pre-B cell colony-enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (NAMPT) is ubiquitously expressed in all tissues (11) and was recently described as a new adipokine up-regulated in visceral fat cells (12). Beside the role in energy metabolism; immunomodulatory and pro-inflammatory properties of visfatin were emphasised recently (13). Visfatin enhances activation of leukocytes, synthesis of adhesion molecules and production of pro-inflammatory cytokines, such as interleukin (IL)-1, tumour necrosis factor (TNF) and IL-6 in monocytes (14) and, in addition, protects fibroblasts and neutrophils from apoptosis (15, 16). Its production is increased in lymphocytes and stimulates their proliferation during polyclonal immune response (15, 17). Visfatin acts as a cytokine that promotes B-cell maturation (18).

Increased levels of visfatin were documented in several chronic autoimmune and inflammatory diseases such as systemic lupus erythematosus (19, 20) or rheumatoid arthritis (RA) (21-23). In RA, visfatin levels correlate with clinical disease activity (24) and predict radiographic progression in established (22) as well as in early disease (25). Visfatin and other adipokines are actively produced by local cells of the rheumatoid joint and by the periarticular adipose tissue (24, 26).

We have recently described increased serum levels of another adipokine resistin in myositis patients compared with healthy controls and their association with systemic inflammation and disease activity, particularly in anti-Jo-1 positive myositis patients (27). However, the role of visfatin in the pathogenesis of myositis has not yet been studied. Therefore, the aim of this study was to compare serum levels of visfatin in anti-Jo-1 positive patients with myositis to levels in healthy individuals and assess their potential relationship with anti-Jo-1 autoantibodies, with myositis activity and with serum levels of B-cell activation factor of the TNF family (BAFF), a crucial cytokine for B-cell maturation and survival, with known association with myositis and anti-Jo-1 autoantibodies (28). We also analysed expression of visfatin in myositis and non-inflammatory muscle biopsies.

Patients and methods

Patients

Thirty-eight anti-Jo-1 positive myositis patients (27 PM and 11 DM) regularly followed at the Institute of Rheumatology in Prague were included in this study. The diagnosis of PM and DM was established based on the Bohan and Peter criteria (29). In 16 patients, longitudinal blood samples obtained during treatment were available (median time between sample collection was 13.4 months, range 7 to 73.6 months). A control group consisted of 35 age- and sex-matched healthy subjects. This study was performed after approval by the local Ethics Committee of Institute of Rheumatology. Written informed consent was obtained from all individuals prior to participation.

Clinical data were collected at the time of serum sampling. Disease activity was evaluated using the Myositis Disease Activity Assessment Tool (MYOACT) according to the International Myositis Assessment & Clinical Studies Group (IMACS), including extra-

muscular, muscular and the physician's score of overall disease activity using visual analogue scales (VAS). Interstitial lung disease (ILD) was defined by either presence of cough or dyspnoea, reduction of lung volumes or changes on radiograph/HRCT as described elsewhere (30).

Laboratory measurements

Serum levels of creatine kinase (CK), myoglobin, lactate dehydrogenase (LDH) and C-reactive protein (CRP) were measured by routine laboratory techniques. Enzyme-linked immunosorbent assay (ELISA) was used for the measurement of serum visfatin (Bio-Vision Research Products, Mountain View, USA) and BAFF levels (R&D Systems, Inc., Minneapolis, USA) according to the manufacturer's protocol. Absorbance was detected using the Sunrise ELISA reader (Tecan, Salzburg, Austria). The sensitivity was 30pg/ml for visfatin and 3.38 pg/ml for BAFF.

Quantitative indirect solid phase ELISA assay (Orgentec, Mainz, Germany) was used for detection of IgG class anti-Jo-1 levels according to manufacturer instructions. Normal range declared by producer was <15 U/ml and borderline values were 15–25 U/ml. Anti Jo-1 positivity was confirmed by line blot assay (Myositis-LIA, IMTEC, Berlin, Germany) and myositis western blot using Anti-Myositis-Antigen EUROLINE-WB kit (Euroimmun, Lubeck, Germany).

Table I. Demographic characteristics, clinical and laboratory data of patients at initial evaluation.

	All n=38	Controls n=35	*p*
F : M	26 : 12	24 : 11	0.99
DM : PM	11 : 27	NA	
Age (year)*	52.5 ± 10.8	50.7 ± 12.5	0.53
Years from diagnosis**	1.92 [0.01 to 18.5]	NA	
ILD†	32 (84%)	NA	
Medication - GCS†	35 (92%)	NA	
- DMARDs†	22 (58%)	NA	
- No therapy†	3 (8%)	NA	
Dose of GCS (mg/d)**‡	17.5 [0 - 80]	NA	
CRP (mg/l)**	2.93 [0.1 to 83.0]	1.42 [0.3 to 7.4]	0.03
CK (μcat/l)**	2.44 [0.26 to 94.5]	ND	
Myoglobin (μg/l)**	96.8 [24.8 to 5313]	ND	
LDH (μcat/l)**	4.14 [1.67 to 22.8]	ND	
ALT (μcat/l)**	0.48 [0.09 to 6.7]	ND	
AST (μcat/l)**	0.43 [0.14 to 5.1]	ND	
BAFF (ng/ml)**	1.41 [0.5 to 20.9]	1.0 [0.42 to 2.1]	0.02
anti-Jo-1 (kU/l)**	183.7 [0.03 to 3604]	ND	
Visfatin (ng/ml)**	1.9 [0.13 to 9.9]	1.51 [0.14 to 5.2]	0.01
MYOACT (VAS mm)**	n=37		
Constitutional	3 [0 to 35]	NA	
Cutaneous	0 [0 to 30]	NA	
Skeletal	0 [0 to 42]	NA	
Gastrointestinal	0 [0 to 17]	NA	
Pulmonary	15 [0 to 86]	NA	
Cardiac	0 [0 to 36]	NA	
Other	0 [0 to 29]	NA	
Global Extraskeletal Muscle	12 [0 to 64]	NA	
Muscle	11 [0 to 82]	NA	
Global	16 [0 to 79]	NA	
MYOACT Score	0.55 [0 to 2.3]	NA	

ALT: alanine aminotransferase (normal levels <0.75 μcat/l for men and <0.57 μcat/l for women); AST, aspartate aminotransferase (normal levels <0.58 μcat/L for men and <0.52 μcat/L for women); BAFF, B-cell activating factor of the TNF family; CK: creatine kinase (normal levels <2.85 cat/l and <2.42 μcat/l); CRP: C-reactive protein (normal levels <5 mg/l); DM: dermatomyositis; DMARD: disease-modifying anti-rheumatic drug; GCS: glucocorticosteroids; ILD: interstitial lung disease (ever present); LDH: Lactate dehydrogenase (normal levels 0.05 – 4.15 cat/L); MYOACT: Myositis Disease Activity Assessment Tool; Myoglobin (normal levels <92 μg/l, for men and <76 μg/l for women); NA: not applicable; ND: not determined; PM: polymyositis. Data are: *mean± s.d.; **median [minimum - maximum] or †number (% from DM, PM or from total number of patients). ‡ Equivalent of prednisolone daily dose.

Immunohistochemistry

Samples for immunohistochemistry were obtained from patients with PM (n=5) and DM (n=5) at the time of diagnostic muscle biopsy, which was guided by positive magnetic resonance imaging (MRI) findings from affected muscles as previously described (31). The biopsies were taken prior to treatment initiation. Muscle tissue samples from patients with myasthenia gravis (MG, n=5) obtained during thymectomy were used as non-inflammatory controls, no lymphocyte infiltrates were present in muscle tissue biopsies from these individuals (32). After the sample collection, 5 μm frozen sections were fixed in acetone and 4% paraformaldehyde and blocked with 0.3% H_2O_2. The sections were incubated with rabbit polyclonal anti- Visfatin antibody (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA, 1mg/ml) at a dilution 1:1000 in Tris Buffered Saline (TBS) buffer for 1 hour. After rinse in TBS buffer, antigen-antibody complexes were visualised with a Histofine detection system (Nichirei Biosciences Inc., Tokyo, Japan) using 3, 3´diaminobensidine as a chromogen. Rabbit IgG (Dako Cytomation, Glostrup, Denmark, 1mg/ml), diluted 1:1000 was used as a negative control. The sections were slightly counterstained with Harris's haematoxylin. All sections were analysed semiquantitatively by two experienced pathologists who were blinded to the clinical data. Visfatin expression was scored semiquantitatively on a four-point scale (- represented negative staining intensity, and scores of + to +++ represented weak, moderate and strong staining intensity) at seven locations (inflammatory infiltrate, perimysial vessels, endomysial capillaries, muscle fibres, regenerating muscle fibres, atrophic muscle fibres and rhabdomyoblasts).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (v. 5.02; GraphPad Software, La Jolla, CA, USA) and SPSS 14.0 (SPSS, Inc., Chicago, Illinois, USA). For analysis of differences between groups, Kruskal-Wallis with

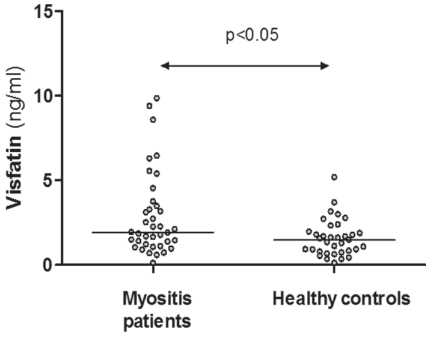

Fig. 1. Serum levels of visfatin are higher in myositis patients compared to healthy individuals. Horizontal bars represent medians; *p*-value of Kruskal-Wallis test is expressed.

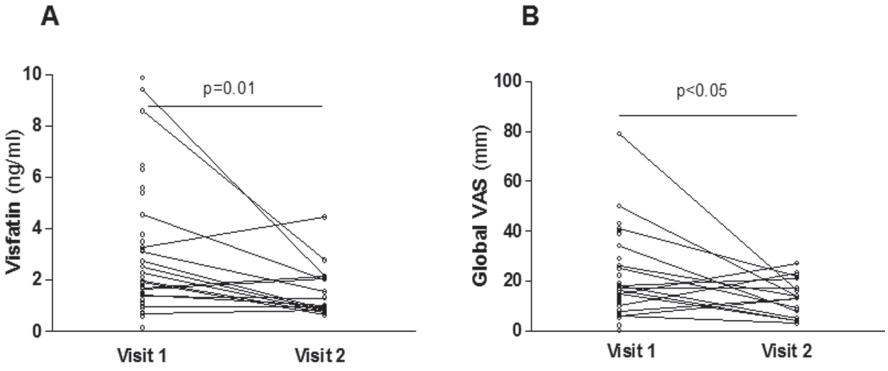

Fig. 2. Visfatin serum levels in paired serum samples decreased between two different time points, median between the two blood withdrawals was 13.4 [7 to 73.6] months (**A**), which was accompanied by global disease activity improvement (**B**). Wilcoxon's paired sample test *p*-values are expressed.

Dunn's post hoc test and non-parametric Mann-Whitney U-test or paired *t*-test with respect to normality of data, were performed. Spearman's rank order test (rs) was used for correlations of parameters. Contingent tables were evaluated with Fisher's exact test. Changes in paired samples were evaluated with Wilcoxon's signed rank test. A *p*-value less than 0.05 was considered to be statistically significant. Unless otherwise stated, data were presented as median [minimum–maximum] values.

Results

Patient characteristics

The demographic characteristics of patients and healthy controls are shown in Table I. Altogether, 35 (92%) patients were treated with glucocorticoids (median dose of prednisone equivalent: 17.5 mg/day; range 0–80 mg/day). 22 patients (58%) were treated with immunosuppressive drugs: 16 received methotrexate, 4 azathioprine, 2 methotrexate in combination with azathioprine, 1 patient used cyclosporine A. Three patients (8%) had no treatment at the time of serum sample collection. There were no significant differences in demographic characteristics, laboratory and clinical disease activity, or treatment between PM and DM patients.

Increased visfatin serum levels in myositis patients

The serum levels of visfatin were significantly higher in myositis patients compared to healthy controls (1.94 [0.13–9.86] *vs*. 1.51 [0.14–5.20] ng/ml; $p<0.05$) (Fig. 1). There were no significant differences in visfatin levels between patients with DM and PM (1.91 [0.13–9.41] *vs*. 1.96 [0.71–9.86] ng/ml; $p=0.89$) or between males and females (1.73 [0.13–6.48] ng/ml vs. 2.28 [0.6–9.86] ng/ml; $p=0.23$). Similarly, no difference was found between visfatin levels in patients double positive for anti-Jo1 and anti-Ro52 (1.96 [0.92–6.31] ng/ml) and patients without anti-Ro52 (1.91 [0.13–9.86] ng/ml; $p=0.68$). The levels of serum visfatin did not correlate with age (r=-0.06, $p=0.75$) or disease duration (r=-0.08, $p=0.63$). Furthermore, visfatin serum levels did not significantly differ between patients treated with glucocorticoids for less or more than one month. Although, serum visfatin levels were comparable between myositis patients with and without conventional immunosuppressive therapy (1.91 [0.1–9.9] *vs*. 2.09 [0.7–6.5] ng/ml; $p=0.89$), the levels of serum visfatin in longitudinal samples significantly decreased during treatment (from 2.12 [0.7–9.4] to 1.16 [0.6–4.45] ng/ml; $p=0.01$) which was accompanied with disease activity improvement in global VAS (from 17.5 [0–79] to 13.5 [3–27]; $p<0.05$) (Fig. 2).

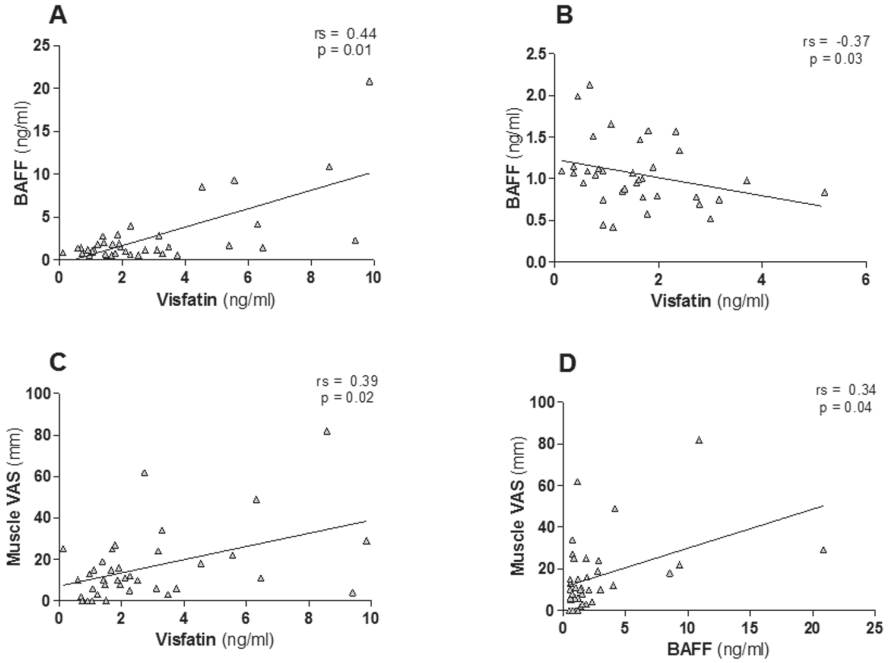

Fig. 3. Correlation between serum visfatin and BAFF levels in myositis patients (**A**) and healthy subjects (**B**). Both serum levels of visfatin and BAFF correlated with muscle disease activity in patients with myositis (**C**, **D**). Spearman's correlation coefficients with p values and linear regression lines are shown.

Association between visfatin, BAFF, autoantibodies and myositis activity

The levels of visfatin (rs=0.39, p=0.02), as well as of BAFF (rs=0.34, p=0.04) correlated with clinical muscle activity assessed by VAS (Fig. 3C and 3D), but did not correlate with laboratory measures of myositis disease activity. There was a trend for correlation of visfatin and BAFF with the global disease activity VAS (rs=0.28, p=0.09 and rs=0.33, p=0.05).

While, there was a moderate correlation between the level of visfatin and LDH (rs=0.39, p<0.02), it did not correlate with other muscle enzymes and myoglobin levels. On the other hand, BAFF levels significantly correlated with serum myoglobin (rs=0.57, p=0.002), CK (rs=0.51, p=0.001) and also with the levels of anti-Jo-1 (rs=0.85, p=0.001). Although there was a trend towards positive correlation between visfatin and anti-Jo-1 autoantibody levels (rs=0.31, p=0.06), no differences were observed in visfatin levels between patients with and without ILD (1.85 [0.13-9.86] *vs*. 1.91 [0.31-4.55] ng/ml; p=0.96). The levels of serum visfatin in myositis patients did not correlate with CRP levels (rs=0.13, p=0.43). There was a correlation between serum BAFF levels and CRP (rs=0.35, p=0.03). Levels of serum visfatin positively correlated with BAFF levels in myositis patients (rs=0.44; p=0.01), but there was a negative correlation in healthy controls (rs=-0.37; p=0.03), (Fig. 3A and 3B).

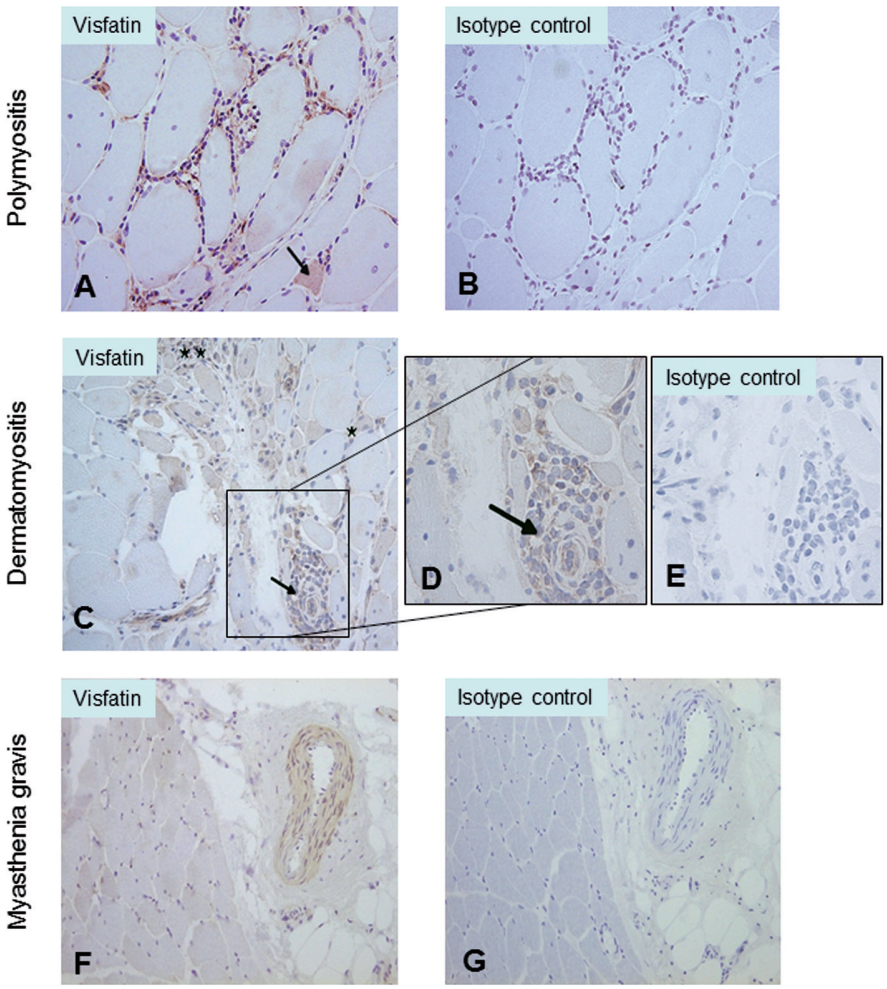

Fig. 4. Endomysial inflammatory infiltrate in a representative case of polymyositis patient express diffuse visfatin immunoreactivity (**A**). Regenerating muscle fibres show slight cytoplasmic immunopositivity (arrow), while muscle fibres of isotype control staining are negative (**B**). A proportion of the perimysial inflammatory cells in a representative case of dermatomyositis (**C**) is immunopositive for visfatin (arrowhead) with detail (**D**). Regenerating muscle fibres are visfatin immunopositive (*). In perifascicular regions, atrophic muscle fibres show cytoplasmatic immunopositivity (**), isotype control staining is negative (**E**). Positivity of visfatin in smooth muscles of perimysial arteries media of a control muscle tissue from MG patient (**F**) compared to isotype control (**G**). Brown colour indicates positive staining. Immunoperoxidase method, counterstain with haematoxylin, magnificaton 200x.

Increased expression of visfatin in muscle tissue of myositis patients

The expression of visfatin in muscle tissue was observed in all myositis patients and was more prominent when compared to control muscle tissues from patients with MG (Fig. 4). Visfatin staining intensity was comparable between muscle specimens from patients with PM and DM. Expression of visfatin was most evident in the endomysial and perimysial inflammatory cell infiltrates in the muscle tissue, although the intensity varied from weak to strong (Table II). Whereas the vast majority of endomysial inflammatory infiltrate in PM was visfatin positive (Fig. 4A), only part of perimysial inflammatory infiltrate was positive in DM (Fig. 4C). Rhabdomyoblasts, when found, were visfatin positive. In all patients with PM and in four out of five patients with DM, regenerating muscle fibres expressed visfatin, a finding not observed in control patients with MG. Similar intensity of visfatin expression was demonstrated in endothelial cells of perimysial vessels or endomysial capillaries in myositis and control muscle tissues.

Discussion

In the present study, we report an association between higher visfatin serum levels and increased muscle disease activity in anti-Jo-1 positive myositis patients. Furthermore, visfatin expression in muscle tissue from myositis patients was up-regulated compared to non-inflammatory muscle controls. This data support a possible role of visfatin in the pathogenesis of idiopathic inflammatory myopathies.

Visfatin, originally discovered as a growth factor for B lymphocyte precursors, plays a significant role in various pathophysiologic processes including inflammation (14, 33). Our study is the first showing elevated serum visfatin levels in patients with anti-Jo-1-positive myositis. Visfatin serum levels were higher in both PM and DM

Table II. Visfatin expression in muscle tissue of patients with dermatomyositis (DM) and polymyositis (PM) compared to non-inflammatory muscle biopsies from patients with myasthenia gravis (MG).

	No.	Inflammatory infiltrate** -/+/++/+++	Perimysial vessels† -/+	Endomysial capillaries†† -/+	Muscle fibres‡ -/+	Regenerating muscle fibres# -/+	Atrophic muscle fibres‖ -/+	Rhabdo myoblasts§ -/+
PM	1	+	+	+	-	+	-	+
	2	+	+	+	-	+	-	+
	3	+++	+	+	+*	+	-	+
	4	-	+	+	-	+	-	+
	5	++	+	+	-	+	-	+
DM	1	++	+	+	-	+	+	+
	2	+	+	+	-	+	+	+
	3	++	+	+	-	-	+	+
	4	+	+	+	-	+	+	+
	5	-	+	+	-	+	+	+
Control muscle (MG)	1	-	+	+	-	-	-	-
	2	-	+	+	-	-	-	-
	3	-	+	+	-	-	-	+
	4	-	+	+	-	-	-	-
	5	-	+	+	-	-	-	-

**The presence and intensity of inflammatory infiltrate: none inflammatory infiltrate present; +: very mild visfatin positive; ++: medium visfatin positive; +++: intense visfatin positive; †Perimysial vessels: - negative; + endothelial cells and vessel wall positivity; ††Capillaries: - negative; + mild positivity; ‡ Muscle fibres: - negative; + positive; #Regenerating muscle fibres: - not present; + present cytoplasmatic positivity; ‖Atrophic muscle fibres: - negative; + cytoplasmatic positivity; § Rhabdomyoblasts: - not present; + present, cytoplasmatic positivity. *Muscle fibres displaying cytoplasmatic visfatin immunopositivity proved to by ragged red fibres via histochemical methods (succinate dehydrogenase, cytochrom C oxidase).

patients compared to healthy controls. We found that visfatin as well as BAFF serum levels were associated with muscle disease activity and there was a trend for correlation with the global disease activity score. This association is supported by evidence that visfatin is associated with several pathological conditions, including cardiovascular diseases, metabolic disorders, inflammatory diseases or malignancies (34). In addition, visfatin levels correlated with disease activity (24, 35) and structural progression of rheumatoid arthritis (22) and ankylosing spondylitis (36). On the other hand, in systemic sclerosis, visfatin levels were not different from that in healthy controls (37, 38) and in systemic lupus erythematosus, visfatin levels were either elevated (20, 39) or comparable (38) to those of healthy subjects.

Although, immunosuppressive therapy did not seem to influence visfatin levels in a cross-sectional analysis, the levels of visfatin significantly decreased during treatment in myositis patients whose disease activity improved. We and others have reported that visfatin levels decrease following successful therapy in rheumatoid arthritis patients (35, 40), a finding which is not consistent in all studies (41).

In this study a positive association between visfatin and BAFF serum levels in anti-Jo-1 positive myositis patients was observed. Both cytokines are involved in B cell maturation into autoantibody producing plasma cells, which provides a possible explanation of the association between visfatin and particularly BAFF levels with that of anti-Jo-1 antibodies. However, we observed an inverse association between visfatin and BAFF levels in healthy subjects, which may potentially represent a counterbalancing mechanism in B cell function under non-inflammatory conditions. The role of these cytokines in myositis pathogenesis may be further supported by the fact that patients with anti-synthetase syndrome respond better to B-cell depleting therapy with rituximab compared to patients with other forms of inflammatory myopathies (42).

Here, we have also shown that visfatin levels did not correlate with muscle enzymes, with the exception of LDH or inflammatory markers in myositis patients. This may suggest that visfatin is more likely associated with immune inflammatory reaction rather than with muscle damage. In this sense it may be hypothesised that visfatin is a biomarker related to the inflammatory aspect of the disease whereas CK levels are more related to the actual muscle cell injury. Finally, we detected increased visfatin expression in muscle tissues from myositis patients compared to non-inflammatory control muscle specimens from MG individuals. Visfatin was predominantly expressed in endomysial and perimysial inflammatory cell infiltrates and also by regenerating muscle fibres in a majority of myositis muscle tissue samples. Since BAFF together with its receptors are up-regulated in myositis muscle tissue (43), and BAFF increases visfatin gene expression in primary B cells *in vitro* (44), it can be hypothesised that BAFF may participate in visfatin up-regulation in muscle tissue inflammatory infiltrates. Increased visfatin expression has been identified in a variety of chronic inflammatory diseases. In rheumatoid arthritis synovial membrane, visfatin was predominantly expressed by fibroblasts, lymphoid aggregates, and interstitial vessels (24, 26, 45). Visfatin can promote synthesis of chemokines, pro-inflammatory cytokines, angiogenic growth factors, and matrix degrading enzymes (24, 26, 45, 46). The expression of visfatin was also demonstrated in skeletal muscle tissue raising the possibility that it may act as a myokine affecting skeletal muscle growth and metabolism (47).

The strengths of our study rely in the homogeneous population of anti-Jo-1 positive patients and the fact that clinical assessments were performed at the time of serum collection, enabling correlative analyses. The limitation of this study is a relatively low number of patients due to the infrequent occurrence of the disease. With this limitation in mind we can still hypothesise that increased amount of visfatin may have an effect on immune as well as muscle cells and it may participate in the disease process in inflammatory anti-Jo-1 positive myositis.

Conclusions

In conclusion, we demonstrated here elevated serum levels of visfatin, similarly to BAFF, in patients with anti-Jo-1 positive myositis. Both cytokines associated positively with muscle disease activity evaluated by MYOACT. In addition, visfatin expression was up-regulated in muscle tissue from myositis patients; however, the exact role of visfatin in the pathogenesis of myositis remains to be determined in further studies.

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RESEARCH ARTICLE Open Access

Resistin in idiopathic inflammatory myopathies

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Abstract

Introduction: The purpose of this study was to evaluate and compare the serum levels and local expression of resistin in patients with idiopathic inflammatory myopathies to controls, and to determine the relationship between resistin levels, inflammation and disease activity.

Methods: Serum resistin levels were determined in 42 patients with inflammatory myopathies and 27 healthy controls. The association among resistin levels, inflammation, global disease activity and muscle strength was examined. The expression of resistin in muscle tissues from patients with inflammatory myopathies and healthy controls was evaluated. Gene expression and protein release from resistin-stimulated muscle and mononuclear cells were assessed.

Results: In patients with inflammatory myopathies, the serum levels of resistin were significantly higher than those observed in controls (8.53 ± 6.84 vs. 4.54 ± 1.08 ng/ml, $P < 0.0001$) and correlated with C-reactive protein (CRP) levels (r = 0.328, $P = 0.044$) and myositis disease activity assessment visual analogue scales (MYOACT) (r = 0.382, $P = 0.026$). Stronger association was observed between the levels of serum resistin and CRP levels (r = 0.717, $P = 0.037$) as well as MYOACT (r = 0.798, $P = 0.007$), and there was a trend towards correlation between serum resistin and myoglobin levels (r = 0.650, $P = 0.067$) in anti-Jo-1 positive patients. Furthermore, in patients with dermatomyositis, serum resistin levels significantly correlated with MYOACT (r = 0.667, $P = 0.001$), creatine kinase (r = 0.739, $P = 0.001$) and myoglobin levels (r = 0.791, $P = 0.0003$) and showed a trend towards correlation with CRP levels (r = 0.447, $P = 0.067$). Resistin expression in muscle tissue was significantly higher in patients with inflammatory myopathies compared to controls, and resistin induced the expression of interleukins (IL)-1β and IL-6 and monocyte chemoattractant protein (MCP)-1 in mononuclear cells but not in myocytes.

Conclusions: The results of this study indicate that higher levels of serum resistin are associated with inflammation, higher global disease activity index and muscle injury in patients with myositis-specific anti-Jo-1 antibody and patients with dermatomyositis. Furthermore, up-regulation of resistin in muscle tissue and resistin-induced synthesis of pro-inflammatory cytokines in mononuclear cells suggest a potential role for resistin in the pathogenesis of inflammatory myopathies.

Introduction

The inflammatory myopathies are a group of acquired skeletal muscle diseases that include polymyositis (PM), dermatomyositis (DM), inclusion body myositis, and overlap and cancer-associated myositis. Inflammatory myopathies are clinically characterised by proximal muscle weakness and skin changes in DM. An autoimmune origin of inflammatory myopathies is supported by their association with other autoimmune diseases, the presence of autoantibodies, the involvement of histocompatibility genes, the evidence of T-cell-mediated myotoxicity or complement-mediated microangiopathy, and their responses to immunotherapy [1]. Both diseases are also characterised by mononuclear inflammatory cell infiltration in skeletal muscle tissue, muscle fibre necrosis and regeneration. The inflammatory infiltrates in the muscle tissue of DM patients primarily include CD4+ T cells, B cells and dendritic cells in predominantly perimysial distribution, while affected muscle tissue in PM patients is characterised by endomysial presence of CD8 + T cells and macrophages, which often surround and

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invade non-necrotic muscle fibres [1]. The pathogenesis of inflammatory myopathies has not yet been completely elucidated, but several cytokines and chemokines produced by immune cells and myocytes have already been shown to be involved in the process of muscle tissue damage during myositis [reviewed in [2]].

Resistin, also known as adipocyte-secreted factor (ADSF) or found in inflammatory zone 3 (FIZZ3), is a member of the adipokine family. Originally, resistin was found in adipocytes to induce insulin resistance in mice. It has been associated with several metabolic disorders but also with cancer, inflammatory and immune-mediated diseases [3]. Resistin is up-regulated by inflammatory mediators in peripheral blood mononuclear cells (PBMC) and induces the expression of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1 and tumour necrosis factor (TNF)-α, angiogenic factors and extracellular matrix metalloproteinases, suggesting a broad contribution to many pathological conditions [4-11].

Therefore, we assessed the serum resistin level and its expression in muscle tissues from patients with idiopathic inflammatory myopathies and studied the potential role of resistin in the pathogenesis of muscle tissue damage.

Materials and methods

Patients characteristics

Our study consisted of 42 patients with inflammatory myopathies and 27 healthy controls. All patients underwent muscle biopsy that was guided by positive magnetic resonance imaging (MRI) findings from affected muscles [12]. The specific pattern of muscle biopsy, including the immunohistological [13,14] and clinical investigation, showed that 17 patients suffered from DM and 25 from other types of myositis. Based on the novel clinicoserological criteria [15], four patients with myositis could be classified as pure PM, while 21 had overlap myositis. These patients had at least one clinical overlap feature and/or an overlap antibody [15].

Patients were recruited from the inpatient and outpatient departments of the Institute of Rheumatology in Prague. Disease activity was assessed using myositis disease activity assessment visual analogue scales (MYOACT) that globally score constitutional, articular, cardiac, pulmonary, gastrointestinal, cutaneous, muscle organs or systems [16]. Manual muscle testing of eight muscle groups (MMT8) was performed and included one axial, five proximal (two upper extremity, three lower extremity), and two distal muscles (one upper, one lower extremity) [17]. Written informed consent from each participant was obtained prior to enrolment, and the study was approved by the local ethics committee.

Laboratory measurements

Peripheral blood was obtained from all patients at the time of clinical assessment and from healthy donors. A routine laboratory analysis was performed on fresh serum of patients with inflammatory myopathies. C-reactive protein (CRP) levels in patients and healthy controls were determined by an immuno-turbidimetric technique using an Olympus biochemical analyser (model AU 400, Tokyo, Japan). Creatine kinase and myoglobin levels were measured by routine laboratory methodology using Olympus analyser. All collected serum was stored at -80°C until further analysis. Serum resistin levels were measured with commercially available ELISA according to the manufacturer's protocol (Biovendor, Brno, Czech Republic). The levels of IL-1β, IL-6, TNFα and MCP-1 (RayBiotech, Norcross, GA, USA), along with the levels of perforin (Abcam, Cambridge, UK) and granzyme (Abcam) in cell culture supernatants were measured using commercially available ELISA kits. The absorbance was measured at 450 nm using an ELISA reader (Tecan Sunrise, Salzburg, Austria).

Immunohistochemistry

Samples for immunohistochemistry were obtained from patients with DM (n = 5) and myositis (n = 5) at the time of diagnostic muscle biopsy. In these patients, the muscle biopsy was guided by positive magnetic resonance imaging (MRI) findings from affected muscle as previously described [12]. Muscle tissue samples from patients with myasthenia gravis (MG, n = 5) were used as non-inflammatory controls. Muscle tissues were snap frozen in isopentane (2-ethylbutane, Sigma-Aldrich, St. Louis, MO, USA) cooled in liquid nitrogen. For the purposes of immunohistochemistry, 5-μm frozen sections were fixed in acetone and 4% paraformaldehyde and blocked with 0.3% H_2O_2. The sections were incubated in tris buffered saline (TBS) buffer and incubated with a monoclonal anti-resistin antibody (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) at a dilution 1:500 for one hour. Afterwards, the slides were rinsed again in TBS buffer. Antigen-antibody complexes were visualised with a Histofine detection system (Nichirei Biosciences Inc., Tokyo, Japan) using 3, 3'diaminobensidine as a chromogene. Rabbit IgG (Dako Cytomation, Glostrup, Denmark), diluted 1:1,000, was used as a negative control. The sections were slightly counterstained with Harris' hematoxylin. All sections were analysed semiquantitatively by two experienced pathologists who were blinded to the clinical data. The intensity of resistin expression was scored on a four-point scale (0 to 3). A staining intensity of 0 represented a negative result, and scores of 1 to 3 represented weak, moderate and strong positive staining intensity, respectively.

Cell culture and stimulation assays

Human skeletal muscle cells (Lonza, Basel, Switzerland) were cultured at a density of 100,000 cells in a six-well plate in 2 ml DMEM (Gibco, Carlsbad, CA, USA). PBMC were isolated by standard Ficoll density gradient centrifugation from blood samples donated by healthy donors. Freshly isolated PBMC were resuspended at a density of 10^6 cells per well in a 12-well plate in 2 ml of RPMI 1640 (Gibco, Carlsbad, CA, USA). Both myocytes and PBMC were stimulated with human recombinant resistin (Biovendor, Modrice, Czech Republic) at a concentration of 10, 100 or 1,000 ng/ml for 6 and 48 hours at 37°C in 5% CO_2 humidified atmosphere. In the initial experiment, PBMC were stimulated with either resistin (1,000 ng/μl) alone or in combination with polymyxin B sulphate (5 ug/ml, Sigma-Aldrich, St. Louis, MO, USA) to exclude possible endotoxin contamination. For the gene expression analysis, cells were lysed in RLT buffer (Qiagen, Hilden, Germany) 6 h after stimulation. The supernatants were collected following 48 h of stimulation. The samples were stored at -80°C until use.

RNA isolation and TaqMan Real-Time PCR

Total RNA was isolated using a MagNA Pure Compact RNA Isolation Kit for the MagNA Pure Compact Instrument (Roche Diagnostics, Mannheim, Germany), and reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using gene expression assays (Applied Biosystems), and the reaction was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Data were analysed using the ddCt method for relative quantification, and 18S was used as an endogenous control.

MTT assay

The evaluation of the proliferation of myocytes after 72 h stimulation with resistin (10, 100, 1,000 ng/ml) was carried out with an MTT test using dimethylthiazol diphenyl tetrazolium bromide (Sigma-Aldrich). The absorbance was measured at 570 nm via ELISA reader (Tecan Sunrise, Salzburg, Austria).

Statistical analysis

Data are expressed as the mean ± SD or mean ± SEM where indicated. The Mann-Whitney U test was used for comparisons between two variables, and the Kruskal-Wallis test, along with Dunn's multiple comparison tests, was used for comparisons among more than two variables. Paired T-tests were used for statistical analyses of gene expression. Spearman's and Pearson correlation coefficients were used to correlate any two variables with non-normal and normal distribution, respectively. *P*-values less than 0.05 were considered statistically significant. The analysis and the graphs were performed using GraphPad Prism 5 (version 5.02; GraphPad Software, La Jolla, CA, USA).

Results

Characteristics of patients

The characteristics of patients and healthy controls included in this study are given in Table 1. Our study group included 17 muscle biopsy-verified patients with DM and 25 with other types of myositis. Out of these, four had pure PM while 21 fulfilled criteria for overlap myositis. The mean duration of myopathy symptoms ranged from 1 to 154 months. Proximal muscle weakness occurred in 95% of patients. Rash was present in 40%, heliotrope rash in 30%, Gottron's papules/signs in 28%, V sign rash in 28%, shawl sign in 14% and mechanics hands in 35% of patients. The Raynaud

Table 1 Characteristics of patients with idiopathic inflammatory myopathies and healthy controls

Characteristic	IIM	HC
N	42	27
Age (years)	53.8 ± 14.2	49.5 ± 8.6
Sex (F/M)	31/11	22/5
CRP (mg/l)	9.3 ± 21.6	1.8 ± 1.5
CK (ukat/l, ULN 2.7)	33.6 ± 58.2	NA
Myoglobin (μg/l, ULN 70)	1345 ± 2422	NA
Clinical activity of myositis		
MYOACT global	0.15 ± 0.11	NA
MMT8 (maximum 80)	65.34 ± 12.31	NA
Autoantibodies (%)		
Anti-Jo-1	24	NA
Anti-Ro-52	37	NA
Anti-Ro-60	2	NA
Anti-PL-7	5	NA
Anti-PM-Scl-100	12	NA
Anti-PM-Scl-75	7	NA
Anti-Mi-2	12	NA
Anti-Ku	2	NA
Anti-SRP	5	NA
Anti-U1RNP	5	NA
Anti-histones	5	NA
Other (AMA, M2, CENP-B)	2	NA
Negative	21	NA
Treatment (%)		
Before therapy	19	0
Treatment less than 1 month	53	0
Treatment more than 1 month	26	0
Glucocorticoids	67	0
Others	19	0

Abbreviations: CK, creatine kinase; CRP, C-reactive protein; F, female; HC, healthy controls; IIM, inflammatory myopathies; M, male; MMT8, manual muscle testing of eight muscle groups; MYOACT, disease activity assessment visual analogue scales; N, number; NA, not analysed

phenomenon was present in 21% of patients, arthritis in 26% and dysphagia in 49%. Myositis-associated interstitial lung disease and cardiac involvement was diagnosed in 44% and 23% of patients with inflammatory myopathies. Eight patients were assessed prior to treatment initiation, and 23 patients were treated for less than one month. All other patients underwent treatment for more than one month. In the 29 patients treated with glucocorticoids, the median dose was 40 mg of prednisone or its equivalent/day ranging from 7.5 to 85 mg/day. Seven patients received methotrexate, one patient received methotrexate in combination with cyclosporine A and one patient was treated with azathioprine.

Increased resistin levels and disease activity in patients with inflammatory myopathies

The serum resistin levels were significantly higher in patients with inflammatory myopathies than in healthy controls (8.53 ± 6.84 ng/ml vs. 4.54 ± 1.08 ng/ml, $P < 0.0001$, Figure 1). Patients with both DM (7.39 ± 3.75 ng/ml, $P < 0.01$) and overlap myositis (9.65 ± 8.88 ng/ml, $P < 0.001$) had significantly higher levels of resistin compared to healthy controls. There were no significant differences in the levels of serum resistin between patients with DM and other types of myositis (7.39 ± 3.75 vs. 9.31 ± 8.30 ng/ml, $P = 0.626$) or between anti-Jo-1 positive and anti-Jo-1 negative patients (9.459 ± 5.948 vs. 8.242 ± 7.153, $P = 0.281$). The serum levels of resistin were comparable between male and female patients (7.160 ± 3.363 vs. 7.864 ± 4.459, $P = 0.761$) and were not affected by age (r = -0.154, $P = 0.331$) or disease duration (r = -0.104, $P = 0.513$). Furthermore, resistin serum levels did not differ significantly between patients treated with glucocorticoids for less and more than one month, and treatment with other immunosuppressive agents also did not affect the serum resistin levels. The resistin levels were not significantly different between patients with and without arthritis or patients with and without interstitial lung disease although these compartments may be a potential source of resistin.

In patients with inflammatory myopathies, but not in healthy controls, the serum resistin levels correlated with the CRP levels (r = 0.328, $P = 0.040$) and, interestingly, positively correlated with the global MYOACT score (r = 0.382, $P = 0.026$). In contrast, resistin levels correlated neither with the creatine kinase and myoglobin levels nor with the MMT8 score in patients with inflammatory myopathies. Furthermore, we found that resistin serum levels significantly correlated with CRP levels (r = 0.717, $P = 0.037$) and MYOACT (r = 0.798, $P = 0.007$) in anti-Jo-1 positive patients, but not in anti-Jo-1 negative patients (CRP r = 0.140, $P = 0.469$; MYOACT r = 0.283, $P = 0.181$). In addition, there was a trend towards correlation between serum resistin levels and myoglobin (r = 0.650, $P = 0.067$) in anti-Jo-1 positive patients.

In patients with overlap myositis, resistin serum levels correlated with CRP (r = 0.511, $P = 0.030$), but not with other measures. In patients with DM, serum resistin levels significantly correlated with MYOACT score (r = 0.667, $P = 0.001$), creatine kinase (r = 0.739, $P = 0.001$) and myoglobin levels (r = 0.791, $P = 0.0003$) and showed a trend towards correlation with CRP levels (r = 0.447, $P = 0.067$). Correlations between the levels of

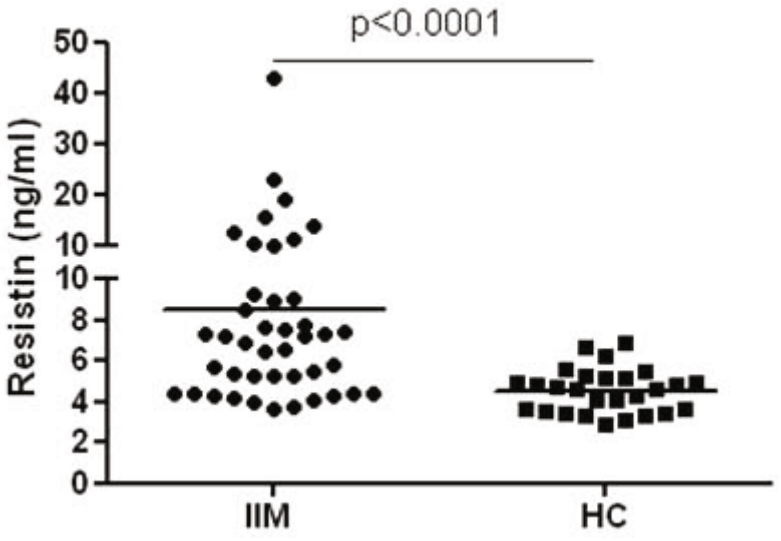

Figure 1 Increased serum resistin levels in patients with idiopathic inflammatory myopathies (IIM) compared with healthy controls (HC).

serum resistin and parameters of clinical myositis activity are summarized in Table 2.

Expression of resistin in muscle tissue

The assessment of resistin staining intensity in inflammatory infiltrates of the sections from muscle biopsies revealed a mean intensity of 1.8 in myositis and 1.6 in DM patients on the 0 to 3 scale. As expected, no lymphocytes were present in control individuals with MG. Therefore, muscle tissue samples from MG patients were used as non-inflammatory controls. We found that resistin expression in muscle tissues from patients with inflammatory myopathies was significantly higher when compared with control muscle tissues from patients with MG (Figure 2). The increased expression of resistin was particularly localised in scattered mononuclear cells and in mononuclear cells in inflammatory infiltrates surrounding large vessels and muscle fibres in patients with inflammatory myopathies. Mild resistin expression was observed in the cytoplasm of some muscle fibres. Resistin staining was observed in regenerating muscle fibres in four-fifths of myositis and three-fifths of DM patients, but not in control patients with MG (Figure 2). Identification of regenerating muscle fibers was based upon hematoxylin and eosin staining. Regenerating muscle fibers were smaller and splitting, with centrally located vesicular myonuclei. The cytoplasm of regenerating muscle fibers appeared basophilic.

Effects of resistin on mononuclear cells

PBMC (n = 8) were stimulated with increasing concentrations of resistin (0, 10, 100, 1,000 ng/ml) for 6 or 48 h. As shown in Figure 3, treatment of PBMC with resistin resulted in a significant and dose-dependent induction of IL-1β (up to 6.5-fold), IL-6 (up to 203-fold) and MCP-1 (up to 10.8-fold). The induction of TNFα at the mRNA level was only mild (up to two-fold). This expression pattern was followed by the increased cytokine release into the cell culture supernatants (Figure 4a-d). Cytokine levels after stimulation with the lowest doses of resistin were comparable with those of unstimulated cells but these values were significantly different after stimulation with higher doses of resistin. To study the effect of resistin on the cytotoxic activity of PBMC, the perforin and granzyme levels were analysed in cell culture supernatants. Only the highest concentrations of resistin resulted in a reduction in the release of both perforin and granzyme (Figure 4e, f).

To exclude endotoxin contamination of human recombinant resistin, PBMC (n = 3) were stimulated with resistin (1,000 ng/ml) alone or in combination with polymyxin B sulphate (5 μg/ml) for 6 h. There were no differences in the expression of the abovementioned cytokines, indicating that there was no nonspecific effect mediated by recombinant resistin (data not shown).

Table 2 Correlations between serum levels of resistin and disease activity in patients with inflammatory myopathies.

			IIM		Myositis		DM
Parameter	Correlation	all	Jo-1 +	Jo-1-	pure	overlap	
MYOACT	r	0.382	0.789	0.283	0.866	0.387	0.667
	p	0.026	0.007	0.181	0.33	0.125	0.001
MMT8	r	-0.055	-0.179	0.002	-0.400	0.020	-0.439
	p	0.765	0.632	0.993	0.750	0.944	0.134
Myoglobin	r	0.233	0.650	0.114	0.200	0.079	0.791
	p	0.159	0.067	0.490	0.917	0.794	0.0003
CK	r	0.145	0.417	0.122	0.200	-0.110	0.739
	p	0.385	0.270	0.528	0.917	0.663	0.001
CRP	r	0.328	0.717	0.14	-0.800	0.511	0.447
	p	0.044	0.037	0.469	0.333	0.030	0.067

Abbreviations: CK, creatine kinase; CRP, C-reactive protein; DM, dermatomyositis; IIM, inflammatory myopathies; Jo-1, anti-Jo-1 antibody; MYOACT, disease activity assessment visual analogue scales

Effects of resistin on myocytes

To assess the ability of resistin to induce a possible inflammatory response in skeletal muscle cells, as it does in mononuclear cells, myocytes (n = 9) were stimulated with increasing concentrations of resistin (0, 10, 100, 1,000 ng/ml). Although the treatment of myocytes with resistin for 6 hours contributed to the slightly increased gene expression of some pro-inflammatory cytokines (Figure 3), the release of these cytokines into the cell culture supernatants after 48 hours from stimulated and unstimulated cells did not significantly differ. There was also no resistin-associated effect of on the expression of several type I interferon induced genes, such as CXCL10, IFI27, IFI44, IFI44L, RSAD2, OAS1, ISG15, IFIT1, MX1 (data not shown). Furthermore, as assessed by MTT assay, resistin did not affect proliferation of skeletal muscle cells *in vitro*.

Discussion

In this study, we report for the first time an association between increased levels of serum resistin and the disease activity of patients with inflammatory myopathies, particularly in anti-Jo-1 positive and dermatomyositis patients. Additionally, we found that the expression of resistin is up-regulated in muscle tissues of patients with inflammatory myopathies. Lastly, we found that resistin may contribute to the increased production of pro-inflammatory cytokines in mononuclear infiltrates, thus indirectly participating in muscle tissue pathology.

Although resistin was initially associated with metabolic disorders, increased levels of resistin and its positive correlation with inflammatory markers and disease

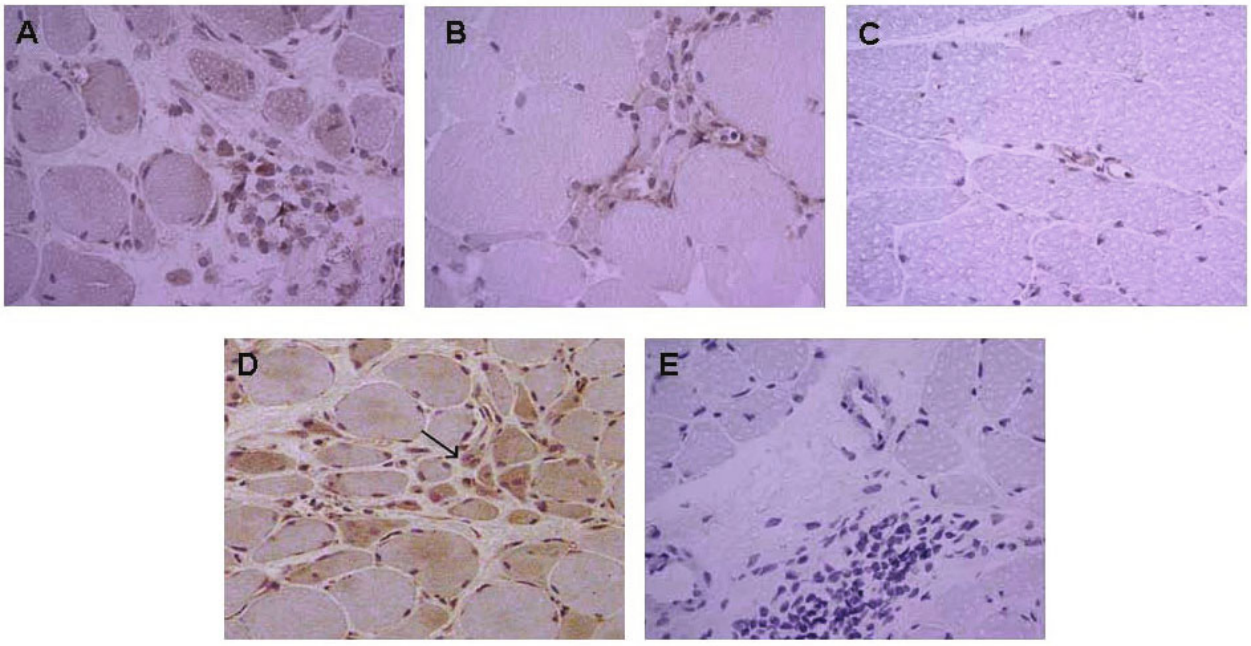

Figure 2 Expression of resistin in muscle tissue. Expression of resistin in affected skeletal muscle tissue from patients with dermatomyositis **(a)** and myositis **(b)** in contrast with no expression in the muscle tissue of myasthenia gravis **(c)**. Expression of resistin in regenerating muscle fibres (pointed with arrow) in a patient with dermatomyositis **(d)**. Corresponding tissue sections stained with isotype antibodies **(e)**. Resistin appears as brown. Nuclei were stained with hematoxylin. Original magnification, ×400 (a-c, e) and ×200 (d).

activity have been previously demonstrated in patients with rheumatoid arthritis (RA) [4,18-20]. In a study by Almehed and colleagues, serum resistin levels did not differ between patients with systemic lupus erythematosus (SLE) and healthy controls [21]; whereas in another study, SLE was independently associated with higher resistin levels [22]. Moreover, in both studies, the levels of resistin positively correlated with inflammatory markers, disease-specific measures and renal dysfunction. In our study, we observed a strong correlation between higher levels of serum resistin and CRP and, most importantly, we observed an association with the global disease activity assessment of inflammatory myopathy. Importantly, we found that serum resistin levels were strongly associated with CRP and global disease activity, and a trend was also observed towards correlation between resistin levels and myoglobin in patients with myositis-specific anti-Jo-1 antibody in contrast to anti-Jo-1 negative patients. Furthermore, resistin levels significantly correlated with global disease activity and muscle enzymes in DM patients. These results are in line with the abovementioned findings, further supporting an association between resistin and autoimmune rheumatic diseases [4,18,22]. On the other hand, serum resistin levels did not correlate with muscle weakness. Thus, it could be hypothesized that serum resistin concentration may reflect global disease activity, including extramuscular organ involvement, rather than functional impairment in inflammatory myopathies. However, disease specific mechanisms can be suggested.

Recently, we demonstrated the increased expression of resistin in immune cells of RA synovial tissue [19]. In the present study, we consistently observed the up-regulation of resistin in the mononuclear cells of inflammatory infiltrates surrounding vessels and muscle fibres in patients with inflammatory myopathies. In contrast, there was no expression of resistin in control non-inflammatory muscle tissue. Importantly, a majority of samples from DM and myositis patients showed resistin staining in regenerating muscle fibres, perhaps implicating a contribution of the muscle tissue to the inflammatory process or influence of resistin on regenerating/immature muscle precursors in the pathogenesis of myositis. The presence of fibre degeneration and regeneration is a typical histopathological picture of inflammatory myositis. Initially, muscle repair is characterized by inflammation and degeneration of damaged muscle tissue. It is followed by activation, proliferation and differentiation of myogenic, so-called satellite cells with subsequent fusion and formation of multinucleated muscle fibres. This process requires a crosstalk between immune and muscle cells, including secreted factors [23,24]. In fact, at the moment it is not possible to distinguish the features of the inflammatory myositis that promote the injury from those that cause muscle

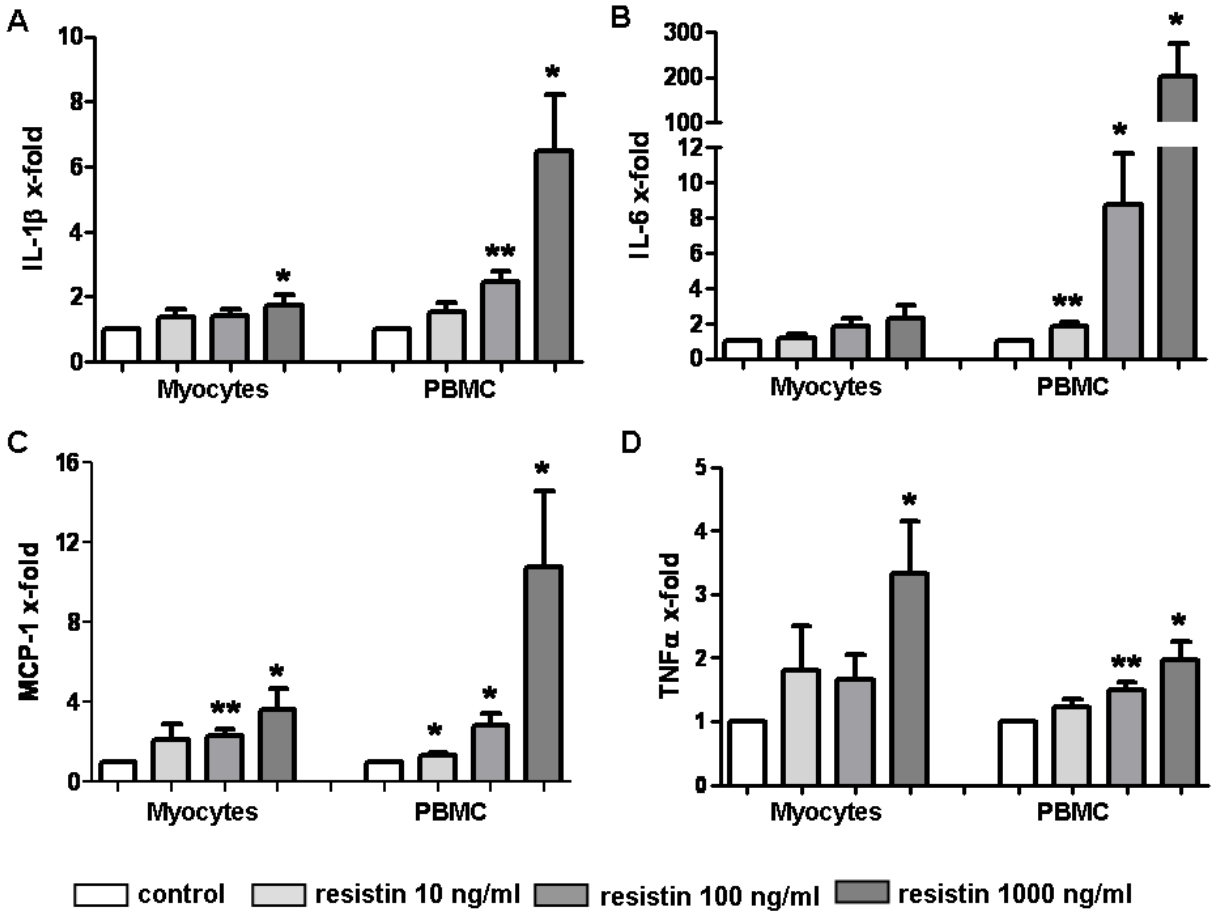

Figure 3 Effects of resistin on inflammatory response in myocytes and peripheral blood mononuclear cells (PBMC). Expression of IL-1α **(a)**, IL-6 **(b)**, MCP-1 **(c)** and TNFα **(d)** mRNA levels after stimulation with human resistin (10, 100, 1,000 ng/ml) for six hours in myocytes and PBMC. Data are shown as fold changes compared to unstimulated controls (in graphs represented by control bar, rated as 1). Bars represent the mean + SEM. *P*-values less than 0.05 were considered statistically significant; * $P < 0.05$, ** $P < 0.01$.

regeneration and repair [25]. Accordingly, the role of resistin in the pathology of muscle tissue in myositis is underpinned by its association with parameters of disease activity in the group of patients with myositis associated autoantibody Jo-1. As we found increased systemic and local levels of resistin in inflammatory myopathies, we examined the effect of resistin on muscle and mononuclear cells *in vitro*. In line with previous reports [8,26], we found that resistin induces expression and synthesis of several proinflammatory mediators in mononuclear cells, thus possibly contributing to muscle tissue pathology. As demonstrated previously, IL-1, IL-6, MCP-1, as well as TNFα, are increased in myositis muscle tissues and contribute to disease pathogenesis [27-29]. However, these cytokines may have dual function and may not only contribute to muscle tissue damage, but also to the regeneration and healing of the muscle tissue [25,30]. Considering the ambivalent role of several mediators in inflammatory myositis, dual functions of resistin may be suggested. In this regard, we observed a trend towards the reduced synthesis of the cytotoxic enzymes perforin and granzyme in resistin-stimulated immune cells.

It has been shown that several Toll-like receptors (TLRs) are expressed in immune cells but are not present in the muscle fibres of patients with inflammatory myopathies [31]. A recent study demonstrated that a TLR4 receptor mediates the proinflammatory effects of resistin in human cells [32]. Therefore, we assume that the lack of resistin-associated effects on myocytes in our study may be due to the absence of TLR4 receptors on the surface of these cells. That being said, resistin did not modulate the expression of several interferon (IFN)-α/β induced genes that have been recently observed in tissue of patients with inflammatory myopathies [33]. Interestingly, immature muscle precursors in myositis biopsy tissues have been recently demonstrated as an

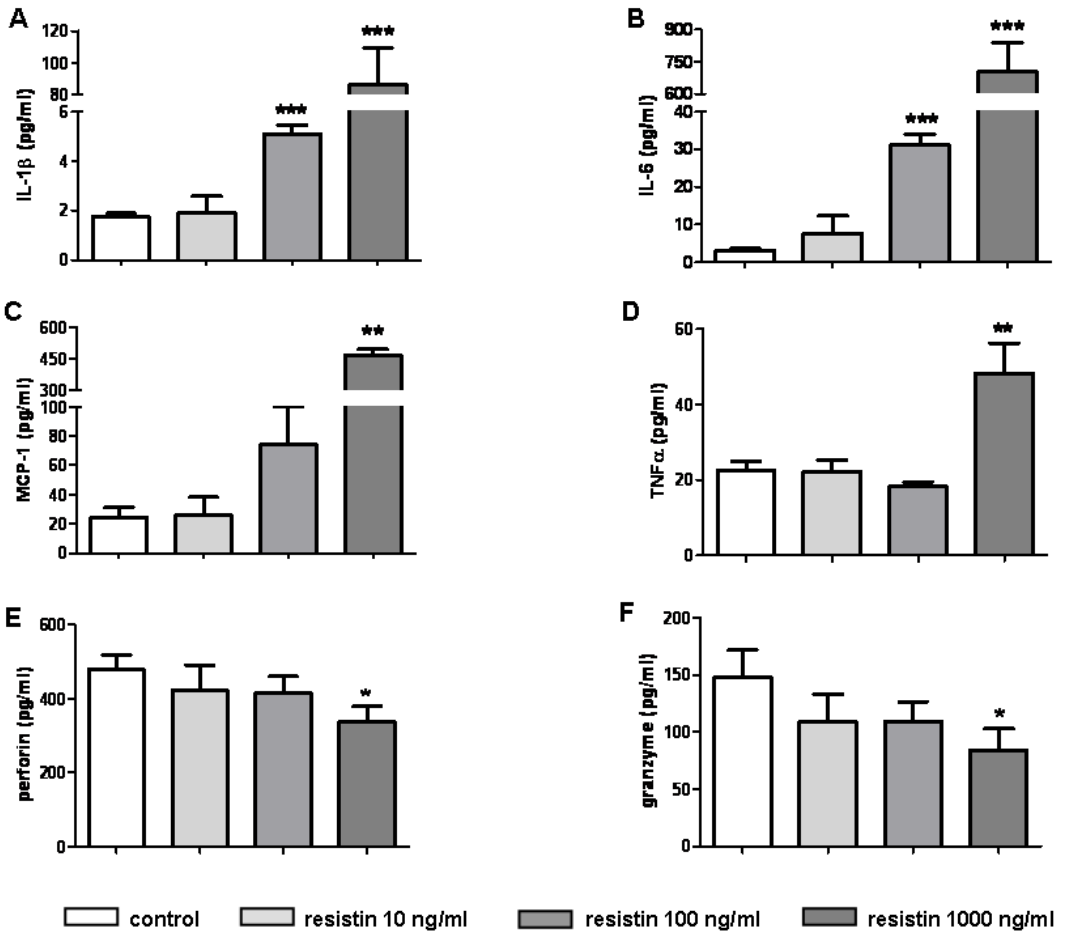

Figure 4 Effects of resistin on peripheral blood mononuclear cells (PBMC). Resistin induces the release of IL-1α **(a)**, IL-6 **(b)**, MCP-1 **(c)** and TNFα **(d)** and reduces the release of perforin **(e)** and granzyme **(f)** from PBMCs into cell culture media after 48 hours. Bars represent the mean + SEM. *P*-values less than 0.05 were considered statistically significant; * $P < 0.05$,** $P < 0.01$, *** $P < 0.001$.

important source of IFN-β, which was, however, mediated by TLR-3 activation [34].

Conclusion

We have demonstrated that increased levels in tissue as well as serum concentration of resistin in patients with inflammatory myopathies correlate with global disease activity. In patients with myositis-specific anti-Jo-1 antibody and, particularly, in dermatomyositis patients, elevated resistin levels associated with disease activity and muscle enzymes. We suggest that resistin indirectly participates in muscle tissue damage by inducing the production of pro-inflammatory cytokines by mononuclear cells. The exact role of resistin in muscle tissue regeneration or destruction in inflammatory myopathies needs further study.

Abbreviations

ADSF: adipocyte-secreted factor; CRP: C-reactive protein; DM: dermatomyositis; F: female; FIZZ3: inflammatory zone 3; HC: healthy controls; IFN: interferon; IIM: inflammatory myopathies; IL: interleukin; M: male; MCP-1: monocyte chemoattractant protein 1; MG: myasthenia gravis; MMT8: manual muscle testing of eight muscle groups; MRI: magnetic resonance imaging; MYOACT: myositis disease activity assessment visual analogue scales; NA: not analysed; PBMC: peripheral blood mononuclear cells; PM: polymyositis; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; TBS: tris buffered saline; TLR: Toll-like receptors; TNF: tumour necrosis factor.

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Authors' contributions

MF performed the majority of the *in vitro* experiments and the statistical analysis, in addition to preparing the manuscript. HH performed the laboratory measurements. KK performed the immunohistochemistry and was involved in enrolling the patients. LAC and MK contributed to *in vitro*

experiments. LP, HM, JZ and JV were involved in enrolling the patients and their clinical data. SG and JV assisted in the design of the study. LS was in charge of the design and conception of the study, helped with the interpretation of the data and with drafting the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Příloha 5

Serum levels of interferon α do not correlate with disease activity in patients with dermatomyositis/polymyositis

Idiopathic inflammatory myopathies include three major diseases: dermatomyositis (DM), polymyositis (PM) and inclusion body myositis.[1–3] The ethiopathogenesis of these entities is still not well understood. Recent studies show an important role of type I interferon (IFN) in the proinflammatory process leading to disease manifestations in muscle tissue.[4–6]

In order to investigate the relationship of soluble IFNα serum levels with clinical and laboratory characteristics we analysed serum samples of 43 patients with DM/PM (24/19 patients with DM and PM, respectively; 32 women/11 men). Patients were selected from a cohort of 81 PM/DM cases with the preference for those who were anti-Jo-1 positive, and had muscle MRI performed. In total, 44% of patients (n=19) were not treated prior to the collection of blood samples (group 1). Patients treated with ≤20 mg of prednisone per day at the time of blood draw were assigned to group 2 (n=15; 35%) and patients treated with prednisone >20 mg per day formed group 3 (n=9; 21%). Methotrexate or other immunosuppressive drugs were used in nine and three patients in groups 2 and 3, respectively. IFNα levels were measured using a bead-based assay (Human IFNα FlowCytomix Simplex; Bender MedSystems, Vienna, Austria; sensitivity 2.2 pg/ml). Results in each group were compared to those of 25 healthy controls (C1) and 6 positive controls with acute viral infection (C2). Anti-Jo-1 antibodies were detected by line blot assay (Inno-Lia ANA Update; Innogenetics, Ghent, Belgium). Data on the presence of interstitial lung fibrosis as detected by high-resolution CT (n=22) or chest x-ray (n=4) were also compared.

Clinical activities of muscle and each organ involvements were determined using the myositis disease activity assessment visual analogue scale (MYOACT) scoring system.[7] Disease activity was also assessed by muscle MRI which was evaluated on the 10 cm visual analogue scales (VAS) for extent, intensity and total MRI affection.[8]

A statistically significant negative correlation was found between IFNα and the intensity of MRI signal (figure 1). None of the clinical or laboratory parameters as assessed by MYOACT showed any correlation with IFNα levels, with the exception of the tendency to higher IFNα levels (p=0.064) in the presence of interstitial lung disease. Significantly lower levels of IFNα were found in patients with myositis in comparison with groups C1 and C2, but no difference was noted between individual patient groups (figure 2). IFNα levels were significantly higher in patients who were anti-Jo-1 positive (n=26; median 117.8 pg/ml, range 70–378) in comparison to patients who were anti-Jo-1 negative (n=17; median 93.4 pg/ml, range 0–199) (p=0.05). There were no significant differences in IFNα levels between patients with PM and those with DM.

IFNα has recently been implicated in the pathogenesis of myositis.[5] We observed higher levels of IFNα in patients who are anti-Jo-1 positive, which is in accordance with described IFNα-inducing capacity of anti-Jo-1 positive serum samples.[9] However, serum levels of IFNα in patients with PM and DM do not reflect suggested increased tissue content[10]; on the contrary, IFNα serum levels in our patients with myositis were lower than serum levels in healthy controls. This is not a consequence of the treatment since the levels of IFNα were not different in patients tested before or during the treatment. Serum levels of IFNα do not seem to be an indicator of clinical activity, rather the opposite; the lower the serum level the more severe the muscle oedema, as demonstrated by the intensity parameter on MRI. Hypothetically, IFNα produced locally could be also locally consumed and not released into the circulation. Alternatively, other type I IFNs and not the IFNα may be responsible for the type I IFN signature that is characteristic for many patients with DM and PM.

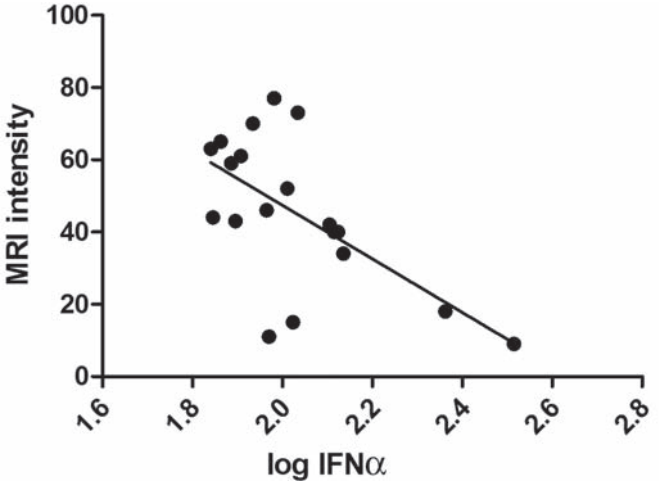

Figure 1 Interferon (IFN) α and MRI intensity in patients showed a negative correlation (Spearman's rank correlation: sr=−0.58; p=0.0095).

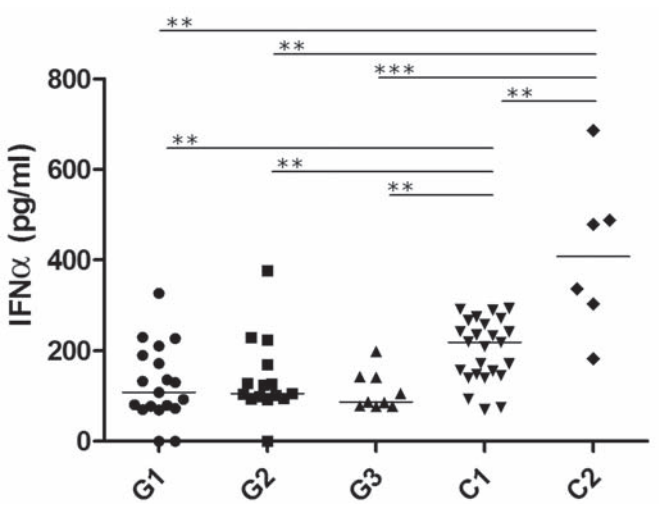

Figure 2 Serum levels of interferon (IFN) α (pg/ml) in three groups (G) of patients with dermatomyositis (DM)/polymyositis (PM) (G1, patients not treated; G2, treated with ≤20 mg prednisone per day; G3, treated with >20 mg prednisone per day) compared to healthy controls (C1) and controls with viral infection (C2). Each group of patients showed a significant difference in comparison to controls (**<0.01; ***<0.001), comparisons between groups of patients were not significant. Horizontal bars indicate median levels. p Values were determined using the Mann–Whitney test.

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Příloha 6

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Autoantibody specificities and type I interferon pathway activation in Idiopathic Inflammatory Myopathies

Running head: Autoantibodies and type I Interferon in Myositis

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Scientific heading: Clinical immunology

Abstract

Myositis is a heterogeneous group of autoimmune diseases, with different pathogenic mechanisms contributing to the different subsets of disease. The aim of this study was to test whether the autoantibody profile in myositis patients is associated with a type I interferon (IFN) signature, as in patients with systemic lupus erythematous (SLE). Patients with myositis were prospectively enrolled in the study and compared to healthy controls and to patients with SLE. Autoantibody status was analyzed using an immunoassay system and immunoprecipitation. Type I IFN activity in whole blood was determined using direct gene expression analysis. Serum IFN inducing activity was tested using peripheral blood cells from healthy donors. Blocking experiments were performed by neutralizing anti-IFNAR or anti-IFNα antibodies. Patients were categorized into IFN high and IFN low based on an IFN score. Patients with autoantibodies against RNA-binding proteins had a higher IFN score compared to patients without these antibodies and the IFN score was related to autoantibody multi-specificity. Patients with dermatomyositis (DM) and inclusion body myositis (IBM) had a higher IFN score compared to the other subgroups. Serum type I IFN bioactivity was blocked by neutralizing anti-IFNAR or anti-IFNα antibodies. Concluding, a high IFN score was not only associated with DM,as previously reported, and IBM, but with autoantibody mono-specificity against several RNA-binding proteins and with autoantibody multispecificity. These studies identify IFNα in sera as a trigger for activation of the type I IFN pathway in peripheral blood, and support IFNα as a possible target for therapy in these patients.

Introduction

Idiopathic inflammatory myopathies (IIM), also known as myositis, are rare chronic autoimmune diseases, characterized by proximal muscle weakness and muscle inflammation and can be subgrouped into polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM) [1, 2]. A

common feature is presence of autoantibodies, which is associated with distinct clinical phenotypes [3]. Some autoantibodies are exclusively found in myositis, so called myositis-specific autoantibodies, e.g., anti-Jo-1 and anti-SRP antibodies, whereas others, myositis-associated autoantibodies, such as anti-Ro52, anti-Ro60, anti-La, anti-PM/Scl, and anti-U1RNP antibodies, are also found in other autoimmune diseases [4]. Autoantibodies can present years before the onset of clinical symptoms indicating a role in the initiation of the disease but the mechanisms for this are as yet unknown [5].

The molecular mechanisms driving inflammation in patients with IIM are not fully understood. An activated type I interferon (IFN) pathway has been demonstrated in many autoimmune diseases [6] and has been proposed to be involved in the pathogenesis in IIM, particularly in the DM subgroup, but the mechanism driving the type I IFN pathway has not been clarified [7-12]. In general, plasmacytoid dendritic cells (pDCs) produce type I IFNs induced by viruses and also by immune complexes (ICs) consisting of autoantibodies directed against nucleic acids or nucleic acid binding proteins [13]. Autoantibodies against RNA-binding proteins have been associated with the type I IFN production in patients with systemic lupus erythematosus (SLE) [14-16]. We have previously demonstrated that sera from patients with PM and DM with anti-Jo-1 or anti-Ro52/60 autoantibodies, together with RNA, may act as endogenous IFN inducers in pDCs [17]. These observations suggest that there may be a role for the type I IFN system not only in DM, but also in other subtypes of myositis, where patients have antibodies against RNA or RNA-binding proteins and that autoantibodies may have a role in driving the type I IFN pathway in subsets of patients with IIM.

The aim of the present study was to test the hypothesis that autoantibodies directed against RNA-binding proteins in patients with myositis are associated with a type I IFN signature and thus potentially could induce IFN production. Moreover, we investigated the nature of the mediator that is responsible for the IFN activity in the blood of IIM patients.

Patients and methods

This cohort consisted of prospectively enrolled patients between 2006 and 2009 from the Rheumatology Unit, Karolinska University Hospital, Stockholm, Sweden, and Institute of Rheumatology, Prague, Czech Republic, and fulfilled the criteria for definite or probable PM/DM [18, 19] or sporadic IBM [20]. Exclusion criteria were presence of overlap syndrome, treatment with biologicals and no available antinuclear antibody (ANA) status. A patient was defined as newly diagnosed when he or she had a maximum disease duration of a month and had no immunosuppressive treatment.

As a comparator group, 47 patients with SLE were recruited at the Rheumatology department, VU University medical center, Amsterdam, The Netherlands. Since the SLE cohort was used as a reference, patients with available SLE Disease Activity Index (SLEDAI) were included (SLEDAI 0, n=24, or SLEDAI >6, n=18). Forty-one healthy controls (HC), (23 (56%) female, mean age 35 years) were recruited at the VU University medical center, Amsterdam, The Netherlands.

This study was approved by the local ethics committees and informed consent was obtained from all subjects in the studies. Ethical permit Karolinska Hospital, Stockholm, Sweden: D-nr 2005/792-31/4 and 2011/1374-32, in VU Medisch Centrum, Amsterdam, the Netherlands: registration number 2007/125 and in Department of Rheumatology, Praque, Czech Republic: ref.nr 3233/2007.

Clinical and laboratory data

The patients' overall disease activity at the time of serum sampling was assessed by the "Disease Activity Core Set Measures" according to the International Myositis Assessment and Clinical Studies (IMACS) Group [21, 22], based on prospectively collected data and retrieved from local myositis

databases at the Karolinska University Hospital and Institute of Rheumatology, Prague and from the web-based Euromyositis register, www.euromyositis.eu. Information on medication, malignancy (within 3 years before or after myositis diagnosis) and interstitial lung disease (ILD) was retrieved from the registries and from patient records. Interstitial lung disease (ILD) was defined as previously described [23]. Creatinine phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels in blood were analyzed as routine tests at the local Departments of Clinical Chemistry, and ANA was analyzed by immunofluorescence as a routine test at the Department of Clinical Immunology, Karolinska University Hospital, Sweden, or in the Laboratory Department of the Institute of Rheumatology, Prague.

Autoantibody assays

Sera in myositis patients were analyzed for autoantibodies against Jo-1, SRP, Mi-2, PM/Scl 70, PM/Scl 75, PM/Scl 100, PL7, EJ, Ku, Ro52, Ro60, La, and U1RNP using a validated line immunoassay system (Euroimmun, Lübeck, Germany), according to manufacturer's instruction. In addition, all patient sera were analyzed for autoantibodies against Jo-1, SRP, Mi-2, PM/Scl, Ku, PL7, PL12, EJ, OJ, Zo, KS, NXP2, TIF1 gamma, SAE, MDA5, RNAPI, RNAPII, RNAPIII, AMA, Topo, Ro60, La, U1RNP and U3RNP, using immunoprecipitation (IP) as previously described [24]. Lineblot is a reliable alternative to immunopreciptiation [25], but as there is some difference in specificities captured by the two methods, e.g. Ro52 we defined a patient as positive for an autoantibody if the autoantibody status was positive in one of the two used methods. Multi-specific antibody status was defined as presence of two or more autoantibodies (all specificities included), ANA not included. All tested antisynthetase autoantibodies (Jo-1, PL7, PL12, EJ, OJ, Zo, KS), in addition to antibodies against U1RNP and Ro60 were defined as autoantibodies against RNA-binding proteins.

IFN signature in whole blood

For whole blood RNA isolation, 2.5 ml blood was drawn in PAXgene tubes (PreAnalytix, GmbH, Germany) and RNA isolation, quantification and purification were performed as previously described [26]. RNA (0.5 µg whole blood RNA derived from PAXgene tubes) was reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions. Real-Time PCR analysis was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, U.S.A), according to the manufacturer's instructions. Relative quantities were calculated using the ddCT method. GAPDH was used as a housekeeping gene and all arrays contained two samples for calibration.

Expression was determined of eight IFN-response genes (IRG), IFI3, IFIT2, MxA, IFI44L, HERC5, IFIT1, RSAD2 and OAS3, (all corrected versus GAPDH, log2 according to earlier studies [10, 12, 27]). Since the IRGs were highly correlative (Pearson $r>0.8$ for all combinations, $p<0.001$), we calculated an IFN score by averaging the expression levels of all IRGs per sample [28-30]. The mean + 2 standard deviations of the IFN score in HC was used as a cut off to define if an IFN signature was present (IFN high) or absent (IFN low).

IFN regulated gene (IRG) induction assay and neutralization

Next we wanted to test if patient sera had the capacity to induce IFN activity. Peripheral blood mononuclear cell (PBMC) isolation from heparinized blood from one healthy donor (National blood bank, Netherlands) was performed using Lymphoprep (Axis Shield, Oslo, Norway) according to the

manufacturer's protocol [26]. After isolation, the PBMCs were cryopreserved in IMDM supplemented with 10% FCS and 10% DMSO and stored in liquid nitrogen until further use. Healthy donor PBMCs (with a cell concentration of $2x10^6$/ml) were incubated with 20% sera from 20 randomly selected IIM patients for 4h or 8h at 37°C and 5% CO_2. To identify the specificity of type I IFN in sera from a second subset of 25 randomly selected samples from IIM patients, 30 ng/ml neutralizing anti-IFNα antibody (#21105, PBL, Piscataway, New Jersey, USA) or 2.5 μg/ml neutralizing anti-IFNα-receptor (IFNAR2) antibody (#21385, PBL, Piscataway, New Jersey, USA) were added to the cultures. After incubation, cells were harvested, washed and lysed in RLT buffer (Qiagen Benelux BV, Venlo, The Netherlands) according to the manufacturer's protocol. The lysates were stored at -20°C until RNA isolation. RNA isolation from cultured cells and reverse transcription of cDNA and Real Time qPCR was performed and assessed as previously described [26], with the exception that the expression levels of target genes were calculated relative to housekeeping gene 18S ribosomal RNA (18SrRNA). To correct for any variations between experiments, all expression values are relative to healthy controls. The overall IRG induction was determined by calculating the average expression of three known IRGs; RSAD2, IFI44L and MX1 [28, 29].

Genotyping

Human leucocyte antigen (HLA) typing was performed for the myositis patients in order to investigate a possible link between HLA type and IFN activity. Sequence-specific primer–PCR (DR low-resolution kit; Olerup SSP, Saltsjöbaden, Sweden) was used for all Swedish patients. The PCR products were loaded onto 2% agarose gels for electrophoresis. An interpretation table was used to determine the specific genotype according to the recommendations of the manufacturer [31]. Allelic polymorphism of HLA-DRB1 and HLA-DQB1 genes for the Czech patients was analyzed by DNA-based typing using commercial sets (OneLambda, Los Angeles, USA) according to manufacturer's instructions.

Statistical analyses

Data were analysed using GraphPad Prism 4 or 5 Software.

The significance of differences between groups was calculated by Mann-Whitney U test or student's t-test when appropriate for continuous variables, or by Pearson's Chi square tests or Fischer's exact test for categorical variables. Differences in IRG induction over time were tested using a paired t-test. Correlation analyses were done using Pearson r or Spearman r tests. P-values <0.05 were considered statistically significant.

Results

Patients

One hundred and eight patients with IIM were included, of which 29 were Czech and 79 were Swedish. Twelve of the Swedish patients were excluded according to the exclusion criteria and four patients based on missing IFN data due to technical issues. The remaining 92 were included. Ten of the Swedish patients and 2 of the Czech patients were newly diagnosed and were on no medications at the time of blood sampling. Patient characteristics at the time of blood sampling are shown in table 1. The most common antibody specificities were Jo-1 (n=23), Ro52 (n=22), Ro60 (n=10).

Antibody specificities in the clinical subdiagnoses of the 92 included patients, are presented in Supplementary Table 1.

IFN activity in whole blood

The IFN activity was significantly higher in patients with myositis compared to HC (p=0.0007), and equal to patients with SLE (Figure 1A). The patients were categorized into two groups, IFN high (n=41, 45%) and IFN low (n=51, 55%), based on the cut off value for the average gene expression in

HC. Patients with DM and IBM had higher IFN scores compared to PM patients (p= 0.04 and 0.04 respectively) (Figure 1B). There were large variations within these clinical subgroups.

IFN signature is related to autoantibody multi-specificity and to autoantibodies against RNA-binding proteins

In order to search for a possible endogenous inducing factor of the type I IFN pathway we analyzed the relationship between the differential activation of the type I IFN pathway and presence of autoantibody specificities in IIM.

Twenty IIM patients had a multi-specific autoantibody status of whom 14 (70%) had an IFN signature, which was significantly more frequent than in patients with only one autoantibody specificity (18 out of 30 (60%), p=0.038) and in patients with no detectable autoantibodies (27 out of 42 (63%), p=0.002). In addition, a significantly higher IFN score was observed in the patients with multi-specific autoantibody status compared to those with only one (p=0.024) or no (p=0.0098) autoantibody specificities (figure 2A).

Autoantibody specificities in the patients with multi-specific autoantibody status are shown in supplementary table 2.

To determine whether the IFN signature was associated with distinct autoantibody specificities, patients with mono-specific autoantibody status were selected (n=30). Autoantibody specificities and clinical subdiagnoses in patients with monospecific autoantibody status are shown in supplementary table 3. The IFN signature was clearly present in patients with mono-specificity for autoantibodies against RNA-binding proteins, such as anti-Jo-1 (55% positive for IFN signature), anti-Ro60 (50% positive) and anti-U1RNP (100% positive) autoantibodies, and was absent in most patients with mono-specificity for autoantibodies against other targets than RNA-binding proteins, such as, anti-Ro52 (20% positive for IFN signature) and anti-PM/Scl (20% positive). The IFN score was also

significantly higher for patients with autoantibodies against RNA-binding proteins compared to the other patients (p=0.011) (Figure 2B), and the frequency of patients positive for an IFN signature was significantly higher in this group (p=0.003).

IFNα in patients' sera is responsible for type I IFN pathway activation

Next we investigated if sera from IIM patients (DM=9, PM=8, IBM =3, in total n=20) had the capacity to activate the type I IFN pathway, i.e., upregulation of IRG expression in healthy donor PBMCs. The average ex-vivo whole blood IRG induction for all samples (1.96) was used to subdivide patients into IFN high and IFN low. Ten of the IIM patients' sera, defined as 'IRG high' showed type I IFN pathway-activating capacity four hours after serum-addition (mean 3.13) whereas significantly lower IRG induction was seen for the ten 'IRG low' patients (mean 1.41) (Figure 3A). The IFN high samples had a significantly higher expression of IRG compared to IFN low samples ($p<0.0001$). Serum-induced IRG expression levels after four hours of incubation correlated positively with the IFN signature in whole blood ($r=0.4$, $p=0.005$). Similar observations were made with serum from SLE patients (data not shown). There were no significant differences in disease activity and autoantibody status between the IFN high and IFN low patients in these experiments. None of the patients' sera induced IRG expression eight hours after serum addition (Figure 3A).

In a second subset of IIM patients (DM=12, PM=7, IBM=6), in total n=25) neutralizing anti-IFNAR or anti-IFNα antibodies were added to the sera and the type I IFN bioactivity was determined after four hours. In IRG high patient samples (DM=2, PM=1, IBM=2), but not in IRG low samples, IFN bioactivity was significantly inhibited by both anti-IFNAR antibodies p=0.0160) and anti-IFNα antibodies (p=0.0095) confirming IFNα being mainly responsible for the type I IFN activity in these patients (Figure 3B). In both these experiments we did not see any significant statistical difference between the distribution of subdiagnoses of IIM in the IRG high and IRG low groups.

Correlation between IFN score and disease activity

To explore whether an IFN signature could be associated with a clinical phenotype we investigated if the IFN score correlated to clinical manifestations. A low degree of correlation between the extent of the IFN score and disease activity (Physician's global disease activity assessment) was found for patients with DM (n=32, r=0.3778, p=0.03) (Figure 4) but not for the whole group with IIM, nor was any correlation seen between disease activity measures and IFN score for PM and IBM. We could not observe a correlation between the IFN score and other clinical or laboratory variables assessed (Table 2).

No association between HLA genotype and IFN signature

We analyzed the association of presence of an IFN signature in patient serum with HLA-DRB1 genotype. No differences in frequencies of haplotypes of HLA-DRB1 were revealed between IFN high and IFN low patients.

Discussion

The results in our study reveal an association between the type I IFN signature and a subgroup of myositis patients with autoantibodies against RNA-binding proteins, in addition to the previously reported association with the subdiagnosis DM. The association between the type I IFN signature and patients with autoantibodies against RNA-binding proteins was stronger in patients with more than one autoantibody specificity. We could demonstrate that IFNα activity is present in sera of subsets of IIM patients of all subdiagnoses and that IFNα can trigger the type I IFN pathway resulting in an IFN signature in peripheral blood of these patients.

The underlying pathogenesis of myositis has not yet been established. IFNα has been identified as a key cytokine predisposing to, and driving, SLE pathology [32] and prior studies suggest a similar phenomenon in myositis, particularly for the DM subgroup [7, 8, 33]. Several case reports on development of PM/DM during IFNα/β therapy have been published [34]. The cause and source of the IFN signature seen, particularly in myositis, have not been clarified [9, 10, 12]. Viral induction has been proposed, but causal relationship is lacking. Another explanation could be an endogenous trigger such as immune complexes as reported in patients with SLE or Sjögren´s syndrome [16]. In juvenile DM (JDM) a correlation was shown between presence of autoantibodies against RNA-containing autoantigens and serum IFNα activity and the capacity to generate interferonogenic immune complexes (ICs) [35]. Similarly, we found that patients with autoantibodies against RNA-binding proteins had a higher IFN score in whole blood than patients without these autoantibodies. In addition, a higher IFN score was observed in the patients with multi-specific autoantibody status compared to those with only one or no autoantibody specificities, suggesting a dose response. The association with multispecificity could in 17 of 20 cases be explained by association to presence of autoantibodies against RNA-binding proteins. Additional support for a potential role of autoantibodies as inducing factors of the type I IFN system is the previously observed higher serum levels of IFNα in anti-Jo-1 autoantibody positive patients compared to anti-Jo-1 negative patients [11], as well as IFNα-inducing capacity of anti-Jo-1 serum samples [17]. These results are in line with reports from studies in SLE which have demonstrated that nucleic acid-containing ICs and their interaction with TLR7/TLR9 may induce the type I IFN pathway, suggesting that ICs containing RNA binding proteins may serve as endogenous inducers of type I IFNs. This could also be the case in patients with myositis [36, 37]. The association between autoantibody status and IFN score might suggest an IFN induction through ICs containing RNA and associated RNA binding proteins. However, some patient with DM and IBM did not have any detectable autoantibodies targeting RNA binding proteins, thus other mechanisms must also be involved. Other factors in sera, e.g., interleukins could also contribute to IFN activity as well as gene variants in the type I IFN signaling pathway [38].

We observed significantly more DM patients in the IFN high compared to the IFN low group, regardless of autoantibody status. Furthermore, patients with DM or IBM had a higher IFN score than PM, which is in line with previous observations [7, 8]. Upregulation of IFN-inducible genes has also been observed in the skin of patients with DM [39], and the presence of pDCs within the epidermis of DM skin suggests that the IFN-mediated processes may take place in the skin, as well as in the muscles of these patients by mechanisms other than the immune complex mediated mechanism discussed above [40].

By using blocking experiments we could demonstrate that IFNα present in the sera of IIM patients was responsible for the majority of the observed *in vitro* IRG induction, and that it is rapidly produced, within a few hours. Thus it is very likely that IFNα is present in the serum of IIM patients. A previous study has also shown a role for IFNß in sera from DM patients [41]. However, in this study IFNß was not measured.

In our study many patients did not display an IFN signature and were categorized as IFN low. One explanation could be that most of the patients were on treatment with glucocorticoids which are inhibitors of IFN production [42]. A study in JDM suggests that IFNα may be important in the early phase of disease [43]. Most patients in our study were, however, not in an early phase of their disease (mean disease duration of 3 years). It is also difficult to compare our IFN scores with previous studies, since different IFN assays have been used in different studies. However, we could show that the IFN score in myositis patients was equal to patients with SLE, a disease previously reported to be associated with a high IFN score [44]. A limitation of our study is the small sample size in the subgroup concerning autoantibody multispecificity, whereby it is difficult to draw complete conclusions.

There was only a weak correlation between the overall disease activity measure and the IFN score in blood for patients with DM, but not for the whole group with IIM, or for PM and IBM. There could be several explanations for this. One is that the overall disease activity was low due to ongoing immunosuppressive treatment. No association was observed between IFN score and age or sex (data not shown), therefore the HCs were not sex and age matched. Nor did we see an association between IFN score and other clinical or laboratory variables assessed (table 2).

No correlation was seen between HLA haplotype and presence of an IFN signature in this study, but this could be due to the limited number of patients. Similar results have been shown in JDM [43]. However, a large proportion of autoimmune disease risk genes are within the type I IFN signaling pathway [45].

In conclusion our study underscores that different molecular pathways may predominate in different subsets of myositis, emphasizing the need for careful molecular phenotyping of patients to gain better understanding of molecular pathogenesis and to improve treatment. The type I IFN pathway is activated in subsets of myositis patients with autoantibodies against RNA-binding proteins and in patients with DM/IBM, regardless of autoantibody status and in patients with autoantibody multispecificity. Thus the mechanisms driving the type I IFN pathway may differ between DM/IBM patients and patients with antibodies to RNA-binding proteins, where the latter group has a potential endogenous factor that can activate pDCs to produce type I IFN, whereas the mechanisms for IFN induction in patients with DM without these antibodies remain to be defined. IFN-blocking agents are on the market, and clinical trials with anti-IFNα monoclonal antibodies suggest beneficial effects in SLE [46], as well as in myositis [27]. The utility of a type I IFN gene signature (IFNGS) as a pharmacodynamic biomarker for assessing response to anti-IFNα monoclonal antibody treatment

has been suggested [27]. Preliminary results show a beneficial effect of target neutralization of the IFNGS and reduction in disease activity in DM and PM patients, but this needs additional confirmation in carefully phenotyped patients [27].

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Tables

Table 1. Patient characteristics at time of blood sampling

Characteristics*	**(n=92)**
Diagnosis, n (%)	
PM	40 (43)
DM	46 (50)
IBM	6 (7)
Sex, n (%)	
Men/women	30 (33)/ 62 (67)
Age, years	58 (49-68)
Disease duration, years	3.0 (0.3-9.6)
Physician's global disease activity assessment, mm	7 (0-23)
Patient's global disease activity assessment, mm	36 (16-64)
MMT8, (0-100 %)	91 (72-99)
HAQ, (0.00-3.00)	0.50 (0.00-1.25)
CPK µkat/liter	2.00 (1.05-8.23)
LDH µkat/liter	3.60 (3.08-4.42)
Extramuscular global assessment, mm	5 (0-14)
ILD, n (%)	32 (35)
Malignancy, n (%)	9 (10)
Immunomodulatory drugs, n (%)	55 (60)
Daily dose of Prednisolone, mg	10 (4-18)

*All data are presented as median (IQR; interquartile range) if not stated otherwise.

PM: Polymyositis; DM: dermatomyositis; IBM: Inclusion Body Myositis; Disease duration: years from diagnosis till sampling date. Physicians global activity assessment (Visual Analogue Scale (VAS), from 0-100 mm); Patients global activity assessment (VAS from 0-100 mm); Extramuscular global assessment (VAS from 0-100 mm); MMT8: Manual muscle test (0-100 %); HAQ: Health Assessment Questionnaire (potential score 0.00-3.00); CPK: creatinine phosphokinase (normal levels: 0.6-3.5 µkat/liter); LDH: lactate dehydrogenase (normal levels <3.5 µkat/liter); ILD: Interstitial Lung Disease; Immunomodulatory drugs includes cyclophosphamide, methotrexate, azathioprine, cyclosporin A, and intravenous immunoglobulin; (n/a): not assessed.

Table 2. Characteristics of interferon (IFN) high and IFN low patients

Variables*	**(n=92)**		**P value**
	IFN high (n=41)	IFN low (n=51)	
Diagnosis, n (%)			
PM	13 (32)	27 (53)	DM vs other 0.2082
DM	24 (59)	22 (43)	
IBM	4 (9)	2 (4)	
Sex, n (%)			
Men/women	10 (24)/ 31 (76)	20 (39)/ 31 (61)	0.1991
Age, years	56 (49-71)	58 (49-68)	0.9896
Disease duration, years	3 (0.2-11.5)	4 (0.4-9)	0.7145
ANA positivity, n (%)	24 (59)	25 (49)	0.4845
Antibody profile†, n			
Jo-1	13	10	
PL7	1	0	

Ku	0	1	
Ro52	13	9	
Ro60	6	4	
PM/Scl 75/100	3	4	
SRP	1	1	
U1RNP	6	2	
La	3	1	
Physician's global disease activity assessment, mm	9 (5-22)	5 (0-28)	0.4426
Patient's global disease activity assessment, mm	29 (16-55)	37 (14-72)	0.4438
MMT8 (0-100 %)	90 (69-99)	86 (68-95)	0.2500
HAQ, (0.00-3.00)	0.44 (0-1.10)	0.75 (0.13-1.25)	0.1772
CPK, µkat/liter	2.25 (1.23-8.90)	1.85 (1.13-7.16)	0.3214
LDH, µkat/liter	3.90 (2.98-4.55)	3.5 (3.18-4.53)	0.4111
Extramuscular global assessment, mm	5 (0-13)	1.5 (0-11.5)	0.3855
ILD, n (%)	15 (37)	17 (33)	0.9161
Malignancy, n (%)	3 (7)	6 (12)	0.7262
Immunomodulatory drugs, n (%)	24 (59)	31 (61)	0.9963
Daily dose of prednisolone, mg	8 (15-19)	10 (3-19)	0.7177

*All data are presented as median (IQR; interquartile range) if not stated otherwise.

IFN (interferon) high and IFN low; PM: Polymyositis; DM: dermatomyositis; IBM: Inclusion Body Myositis; Disease duration: years from diagnosis till sampling date; ANA: antinuclear antibody analysed by immunofluorescence; Physicians global disease activity assessment (Visual Analogue Scale (VAS) from 0-100 mm); Patients global activity disease assessment (VAS from 0-100 mm); Extramuscular global assessment (VAS from 0-100 mm); MMT8: Manual muscle test (0-100); HAQ: Health Assessment Questionnaire (potential score 0.00-3.00); CPK: creatinine phosphokinase (normal levels: 0,6-3,5µkat/liter); LDH: lactate dehydrogenase

(normal levels <3.5 µkat/liter); ILD: Interstitial Lung Disease; Immunomodulatory drugs includes cyclophosphamide, methotrexate, azathioprine, cyclosporin A, intravenous immunoglobulin; (n/a): not assessed.

† Autoantibodies to Jo-1; PL7; Ku; Ro52; Ro60; PM/Scl 75/100; SRP; U1RNP; La. The other tested autoantibodies were negative. A patient is considered autoantibody positive if positive response in either the line immunoassay system or the immunoprecipitation assay. One patient could have several autoantibody specificities.

Figure legends

Figure 1 The interferon (IFN) score in idiopathic inflammatory myopathies (IIM) patients, systemic lupus erythematosus (SLE) patients and healthy controls (HC). Gene expression levels of 8 IFN-response genes (IRG) were averaged to calculate the IFN score. The IFN score was measured in whole blood cells of IIM patients. The mean + 2*standard deviations (SD) of the IFN score in HC (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line) **A)** The extent of the IFN score was compared between HC, SLE (n=47) and IIM patients (n=92) and an increased IFN score was observed in a subset of SLE and IIM patients. **B)** The extent of the IFN score was compared between IIM patients with different subdiagnosis, i.e. dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM). An IFN signature was observed in individual patients, irrespective of their subdiagnosis, but the IFN score was higher in DM (n=46) and IBM (n=6) patients compared to PM patients (n=40) (p= 0.0415 and 0.0415, respectively).

Figure 2 Interferon (IFN) score in idiopathic inflammatory myopathies (IIM) patients with multi-specificity and RNA-binding autoantibodies. Gene expression levels of 8 IFN-response genes were averaged to calculate the IFN score. The IFN score was measured in whole blood cells of IIM patients.

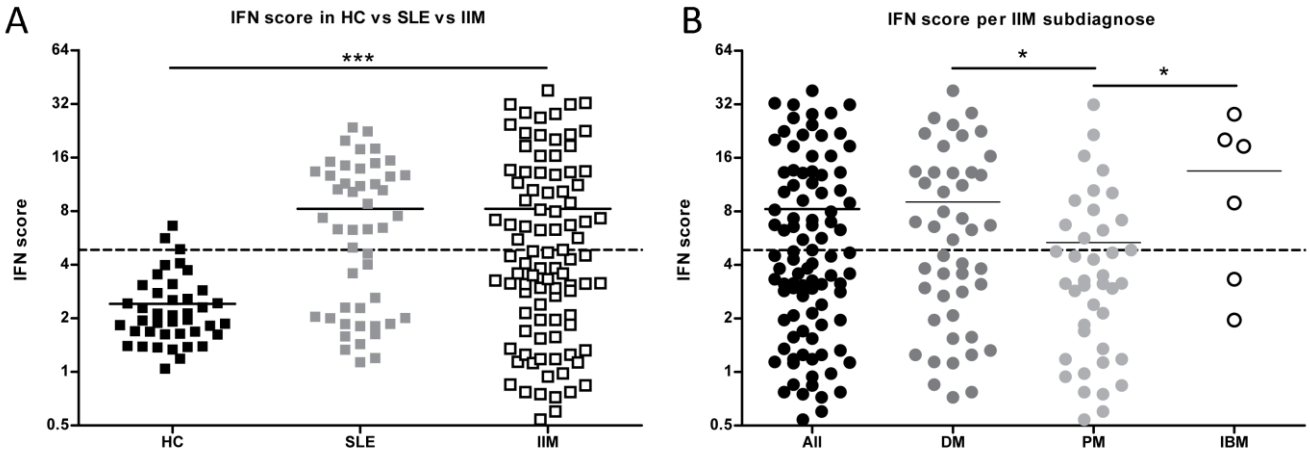
The mean + 2*standard deviations (SD) of the IFN score in healthy controls (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line). **A)** The IFN score was compared between patients with multi-specific autoantibody profile (n=20) versus patients with mono-specific autoantibody profiles (n=30) and patients without autoantibodies (n=42). Almost all patients with multi-specific autoantibody profiles have an IFN signature and the IFN score was higher in those patients compared to patients with mono-specific autoantibody profiles (p=0.0240) and patients without autoantibodies (p=0.0098). **B)** Patients with autoantibodies against RNA-binding (n=17) proteins had significantly higher IFN-score compared to other patients (n=13) (p=0.011).

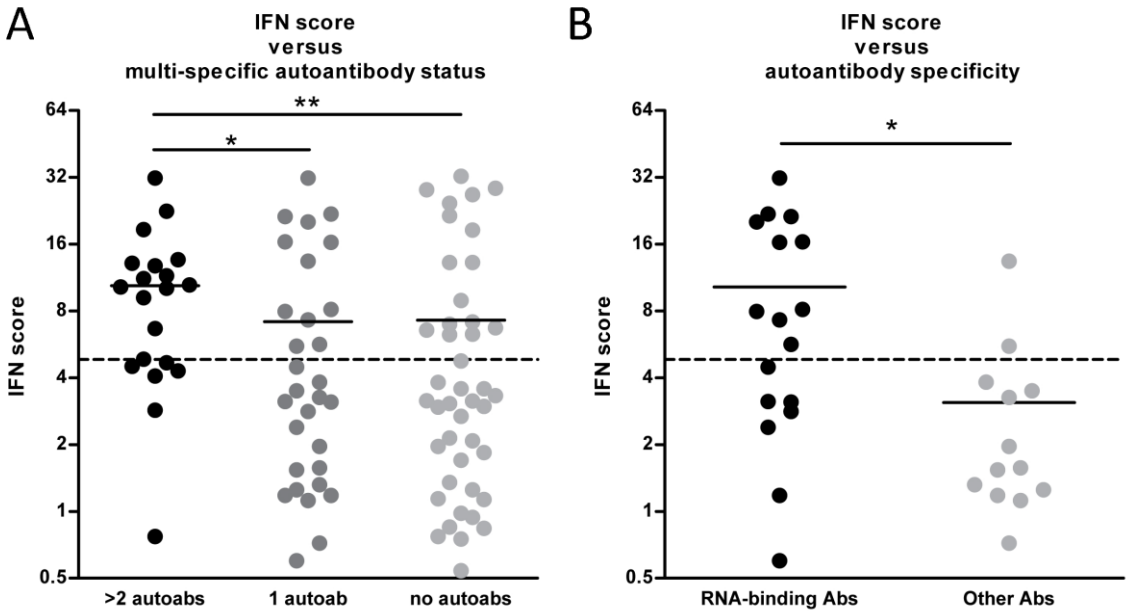
Figure 3 Interferon (IFN) bioactivity in serum of idiopathic inflammatory myopathies (IIM) patients.

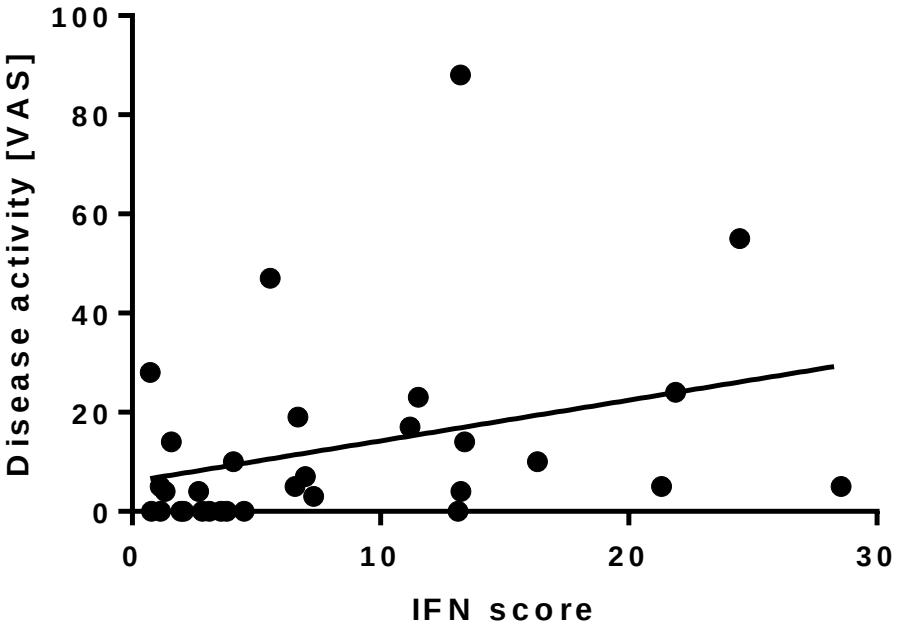
A) To investigate the origin of the trigger or interferogenic component that is responsible for activation of the type I IFN pathway in sera of IIM, we tested sera of IIM patients for its capacity to activate the type I IFN pathway. In vitro upregulation of interferon gene regulated (IRG) expression was determined as a measure for the presence of type I IFN pathway-activating capacity in serum. Sera from IFN high patients induced IRG expression in peripheral blood mononuclear cells (PBMCs) from healthy donors after four hours of incubation, whereas sera from IFN low patients induced significantly lower IRG induction. None of the patients´ sera induced IRG-expression at eight hours after serum-addition. "Baseline" indicates the basal IRG induction (0.27) in PBMCs that were not exposed to serum. The bars in the graph represent the mean of the three IRG genes, of all samples within the group, and the error bars represent the standard error of the mean. IRG high n=10, IRG low n=10.

B) To investigate whether type I IFNs are responsible for the observed activation of the type I IFN pathway in a subset of IIM patients (n=25), anti-IFNα-receptor (IFNAR) antibodies were added to the sera and the type I IFN bioactivity was determined after four hours. Serum-induced IRG expression, at four hours of incubation, was blocked when anti-IFNα or anti-IFNAR antibodies were added to the serum. A significant effect was observed for both anti-IFNα and anti-IFNAR antibodies in IRG high patients, but not in IRG low patients, indicating that IFNα in serum was responsible for the observed IRG induction. "Baseline" indicates the basal IRG induction (0.34) in PBMCs that were not exposed to serum. The bars in the graph represent the mean of the three IRG genes, of all samples within the group, and the error bars represent the standard error of the mean. IRG high n=5 and IRG low n=20.

Figure 4 Correlation between Interferon (IFN) score and disease activity in patients with dermatomyositis (DM). The IFN score was correlated to clinical manifestations. The mean + 2 standard deviation of the IFN score in healthy controls (HC) was used as a cut off to define if an IFN signature was present (IFN high) or absent (IFN low) and disease activity (Physician's global disease activity assessment) measured as VAS (visual analogue scale) from 0-100mm, was observed for patients with dermatomyositis (DM) (r=0.3778, p=0.03, n=32), but not for polymyositis (PM) and for inclusion body myositis (IBM), nor for the whole group of patients.







Příloha 7

Microarray analysis of circulating micro RNAs in the serum of patients with polymyositis and dermatomyositis reveals a distinct disease expression profile and is associated with disease activity

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Abstract

Objective

The aim of this study was a large scale investigation of myositis-associated circulating miRNA molecules and also determination of expression of these candidate molecules in relation to clinical activity of myositis.

Methods

RNA, containing also miRNAs, was isolated from sera of 28 patients suffering from idiopathic inflammatory myopathies (IIM) and 16 healthy controls. Expression of miRNAs was determined using a miRNA microarray method. Statistical analysis of miRNA expression was carried out using Arraystar software.

Results

Our results showed 23 significantly differentially expressed miRNAs. Six miRNAs were differentially expressed in IIM compared to healthy controls. In dermatomyositis (DM) we found 3 and in polymyositis (PM) 6 differentially expressed miRNAs compared to controls. Three miRNAs were up-regulated in patients with highly active disease compared to patients with low disease activity. Furthermore, we found 26 significantly differentially expressed miRNAs in SLE patients compared to IIM, DM and PM patients.

Conclusion

This is the first study that comprehensively describes expression levels of circulating miRNAs in serum of patients suffering from IIM. It can be expected that some of these deregulated miRNA molecules are involved in aetiology of IIM and may potentially serve as molecular markers for IIM development or for monitoring of disease activity.

Key words

circulating microRNA, idiopathic inflammatory myopathies, serum

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Introduction

Idiopathic inflammatory myopathies (IIM) belong to a group of autoimmune diseases characterised by chronic muscle inflammation that can lead to a dysfunction and/or destruction of muscle cells. The aetiology of these diseases is unknown and recent findings suggest that both, immune and non-immune mechanisms are involved in the pathogenesis of myositis (1, 2). Moreover, in different subsets of myositis, different molecular pathways might predominate (3). Differential diagnosis may be complicated in IIM; diagnostic errors, late detection of the disease and prolonged treatment initiation can significantly worsen the prognosis and result in irreversible tissue damage (4). Current data suggest that the presence of MHC haplotype A1-B8-Cw7-DRB1*0301/DQA1*0501 is a strong genetic risk factor for IIM development in a large subset of white patients (5, 6). Besides known genetic risks located within the MHC complex, also epigenetic regulations including changes in miRNAs expression profiles have been implicated recently in the pathogenesis of autoimmune diseases (7-9).

MicroRNAs (miRNAs) are small, non-coding, evolutionarily conserved RNA molecules posttranscriptionally regulating gene expression. Epigenetic mechanism of RNA interference (RNAi) influences the stability and translational efficiency of the target mRNA. MiRNAs can be found in most organisms and they form about 1–2% of eukaryotic genomes. They have the ability to control the expression of approximately half of the protein-coding genes (10). MicroRNA expression profiles are typically tissue- and cell- specific and are strictly controlled (11). Aberrant expression patterns are included in the pathogenesis of a wide range of diseases including systemic autoimmune diseases (12). A specific group of miRNAs biomarkers represent extracellular circulating miRNAs (c-miRNAs) which may provide a new insight also into the pathogenesis of autoimmune diseases (13). Growing evidence indicates that c-miRNAs may serve as biomarkers to assess pathophysiological status (14). Indeed, in muscular dystrophies significantly changed c-miRNAs levels have been described as well as in patients with chronic obstructive pulmonary disease, who often exhibit reduced muscle fibre size (15). It was recently suggested that c-miRNA may move into other organs or muscle itself and may regulate their functions (16). Several studies investigated expression of miRNAs in the muscle tissue (13, 17-19) and few also looked at serum levels of miRNAs (20-22). A single miRNAs were selected for each of these studies and, so far, no report analysed comprehensive status of miRNAs in patients with polymyositis and dermatomyositis (8, 23-25). Therefore in the current study we aimed to investigate myositis associated circulating miRNA molecules on a large scale and also to determine the relation of expression of these candidate molecules to clinical course of myositis.

Methods

Group of patients

Twenty-eight patients suffering from idiopathic inflammatory myopathies (IIM), who fulfilled Bohan and Peter criteria for the diagnosis of polymyositis (PM) or dermatomyositis (DM) were investigated in this study. Basic characteristics of patients with myositis are shown in Table I. A group of healthy individuals (7 females, 9 males; mean age 41.3±11.3 years) and a group of patients with systemic lupus erythematosus (16 females, mean age 44.1±15.1) served as controls.

Disease activity was assessed using visual analogue scale (Physician's Global Assessment [PGA]) (range 0–100 mm). For the purpose of this study the cut-off for disease activity was selected at 40. Patients below this cut-off were considered to have low activity; patients above the cut-off were assigned as having a highly active disease. Written informed consent was obtained from all participants, and the study was approved by local ethics committee.

Collection of biologic material and RNA extraction

Peripheral blood samples were collected into vacuum plastic tubes containing spray-coated silica. Samples were handled according standardised laboratory

Table I. Demographic and basic clinical data of patients with idiopathic inflammatory myopathies.

Clinical data of patients with idiopathic inflammatory myopathies	IIM
Sex (female/male)	20/8
Age (yrs)	
Mean ± SD	54.5 ± 15.0
Diagnosis	
DM (n)	14
PM (n)	14
Disease duration	
Mean ± SD	8.2 ± 4.0
Physician's Global Disease Assessment [a]	35.2 ± 25.4
- high global disease activity >40mm [b]	n=18; 51.9 ± 13.8
- low global disease activity <40mm [b]	n=10; 5.1 ± 3.0
HAQ	1.2 ± 0.9
CK	24.6 ± 42.7
LDH	7.3 ± 5.5
CRP	15.2 ± 31.8
Disease activity[a]	
Constitutional	11.5 ± 16.4
Cutaneous	9.7 ± 16.8
Skeletal	4.8 ± 10.6
Gastrointestinal	10.5 ± 17.4
Pulmonary	15.9 ± 21.2
Cardiovascular	3.6 ± 9.8
Extramuscular	21.6 ± 21.8
Muscle	36.7 ± 30.2

[a]Shown as mean ± SD (in mm on visual analogue scales). IIM: idiopathic inflammatory myopathy; [b]number of patients, mean±SD; PM: polymyositis; DM: dermatomyositis. Upper normal limits: CK 2,85 μkat/l; LDH 4.13 μkat/l; CRP 5 mgl.

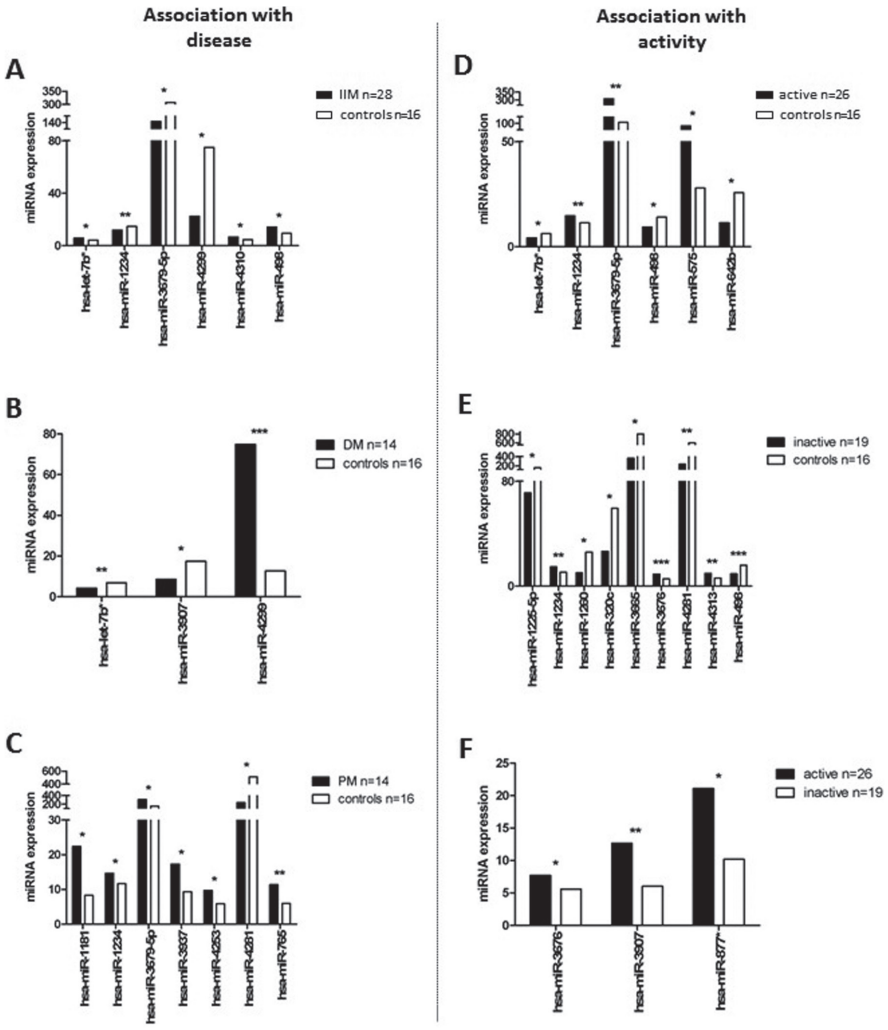

Fig. 1. Association of microRNA expression pattern with IIM diagnoses and with disease activity. Six microRNAs are significantly differentially expressed in patients with myositis compared to healthy controls.
A. After we divided the patients according to their diagnosis into two groups (DM and PM), we found in total 3 differentially expressed miRNAs in DM patients compared to controls.
B. and 7 microRNAs differentially expressed in PM patients compared to controls.
C. The analysis of microRNA expression pattern and relation to the disease activity revealed additional molecular associations. Six miRNAs were differentially expressed in patients with active stage of the disease when compared to healthy controls.
D. and 9 miRNAs were differentially expressed in patients with inactive stage of the disease when compared to controls.
E. Furthermore, comparison of microRNA expression profile between patients with active and inactive stage of the disease showed 3 miRNAs that are significantly differentially expressed.
F. The disease activity was assessed using Physician's Global Disease Assessment scale. For the purpose of this study the cut-off for disease activity was selected at 40. Patients below this cut-off were considered to have lower or no activity, patients above the cut-off were assigned as having active disease. Expression levels are displayed as average expression values from all patients within one group. *P*-values were corrected using FDR (Benjamini Hochberg) method. $^{*}p<0.05$; $^{**}p<0.001$; $^{***}p<0.0001$.

procedures and were processed within 4 hours after blood withdrawal.

Isolation of RNA from sera, containing also miRNAs, was performed according to Filková *et al*., 2013 (26). In brief, 500 μl of individual serum sample was homogenised with 500 μl of Trizol® LS reagent (Life Technologies, USA) and incubated for 5 minutes at room temperature. The samples were cleared by centrifugation at 12,000 × g for 10 minutes at 4°C. The supernatant containing RNA was then processed 3 times by acid phenol-chloroform (Life Technologies, USA) extraction and aqueous phase was separated by centrifugation at 12,000 × g for 5 minutes at 4°C. RNA was precipitated by adding of 100 μg of RNase-free glycogen (Roche Diagnostics, Germany) and 100% isopropanol, incubated for 10 minutes at room temperature with subsequent centrifugation at 12,000 × g for 10 minutes at 4°C. RNA pellet was then washed with 75% ethanol, spin at 7,500 x g for 5 minutes at 4°C and air dried. The extracted RNA was dissolved in RNase-free water. The quality and quantity of extracted RNA samples were controlled using the NanoDrop 2000 (Thermo Fisher Scientific, USA) and Bioanalyzer 2100 with the Small RNA Kit (Agilent Technologies, USA).

Determination of disease specific miRNA expression signature using the microarray technology

Expression of miRNAs was determined using a single-channel platform

Table II. Differentially expressed miRNAs in patients with IIM compared to healthy controls.

SystematicName	Myositis	Controls	Fold change	*p*-value
hsa-miR-1234	11,822	14,659	-1,24	0,0078
hsa-miR-498	14,159	9,328	1,517	0,0113
hsa-miR-3679-5p	141,668	307,403	-2,169	0,0152
hsa-miR-4299	22,444	74,922	-3,338	0,0255
hsa-let-7b*	5,874	4,251	1,381	0,0412
hsa-miR-4310	6,542	4,537	1,442	0,0451

Relative expression data of patients (myositis) and controls, fold change and *p*-values are shown here. $p<0.05$ was considered as significant. P-values were corrected using FDR (Benjamini Hochberg) method.

of 8x60K high density human miRNA microarray method (Agilent Technologies, USA). Three hundred nanograms of total RNA were used as a starting material to prepare cDNA. Total RNA samples were dephosphorylated, 3´ end- labeled with Cy3-pCp, purified on Micro Bio-Spin columns, dried, and hybridised to the microarrays. The hybridisation was performed for 20 hours at 55°C. After washing, Cy3 was detected by one-color scanning using a DNA microarray scanner type G2505B (Agilent, USA) at 5 micron resolution. Scanned image files were visually inspected for artefacts. The data were extracted from the scanned images using Feature Extraction software (Agilent Technologies, USA).

Statistical analysis

Statistical analysis of miRNA expression was carried out using Arraystar software (Lasergene, USA). The signal intensities of the samples were transformed into $\log^2$-ratio data. The array data were normalised by the averaging summarisation of global medians across all arrays. The Student's *t*-test was used to identify differentially expressed miRNA molecules. A miRNA was defined as being differentially expressed if $p<0.05$ was observed between two data sets. *P*-values of the entire analysis were corrected using FDR (Benjamini Hochberg) method.

Results

Idiopathic inflammatory myopathies (IIM) have a distinct miRNA expression signature in serum

The miRNA microarray used in this study could analyse 1,673 human microRNA molecules. Six miRNAs (let-7b*, miR-1234, miR-3679-5p, miR-4299, miR-4310, miR-498) had significantly different expression levels in serum of IIM patients in comparison with healthy controls ($p<0.05$). The expression of miR-1234, miR-3679-5p, and miR-4299 was significantly down-regulated and the expression of let-7b*, miR-4310, and miR-498 was up-regulated in the serum of patients in comparison with control samples (Fig. 1A) (Table II).

The analysis of serum miRNA content in patients with DM revealed a differential expression of 3 miRNAs; one (miR-4299) was down-regulated and two (let-7b*, miR-3907) were up-regulated in DM compared to healthy controls ($p<0.05$) (Fig. 1B). Similarly, when we compared PM patients with healthy controls we found that 6 miRNAs (miR-1181, miR-1234, miR-3679-5p, miR-3937, miR-4253, miR-765) were significantly down-regulated and one miRNA (miR-4281) was up-regulated in PM patients ($p<0.05$) (Fig. 1C). No difference was found in serum miRNA expression when PM and DM patients were compared.

After combining both, $p<0.05$ and fold-change >1.5, we found three differentially expressed miRNAs (hsa-miR-498, hsa-miR-3679-5p and hsa-miR-4299) in all IIM patients compared to controls; 6 miRNAs (hsa-miR-1181, hsa-miR-3679-5p, hsa-miR-3937, hsa-miR-4253, hsa-miR-4281 and hsa-miR-765) in PM and 3 miRNAs (hsa-let-7b*, hsa-miR-3907 and hsa-miR-4299) in DM were differentially expressed when compared to healthy controls.

Differential expression of several miRNAs is associated with disease activity

Three miRNAs (miR-3676, miR-3907, miR-877*) were significantly up-regulated in patients with highly active disease (defined as physician's global activity >40 mm) compared to patients with low disease activity ($p<0.05$) (Fig. 1F). When compared with healthy controls, 6 miRNAs were differentially expressed in patients with highly active disease; three miRNAs (miR-1234, miR-3679-5p, miR-575) were down-regulated and 3 miRNAs (let-7b*, miR-498, miR-642b) were up-regulated in patients ($p<0.05$) (Fig. 1D). In patients with low or inactive disease 3 miRNAs (miR-1234, miR-3676, miR-4313) were down-regulated and 6 miRNAs (miR-1225-5p, miR-1260a, miR-320c, miR-3665, miR-4281, miR-498) up-regulated when compared to healthy controls ($p<0.05$) (Fig. 1E).

Furthermore, we made a comparison of DM and PM in active and inactive patients. Three miRNAs (hsa-let-7b*, hsa-miR-548d-5p and hsa-miR-4299) were found to be differentially expressed in active DM compared to active PM patients ($p<0.05$). One miRNA (hsa-miR-3648) was found to be up-regulated in inactive DM compared to inactive PM patients ($p<0.05$).

The overview of expression data of all analysed miRNA molecules is shown in Table III.

Furthermore, after taking into consideration both, $p<0.05$ and fold-change >1.5, two miRNAs (hsa-miR-3907 and hsa-miR-877*) were found to be differentially expressed in patients with highly active disease compared to patients with low disease activity. When compared with healthy controls, four miRNAs (hsa-miR-3679-5p, hsa-miR-498, hsa-miR-575 and hsa-miR-642b) were differentially expressed in patients with highly active disease and 8 miRNAs (hsa-miR-3676, hsa-miR-498, hsa-miR-4313, hsa-miR-4281, hsa-miR-3665, hsa-miR-1225-5p, hsa-miR-320c and hsa-miR-1260) were differentially expressed in patients with low or inactive disease.

Association of biological pathways regulated by detected miRNAs

An analysis of the associated biological pathways regulated by the detected miRNAs revealed that among all 23

Table III. Overview of microRNA expression in different analyses.

miRNA	patients *vs.* controls	PM *vs.* DM	PM *vs.* controls	DM *vs.* controls	active *vs.* inactive	active *vs.* controls	inactive *vs.* controls	DM_act *vs.* PM_act	DM_inact *vs.* PM_inact
hsa-let-7b*	↑			↑		↑		↑	
hsa-miR-1181			↓						
hsa-miR-1225-5p							↑		
hsa-miR-1234	↓		↓			↓	↓		
hsa-miR-1260							↑		
hsa-miR-320c							↑		
hsa-miR-3648									↑
hsa-miR-3665							↑		
hsa-miR-3676					↑		↓		
hsa-miR-3679-5p	↓		↓			↓			
hsa-miR-3907				↑	↑				
hsa-miR-3937			↓						
hsa-miR-4253			↓						
hsa-miR-4281			↑				↑		
hsa-miR-4299	↓			↓				↓	
hsa-miR-4310	↑								
hsa-miR-4313							↓		
hsa-miR-498	↑					↑	↑		
hsa-miR-548d-5p								↓	
hsa-miR-575						↓			
hsa-miR-642b						↑			
hsa-miR-765			↓						
hsa-miR-877*					↑				

miRNAs found to be significantly differentially expressed, 11 (48%) (miR-1260, miR-320c, miR-498, miR-575, miR-765, miR-3665, miR-3679-5p, miR-3907, miR-4253, miR-4281, let-7b*) are predicted to regulate immune response; 4 (17%) miRNAs (let-7b*, miR-1225-5p, miR-498, miR-765) are related to pathways of actin and myosin; and 2 (9%) miRNAs (miR-1225-5p, miR-3937) are known to play a role in autoimmune diseases. Stat3 protein is probably regulated by miR-1234 (27). MiR-1181 plays a vital role in inhibiting cancer stem cell-like phenotype in pancreatic cancer (28) and miR-642b was among the three serum markers identified with high diagnostic accuracy for early stage of pancreatic cancer (29).

MiRNA genes are distributed across chromosomes either individually, or in clusters. A miRNA cluster is a group of miRNA genes located within a short distance on a chromosome. Based on the miRBase database (http://www.mirbase.org) definition, clustered miRNAs are a group of miRNA genes located within 10 Kb of distance on the same chromosome. We examined the genomic location of all of the 21 miRNA molecules that were differentially expressed in patients with IIM. We have found that 4 of the differentially expressed miRNAs were located in the known miRNA clusters and 16 miRNAs were individually distributed. We did not find any 2 or more miRNAs that would locate into the same cluster.

MicroRNA expression pattern found in IIM is different from serum miRNAs in SLE

In order to see whether the significantly differentially expressed miRNA molecules found in IIM patients are disease specific, we have performed a similar analysis in SLE patients (Table IV). The microarray expression analysis showed that the expression of 19 miRNA molecules was significantly changed in SLE patients compared to healthy controls ($p<0.05$). Of them, 6 miRNAs (miR-1281, miR-1825, miR-3679-5p, miR-3907, miR-4313, miR-575) were down-regulated and 13 miRNAs (miR-22, miR-24, miR-33b*, miR-3610, miR-3663-3p, miR-3937, miR-4257, miR-4271, miR-4298, miR-584, miR-642b, miR-671-5p, miR-4306) were up-regulated in SLE patients compared to controls. One miRNA (miR-3679-5p) was down-regulated in SLE patients similarly as in sera of patients with IIM compared to controls. The remaining 18 molecules differentially expressed in SLE were not differentially expressed in IIMs, when both compared to healthy controls.

Of the 19 differentially expressed miRNAs in SLE, 15 molecules (hsa-miR-3679-5p, hsa-miR-671-5p, hsa-miR-4271, hsa-miR-575, hsa-miR-584, hsa-miR-4298, hsa-miR-642b, hsa-miR-22, hsa-miR-3937, hsa-miR-3663-3p, hsa-miR-4257, hsa-miR-3907, hsa-miR-3610, hsa-miR-24 and hsa-miR-4306) have reached fold-change >1.5.

Further analysis determining the expression of miRNA molecules in SLE compared to IIM, DM and PM patients was performed. Nineteen miRNAs were significantly differentially expressed in SLE patients compared to IIM patients ($p<0.05$) (Fig. 2; Table V). Only two of them (miR-1234 and miR-3679-5p) were similarly regulated when considering the results obtained from SLE *vs.* IIM and IIM vs controls comparisons. Ten miRNAs were found to be differentially expressed in SLE patients compared to DM patients and 20 miRNAs were found to be differentially expressed in SLE patients compared to PM patients.

The overview of expression data of all analysed miRNAs is shown in Table V. After combining both, $p<0.05$ and fold-change >1.5, we found 16 miRNAs (hsa-miR-584, hsa-miR-671-5p, hsa-miR-4271, hsa-miR-3937, hsa-miR-4257, hsa-miR-3907, hsa-miR-320b, hsa-miR-3679-5p, hsa-miR-1290, hsa-miR-4298, hsa-miR-575, hsa-miR-1275, hsa-miR-939, hsa-miR-197, hsa-miR-3620 and hsa-miR-3610) to be differentially expressed in SLE patients compared to IIM patients.

Discussion

Idiopathic inflammatory myopathies are autoimmune diseases characterised by chronic muscle inflammation that can lead to a dysfunction and/or destruction of muscle cells. It is known that some microRNAs play role in muscle differentiation and thus also epigenetic regulations may be implicated in

Table IV. Differentially expressed miRNAs in patients with SLE compared to healthy controls.

SystematicName	SLE	Controls	Fold change	*p*-value
hsa-miR-3679-5p	61,674	307,403	4,984 down	7,23E-06
hsa-miR-671-5p	52,557	16,085	3,267 up	0,00011
hsa-miR-4271	56,48	23,365	2,417 up	0,00048
hsa-miR-1281	8,179	11,299	1,381 down	0,00061
hsa-miR-575	14,369	81,02	5,638 down	0,00155
hsa-miR-584	41,569	9,63	4,316 up	0,00196
hsa-miR-4298	39,395	14,402	2,735 up	0,00213
hsa-miR-642b	32,972	11,337	2,908 up	0,00301
hsa-miR-22	47,177	16,362	2,883 up	0,00488
hsa-miR-3937	39,641	17,316	2,289 up	0,00722
hsa-miR-3663-3p	112,625	33,638	3,348 up	0,00821
hsa-miR-1825	7,677	9,422	1,227 down	0,0115
hsa-miR-4313	8,09	9,701	1,199 down	0,0159
hsa-miR-4257	18,579	10,438	1,780 up	0,0179
hsa-miR-3907	3,944	8,643	2,191 down	0,0218
hsa-miR-3610	42,85	16,43	2,608 up	0,0225
hsa-miR-33b*	6,498	5,339	1,217 up	0,0399
hsa-miR-24	18,46	8,65	2,133 up	0,0481
hsa-miR-4306	12,427	5,173	2,402 up	0,0492

Relative expression data of patients (SLE) and controls, fold change and *p*-values are shown here. $p<0.05$ was considered as significant. *p*-values were corrected using FDR (Benjamini Hochberg) method.

Table V. Differentially expressed miRNAs in patients with SLE compared to IIM, DM and PM.

miRNA	SLE *vs*. IIM	SLE *vs*. DM	SLE *vs*. PM
hsa-miR-1224-5p		↓	
hsa-miR-1234	↓		↑
hsa-miR-1275	↑	↓	
hsa-miR-1280			↓
hsa-miR-1281	↑		↓
hsa-miR-1290	↓	↑	
hsa-miR-1825	↑		↓
hsa-miR-197	↑		
hsa-miR-2276			↑
hsa-miR-320b	↑	↓	
hsa-miR-320d		↓	↑
hsa-miR-3610	↓		↑
hsa-miR-3620	↓		↑
hsa-miR-3663-3p			↑
hsa-miR-3679-5p	↑		↓
hsa-miR-3907	↑	↓	↓
hsa-miR-3937	↓		↑
hsa-miR-4253			↑
hsa-miR-4257	↓	↑	↑
hsa-miR-4271	↓	↑	↑
hsa-miR-4298	↓		↑
hsa-miR-575	↑		↓
hsa-miR-584	↓	↑	↑
hsa-miR-601			↓
hsa-miR-671-5p	↓	↑	↑
hsa-miR-939	↑		

Relative expression data of significantly differentially expressed miRNAS of patients with SLE and IIM, fold change and *p*-values are shown here. $p<0.05$ was considered as significant.

pathogenesis of this disease. Recently, growing evidence has shown that some miRNAs persist in circulation. In 2007, Valadi *et al*. demonstrated that miRNAs can be taken up into intracellular vesicles and afterwards released into circulation. Many studies have shown the existence of circulating miRNAs (c-miRNAs) in various human body fluids (serum, plasma, breast milk, urine, saliva, etc.) (11). A significant relation between changed expression of circulating microRNAs and many autoimmune diseases has been described (30).

In our study, we have found in total 21 miRNA molecules that were significantly differentially expressed in patients when compared to control cohorts. Most of these miRNAs (miR-1234, miR-4299, miR-1181, miR-4281, miR-765, miR-575, miR-642b, miR-1225-5p, miR-1260, miR-320c, miR-3665, miR-877, miR-498) were studied in diseases and medical conditions including cancer, cardiovascular complications, or infections. So far, none of these miRNAs were found to be differentially expressed in autoimmune diseases.

To date, there are only few studies that describe the relationships between miRNAs and IIM, mostly in DM. Shimada *et al*. (25) hypothesised that if the miR-21 expression in the muscle tissues is significantly elevated in DM patients then serum miR-21 levels might be a potential biomarker for diagnosis and monitoring of disease activity in DM patients. Their results showed that serum levels of miR-21 were up-regulated in DM patients and related to the disease activity. This indicated that serum miR-21 might be involved in the pathogenesis of this disease. Oshikawa *et al*. (23) analysed miR-7 levels in serum of patient suffering from IIM. They have demonstrated that serum levels of miR-7 are specifically down-regulated in DM patients as well as in PM patients or CADM patients. However, levels of miR-7 were not decreased in patients with other autoimmune diseases such as SSc. Thus they indicate that serum miR-7 levels could possibly be used as a diagnostic marker for PM/DM. Results of our study could not support these findings, since both miR-21 and miR-7 were not differentially present in the serum of patients and controls or were present in different quantities in patients with active or inactive disease. Different miRNA molecules were found in analyses of patients with SLE. Wang *et al*. (31) investigated the levels of miR-146a and miR-155 in the serum of SLE patients. This was the first description of circulating miRNAs as biomarkers. They revealed that serum levels of miR-146a and miR-155 were decreased in SLE, and miR-146a was inversely associated with proteinuria

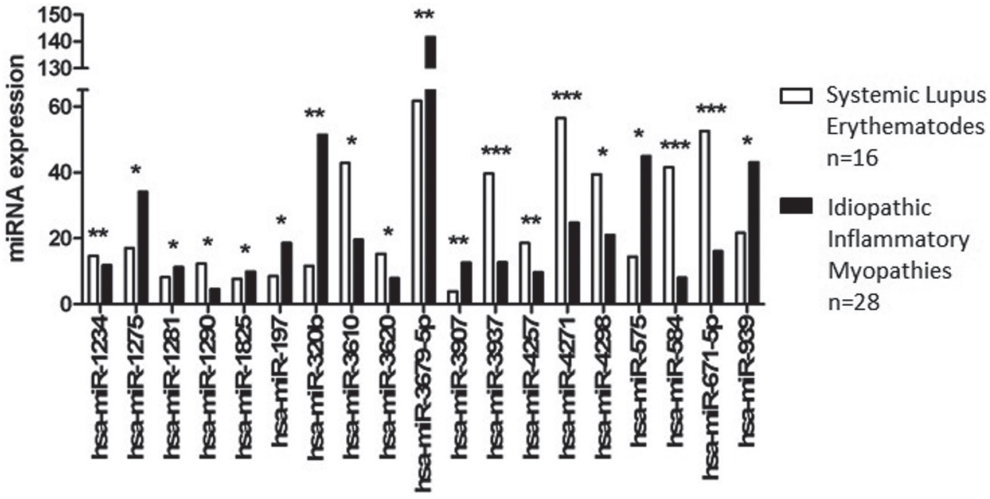

Fig. 2. Expression of microRNAs in different analyses using high density human miRNA microarray. In total, nineteen microRNA molecules have been found to be significantly differentially expressed in patients with myositis when compared to patients suffering from SLE. Expression levels are displayed as average expression values from all patients within one group. *P*-values were corrected using FDR (Benjamini Hochberg) method. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

and SLE Disease Activity Index (SLEDAI). These findings indicated that serum miR-146a and miR-155 may participate in the pathophysiology of SLE. The same group later conducted a pilot study in order to find a specific miRNA signature of SLE. They found that the serum levels of 6 miRNAs (miR-200a, miR-200b, miR-200c, miR-429, miR-205 and miR-192) were decreased in SLE patients compared to HCs (32). In 2012, Wang *et al*. (21) identified circulating miRNAs that were altered specifically in patients with SLE compared with RA and HCs. Based on these results 8 miRNAs were selected for continued clinical study. These molecules may be important regulators of immune cell development, playing vital roles in the inflammatory response and as key players in the pathogenesis of SLE (33). Carlsen *et al*. (34) found 7 miRNAs that were significantly differentially expressed in patients with SLE compared to patients with RA and healthy controls. MiR-142-3p and miR-181a were increased; miR-106a, miR-17, miR-20a, miR-203 and miR-92a were decreased in patients suffering from SLE.

None of these miRNAs were revealed as significantly regulated in our study. The comparison of sera from patients with SLE to healthy controls showed 19 miRNA molecules (miR-1281, miR-1825, miR-3679-5p, miR-3907, miR-4313, miR-575, miR-22, miR-24, miR-33b*, miR-3610, miR-3663-3p, miR-3937, miR-4257, miR-4271, miR-4298, miR-584, miR-642b, miR-671-5p, miR-4306) with significantly changed expression. Molecules found to be significant for IIM patients differed from molecules found to be deregulated in SLE patients except one (miR-3679-5p). This means that the expression of circulating miRNAs in the serum is largely different in the two diseases, perhaps forming a specific pattern for the respective disease. MiR-3679-5p was found down-regulated in the serum of patients with IIM, particularly in PM and active disease, as well as it was significantly lower in the serum from patients with SLE. There is not yet published information available on the role of miR-3679-5p in human organism; a number of predicted targets were identified for this miRNA with a putative role of targeted genes in immune system and in different molecular and structural reactions.

We have analysed also the expression of miRNA according to the disease severity. Disease activity was measured in every patient, cut-offs were set and patients with high and low or no activity were compared. Three miRNAs were found to be significantly differentially expressed (miR-3676, miR-3907, miR-877*) between patients with high and low activity. It remains to be determined for the future whether the levels of these miRNAs fluctuate longitudinally with the changes of disease activity and if so, whether they can be used as biomarkers for the disease. There were also a number of miRNAs differentially expressed in patients with active or inactive disease in comparison with healthy controls. It will be interesting to find out any relations with other disease parameters, particularly disease damage, where we do have a very limited spectrum of tools for muscle damage evaluation.

In summary, we provide here the first analysis of circulating serum miRNA gene expression profile in patients suffering from IIM. The results of this study indicate that the miRNA expression profile in serum of patients suffering from IIM is disease specific and creates a disease specific signature. It is expectable that some of these deregulated miRNA molecules are involved in aetiology of IIM. Additionally, we have shown that the detection of miRNA molecules in human serum is fast and easy approach in laboratory medicine. Therefore deregulated microRNA molecules shown in this work can potentially serve as molecular markers for IIM development or for monitoring of disease activity.

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