

# Cardiac Function and Cytokine Profile in Juvenile Dermatomyositis

Thesis by  
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2014



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*Series of dissertations submitted to the  
Faculty of Medicine, University of Oslo  
No. 1790*

ISBN 978-82-8264-827-1

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Cover: Inger Sandved Anfinsen.  
Printed in Norway: AIT Oslo AS.

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## ACKNOWLEDGEMENTS

The present work has been carried out at the Institute of Experimental Medical Research, Oslo University Hospital Ullevål, University of Oslo in the years 2009-2014, while I was working as a research fellow/lecturer at the University of Oslo. It has been five inspiring years, getting introduced to the research world and way of thinking; I thank the University of Oslo for giving me this opportunity.

I am most grateful to my main supervisor Ivar Sjaastad for his active involvement in my work. His extensive knowledge within the research field, bright ideas and ability to see things clearly, never fail to impress me. Despite his numerous responsibilities and busy schedule, he has all along been available with insightful feedback and advices on short notice. Also, I have never met anyone more deserving to be entitled *translational researcher* with expertise spanning from echocardiography, MRI and clinical medicine, to cytokine analyses, molecular biology and focal microscopy.

A large part of this thesis is based on the data my co supervisor Helga Sanner collected for her PhD at Department of Rheumatology, Oslo University Hospital Rikshospitalet, in 2011. It has been a privilege to work with her. Her deep insight in the material has made the work and analyses a lot easier. The rewarding words I have received along the way regarding patient- and material collection is entirely her profit. She also introduced me to the field of rheumatology. Thanks also to my second co supervisor, Berit Flatø with her extensive knowledge in rheumatological medicine and research. Her constructive and encouraging feed back has been greatly appreciated.

I thank my fellow researchers Birgit Nomeland Witczak and Zoltan Barth for inspiring and useful discussions; from now, they will carry on with the project.

A special thank goes to the patients, control persons and their parents for making the study possible.

I want to thank my co authors: Maria Vistnes for analysing the cytokines; also, I thank her and Geir Christensen to help me gain knowledge in this, for me, confusing and complicated field of

medicine. I thank Ola Gjesdal for highly constructive conversations in the hospital corridors; I also thank him and Trygve Husebye for sharing their knowledge on echocardiographic research with me.

For all the hours in front of the scanner, teaching me how echocardiography *really* should be performed at Ullevål echo-lab, I am most grateful to Ivar Sjaastad, Gunnar Smith and Reidar Bjørnerheim. I also thank the staff at the echo-lab for their cooperation on the project.

I thank Hilde Dishington for running the cytokine analyses and Ståle Nygård for highly useful statistical advices. I thank Per Andreas Norseng, Vidar Skulberg and Roy Trondsen for invaluable and instant help whenever any issues occurred to my computer or software and for technical support. I thank Head of the Department Ole Sejersted, Joselan Fabe Larsen, Lisbeth Hagen Winer, Magnus Aronsen, the “lunch klubb” and the rest of the people at the institute for a social and inspiring work environment.

Thanks to my dear parents, grandmother, sisters and mother-in-law for always and enthusiastically paying attention and interest to my doings in life; whether of scientific or non-scientific character. Also, thanks to my friends for providing me fun and social fuel.

At last, thank you May, my wife, best friend and sparring-partner in all important aspects of life, for unconditional love and support. Gaute, Jesper and Julia: my number one kids and clever conversers on any topic that pops up during dinner; thank you for filling my spirit with happiness and for patiently keeping up with early morning- and week-end writings and my never ending talk about this rare disease called JDM.

Oslo, May 2014

*Thomas Schwartz*

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## LIST OF PAPERS

### Paper I

**Cardiac dysfunction in Juvenile Dermatomyositis: a case control study.**

Schwartz T, Sanner H, Husebye T, Flato B, Sjaastad I.

Ann Rheum Dis 2011 May;70(5):766-71.

### Paper II

**In juvenile dermatomyositis cardiac systolic dysfunction is present after long-term follow-up, and is predicted by sustained early skin activity.**

Schwartz T, Sanner H, Gjesdal O, Flatø B, Sjaastad I.

Ann Rheum Dis published 23 July 2013; doi:10.1136/annrheumdis-2013.

### Paper III

**Increased levels of eotaxin and MCP-1 in juvenile dermatomyositis median 16.8 years after disease onset; associations with disease activity, duration and organ damage.**

Sanner H and Schwartz T, Flatø B, Vistnes M, Christensen G, Sjaastad I.

In Revision.

### Paper IV

**In active state of juvenile dermatomyositis: elevated eotaxin and MCP-1, and cholesterol level in the upper normal range are associated with cardiac dysfunction.**

Schwartz T, Sjaastad I, Flatø B, Vistnes M, Christensen G, Sanner H.

In Revision.

## SELECTED ABBREVIATIONS

|        |   |
|--------|---|
| A      | = late diastolic transmitral velocity of blood              |
| CHAQ   | = Child Health Assessment Questionnaire                     |
| CMAS   | = Childhood Myositis Assessment Scale                       |
| DAS    | = disease activity score                                    |
| DM     | = dermatomyositis   |
| E      | = early diastolic transmitral velocity of blood             |
| e'     | = early diastolic tissue velocity                           |
| ECG    | = electrocardiogram   |
| EF     | = left ventricular ejection fraction                        |
| HAQ    | = Health Assessment Questionnaire                           |
| IFN    | = interferon  |
| IIM    | = idiopathic inflammatory myopathy                          |
| IL     | = interleukine  |
| IP-10  | = interferon-inducible protein 10, CXCL10                   |
| JDM    | = juvenile dermatomyositis                                  |
| LV     | = left ventricle/left ventricular                           |
| MCP-1  | = monocyte chemoattractant protein-1, CCL2                  |
| MDI    | = Myositis Damage Index                                     |
| OUS    | = Oslo University Hospital                                  |
| PM     | = polymyositis  |
| PRINTO | = Paediatric Rheumatology International Trials Organization |
| RA     | = rheumatoid arthritis                                      |
| SF-36  | = Short Form-36   |
| SLE    | = systemic lupus erythematosus                              |
| TDI    | = tissue Doppler imaging                                    |
| TNF    | = tumor necrosis factor                                     |

# INTRODUCTION

## Juvenile dermatomyositis

Juvenile dermatomyositis (JDM) is a rare disease with annual incidence of 1.9 -3.2 /million children reported in population based studies in the UK and US ( 1; 2); still, it is an important systemic connective tissue disease in childhood. In Norway, the population below 18 years is approximately 1 million, which corresponds with 2-4 new cases diagnosed each year. Previously, disease course was serious with high mortality. In the 1960's Bitnum introduced "the rule of thirds" – 1/3 of the patients died, 1/3 developed severe long term functional disabilities and 1/3 recovered ( 3). The last decades, prognosis has improved ( 4) and the mortality rate is now 2- 3% ( 5; 6), presumably due to treatment with corticosteroids and other immunosuppressive agents. However, knowledge of the long term consequences of JDM has been limited. Still 30-61% of patients have signs of sustained disease activity and 60-90% develop organ damage 7.2-16.8 after disease onset ( 4; 5; 7).

Juvenile and adult dermatomyositis (DM) and polymyositis (PM) are idiopathic, inflammatory myopathies (IIM). DM and PM are characterized by skeletal muscle weakness and inflammation. In contrast to PM, DM has a variety of skin manifestations. A number of classification criteria of IIM's have been published ( 8); other conditions classified as IIM's are overlap myositis, inclusion- body myositis, amyopathic myositis and cancer associated myositis. While new, revised criteria are under development, Bohan and Peter's diagnostic criteria for JDM from 1975, are still in use ( 9), based on proximal muscle weakness and pathognomonic rash (heliotropic rash in face and Gottron's papules on fingers/knuckles). However, multiple organ affection is well known.



Calcinosis is a manifestation of JDM where calcium deposits in tissue can cause contractures and pain from nerve entrapment. Ulcerations of the skin and in the gastrointestinal tractus can occur and pulmonary dysfunction is also shown often in a restrictive, interstitial pattern ( 10). In the pre-cortisone era, gastrointestinal haemorrhage with perforation, together with bronchopneumonia and respiratory paralysis were the major causes of death in JDM ( 3). Contrary to adult DM, JDM is not associated with malignancy ( 11).

Although a specific mechanism or agent is yet to be confirmed, immune reactions initiated by environmental factors in genetically susceptible individuals ( 12) and infectious triggers ( 13), have been suggested. Skeletal muscle and skin are primary targets in JDM, but with the systemic nature of the disease, other organs are affected as well. It is a vasculopathic disease where the small vessels are targets for inflammation and immune responses, both humoral and cellular ( 14). Many autoantibodies related to JDM have been identified. Some are shared with overlap syndromes and other autoimmune diseases (myositis-associated antibodies; MAA), for instance anti-Ku and anti-Ro 52. Others are highly specific to IIM (Myositis-specific antibodies; MSA). Anti-Mi2, anti-signal recognition particle and anti-synthetases (e.g. anti-Jo-1) are examples that are well known. More recently, melanoma differentiation-associated gene 5 (MDA5), transcriptional intermediary factor 1 $\gamma$  (TIF  $\gamma$ ) and nuclear matrix protein 2 (NXP2) have been described and added to the list of MSA's ( 15; 16). Autoantibodies are seen in 40%-80% of JDM patients; a number rapidly increasing along with research in the area. Many of the MSA's are associated with specific clinical subgroups of IIM and will probably play an important role in future classification – and treatment – of IIM.

## **Cardiac dysfunction and heart failure**

### *Systolic and diastolic heart failure*

Traditionally the term “heart failure” has been equivalent to the hearts’ inability to serve the organism with sufficient blood perfusion. A history of dyspnoea and fatigue and findings of pulmonary congestion, peripheral edema, neck vein distention, enlargement of the liver and a murmur or third heart sound on cardiac auscultation are among the commonest symptoms and physical signs. In many cases this is due to chamber dilatation and impaired contractile function of the heart, and on echocardiography, left ventricular (LV) ejection fraction (EF) is typically reduced ( 17). This is called systolic heart failure or Heart Failure with Reduced Ejection Fraction (HFREF).

However, the last two decades, increasing awareness has been paid to those (up to 50%) with clinical signs and symptoms of heart failure but with an intact contractility of the heart ( 18; 19). It is defined as diastolic heart failure or “Heart Failure with Preserved/Normal Ejection Fraction” (HFPEF/HFNEF) ( 20; 21). Other mechanisms are believed to be responsible than in those with systolic heart failure. In diastolic heart failure, the pattern of LV myocardial remodeling is characterized by normal chamber size and increased wall thickness. This results in impaired relaxation and increased stiffness of the myocardium. By some authors, impaired systolic and diastolic function are seen as two separate pathways to a failing heart ( 22). A strong argument for this is the lack of treatment effect in diastolic heart failure, by therapies developed for systolic heart failure. Still, no therapy has been proven effective in diastolic heart failure. However, other authors consider impaired systole and diastole as a continuum of cardiac damage since systolic heart failure is associated with impaired diastolic parameters, as well as abnormal longitudinal systolic function can be seen in patients

predominantly with diastolic heart failure ( 23).

### *Cardiac dysfunction*

As mentioned above, *heart failure* is a clinical syndrome with characteristic symptoms and physical findings. The term *cardiac dysfunction* is less clearly defined, however; in literature it is most often based upon echocardiographical measurement of EF, when assessing *systolic function*. In the SOLVD study, 4228 asymptomatic patients with LV dysfunction (EF  $\leq$ 35%) were examined ( 24). Other less strict definitions have been used ( 25), such as asymptomatic patients merely with a subnormal EF ( $\leq$ 54%), based on current guidelines ( 17). The prevalence of cardiac dysfunction reported varies (1.8 to 12.5% in adult populations above 45 years) depending on the definition used ( 25; 26; 27). As will be addressed in the Material and methods section, there are a number of other highly suitable indices to estimate systolic function such as strain, strain rate, MA plane movement, contrast ventriculography and cardiac MRI. In neither of these methods are there generally agreed cut-off values for systolic dysfunction.

While *diastolic heart failure* has remained a somewhat controversial field, a consensus exists on the definition of *diastolic dysfunction*; this is based on transmitral Doppler inflow velocity and tissue Doppler of the mitral annulus (MA). According to current (2007) guidelines, the cut-off value for diastolic dysfunction is  $E/e' > 15$ ; when  $E/e'$  is between 8 and 15, it is considered as *possible* diastolic dysfunction ( 21). Invasive measurements of LV filling pressure can also be used and, today considered less reliable, transmitral Doppler inflow velocity patterns (E/A-ratio). As in systole, absence of symptoms distinguishes dysfunction from heart failure.

As expected, development of heart failure is much more common in patients with

cardiac dysfunction than in the normal population ( 27). In the SOLVD study, the incidence of heart failure and the rate of hospitalization were reduced in patients receiving enalapril treatment compared to the placebo group. Hence, cardiac dysfunction may be seen as asymptomatic state on the road to clinically overt heart failure.

### *Cardiac dysfunction and inflammation*

The last two decades, strong links between inflammation and heart disease have been established ( 28; 29). In atherosclerosis, inflammation was noted through histologic observations ( 30). In the presence of endothelial dysfunction, adhesion molecules enable monocytes to enter the intima where they develop into macrophages. Together with T-cells, dendritic cells and mast cells, the activated macrophages (foam cells) contribute in the formation of atherosclerotic lesions through release of pro-inflammatory substances such as monocyte chemoattractant protein 1 (MCP-1, CCL2), interleukine 1 (IL -1), IL-6, IL-18 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) ( 31) . In the progression of an atherosclerotic lesion, proliferation of smooth muscle cells, increased synthesis of collagen and accumulation of lipids, occurs. MCP-1, also produced by activated endothelial cells, plays an essential role in this development ( 32). The lipid cores of the foam cells increase in size and necrotize. The fibrous cap of the plaque is thinned, enhanced by oxidized LDL cholesterol, over-expression of matrix metalloproteinases (MMP) and TNF- $\alpha$  ( 28; 31). These final changes make the lesion increasingly vulnerable to plaque rupture and subsequently thrombosis and a cardiovascular event. Hence, inflammation is involved in all steps of atherosclerosis development. In the development of systolic cardiac failure, increased serum-levels of pro-inflammatory cytokines are seen with increasing NYHA-class ( 29). The cytokines have

been shown to affect myocardium through a number of mechanisms. TNF- $\alpha$  is involved in systolic heart failure with negative inotropic effect and promotion of LV remodelling leading to hypertrophy and dilatation ( 33). MCP-1 and IL-6 also induce hypertrophy and myocardial fibrosis ( 29).

The role of inflammation in diastolic heart failure is less studied. However, IL-6 ( 34), IL-18 ( 35) and MCP-1 ( 36) are all associated with increased synthesis of collagen, fibrosis and myocardial stiffness in experimental models. Increased levels of TNF- $\alpha$  and IL-6 have been shown in patients with diastolic heart failure ( 37). In the post-myocardial infarction reparative, fibrotic process, the significance of MCP-1 and other cytokines are well documented ( 36).

*Cardiac dysfunction in rheumatic diseases, included idiopathic inflammatory myopathies*

There has been a growing attention to cardiac function in the field of rheumatic diseases. It is well documented that RA and SLE carries an increased mortality and morbidity from cardiac disease ( 38; 39; 40; 41; 42). In fact, RA patients die prematurely compared to the general population, probably largely as a result of increased cardiovascular death. They have a higher risk of sudden death and silent myocardial infarction and a worse outcome associated with heart failure ( 43). Similarly, patients with SLE have increased mortality and morbidity from premature ischemic heart disease, severe hypertension, heart failure and peri- and myocarditis ( 44). More recently, it has also been shown that patients with RA and SLE have diastolic dysfunction, compared to healthy controls ( 45; 46). It is likely that a high level of inflammation, present in all rheumatic diseases, plays a leading role in the pathogenesis in cardiac diseases in these patients as well ( 43).

In adult PM/DM, increased cardiac morbidity has during the last decades been recognised, although it has been considered mainly subclinical ( 47). However, 162 Hungary patients with PM/DM were followed over median 5 years, 20 deaths occurred; of these 11 were due to cardiac causes: congestive cardiac failure, electromechanical conduction abnormalities and ischemic heart disease ( 48). This is similar to the pattern seen in RA and SLE. Recently in a large Swedish population based study, Zoller et al showed that there was a 4-fold increased risk of ischemic heart disease during the first year after hospitalisation from PM/DM – comparable to patients hospitalized from RA and SLE ( 49). Studies on the pathogenesis of cardiac involvement in PM have shown pathological ECG in 33 - 72% ( 50; 51; 52; 53; 54). In two autopsy series from 1979 and 1982, myocarditis was found in 6 of 20 and 4 of 16 patients with PM ( 52; 55); only two children were included in each study and none of these had cardiac disease. Pericardial effusion has also been reported ( 56; 57), but no controlled studies have been carried out. The suggested underlying mechanisms of cardiac involvement in adult PM/DM are atherosclerosis in coronary arteries and perhaps small vessel, and myocarditis.

To document cardiovascular events in JDM, a longer observational period is needed due to the young age of the patients; hence the data are scarcer. Interest has been paid to whether inflammation similar to what is seen in skeletal muscle also can occur in the heart. In theory, several factors can suggest cardiac affection in JDM: Inflammation of skeletal muscle is a key feature of JDM; the inflammation has also been shown to be systemic in nature ( 14; 58). Given this knowledge it is not unlikely that inflammation can affect the myocardium in JDM patients. Publications from two large, but uncontrolled

cohorts of JDM patients have suggested that cardiovascular complications are rare ( 5; 59). Since early changes in cardiac function can be subtle and subclinical, a control group is essential for detecting these. Up to now, no controlled study addressing cardiac function in JDM – or adult myositis- has been carried out, and the need for this has been raised by researchers in the field ( 47).

## **Cytokines**

### *Classification of cytokines*

Cytokines are small signal molecules, produced by endothelial- immune- and muscle cells. They modulate, activate or inhibit immune responses and inflammatory reactions through a number of mechanisms. TNF- $\alpha$  can direct phagocytizing neutrophils to an inflamed area through up-regulation of adhesion molecules. Also MCP-1 is responsible for activation of macrophages and differentiation into phagocytizing cells. Furthermore, activation of B- and T- cells in the adaptive immune system is also cytokine driven, among others by IL-6 ( 60; 61).

Classification of cytokines can be done on basis of *structural homology*. An example is CXC– and CC-chemokines; interferon inducible protein-10 (IP-10, CXC10) and MCP-1 (CCL2) and eotaxin (CCL11) representing the two groups. These are structurally distinguished by one amino acid separating the two cysteins near their amino terminus. Another example is cytokines categorized from a three-dimensional structure, hence termed the four helix bundle families.

There are many classifications on basis of *functional properties* and in the literature, this can be confusing. The term *chemokine* are used by cytokines promoting chemotaxis

between cells. Cytokines can be distinguished by those which are produced by *Th1*-lymphocytes (IFN- $\gamma$ , IL-2 and IL-18) and those produced by *Th2* - lymphocytes (IL-4, IL-5, IL-10 and IL-13) ( 61; 62). The *Th1*- cytokines, along with TNF- $\alpha$ , IL-1, IL-6, IL-8, IL-12 and, dominate in the acute phase of inflammation and are often termed *pro-inflammatory* cytokines, while *Th2* – cytokines and TGF- $\beta$  are involved in the late, recovery phase and are therefore considered *anti-inflammatory* ( 63). Another commonly used distinction is *type 1 and type 2 interferon driven* cytokines or signatures. This is referring to cytokines induced by type 1 interferons (IFN- $\alpha$  and IFN- $\beta$ ) as opposed to those induced by type 2 interferons (IFN- $\gamma$ ). Examples of cytokines known to be type 1 interferon-induced are IP-10, MCP-1 and IL-6. When the term *signature* is used, it most often refers to induction on a gene- and not protein level.

### *Cytokines in JDM*

Cytokine-inducible receptors, enhancing inflammation have become central pharmacological targets in rheumatic diseases such as adult and juvenile idiopathic arthritis (JIA) ( 64; 65; 66). This has made the role of cytokines in the pathogenesis in myositis to an area of interest ( 67; 68; 69). Studies have been performed on gene expression level, both from muscular tissue and peripheral blood, and type 1 interferon signature is present and associated with disease activity in DM ( 70; 71). Increased plasma levels of IL-18 ( 72) and IL-15 ( 73) are reported in patients with DM/PM early in the disease course (first year and median 1 year, respectively). IL-15 also correlated with disease activity ( 73). In a controlled study on 37 DM and 19 JDM patients (median disease duration 2 years), several chemokines including MCP-1 and IP-10 were increased ( 74). However, the area is confusing; most studies performed on cytokines consist of mixed patients groups with PM, adult and juvenile DM, with variable disease



duration, in particular, few have long term follow-up. If controlled, the studies have been small. Despite numerous reports, yet no definite biomarker of JDM has been found and the knowledge about cytokine abundance remains limited.

#### *Cytokines, lipids and cardiac dysfunction in rheumatic diseases*

Increased atherosclerotic burden in RA ( 38) despite decreased or normal cholesterol levels ( 75; 76) has been raised as a paradox ( 77). This reduced threshold of unfavourable cholesterol levels could be due to cytokine-driven inflammation.

Documentation is scarce; though, some studies suggest that pro-inflammatory cytokines are involved in development of cardiovascular complications in rheumatic diseases. A recent meta-analysis on rheumatoid arthritis (RA), showed that anti-TNF- $\alpha$  treatment reduced the risk of cardiovascular events ( 78), and in 23 RA patients, IL-1 antagonists improved LV function ( 79). Although studies have reported impaired glucose- and triglyceride metabolism in JDM ( 80), the impact of circulating lipid- and cytokine levels on cardiac function in patients with myositis has to our knowledge not been addressed.

# AIMS OF THE STUDY

## Main aim

To examine possible cardiac dysfunction in JDM patients, and investigate the association between patient characteristics, disease variables, cardiac function, lipids and specific cytokines.

## Specific aims

- To assess left ventricular diastolic function and to investigate whether diastolic cardiac function is associated with patient characteristics and disease variables (paper I).
- To assess left ventricular systolic function and to investigate whether cardiac function is associated with patient characteristics and disease variables (paper II).
- To examine serum cytokine profile after medium to long disease duration, and to explore possible relationship with patient characteristics and disease variables (paper III).
- To examine the relation between chemokines (eotaxin, MCP-1 and IP-10) lipid parameters and cardiac function, both in patients with active and inactive disease (paper IV).
- To investigate possible early predictors of cardiac dysfunction and cytokines at follow-up in patients (paper I+II+III).

## **MATERIAL AND METHODS - CONSIDERATIONS**

### **Study population and data collection**

A retrospective inception cohort of 66 patients with a probable or definitive diagnosis of JDM (9) was identified. In the medical records from January 1970 and June 2006, both manual and electronic searches were performed. Of 66 identified patients, 4 were deceased; the remaining 62 patients could all be tracked through the Norwegian Population Register and 59 (95%) agreed to participate in the study. Based on these data, the average annual incidence in Norway in the period was 2.9/mill.; this corresponds with literature from other countries (1; 2). Oslo University Hospital(OUS) has been a national referral centre for all JDM patients in Norway. Also, OUS is responsible for the care of children with rheumatic diseases in a region comprising 55% of the Norwegian population. Therefore, we believe that our study population represents by far most of the JDM patients in the country from this period.

Because of large variation in age among our patients, one sex- and age-matched control per patient was randomly drawn from the National Population Register. This case-control design helps us to determine whether subclinical findings are specific to JDM or not. Exclusion criteria were mobility problems, other inflammatory rheumatic diseases, autoimmune diseases treated with immunosuppressive agents, heart- or lung disease, except for mild asthma.

## **Clinical examination - disease activity, organ damage and other clinical measures**

Clinical examination of all patients and matched controls was performed at OUS in the period September 2005 - May 2009 by Dr. Helga Sanner, this was defined as time of follow-up. A number of clinical measures were assessed:

- Disease activity was measured by Disease Activity Score (DAS) for JDM ( 81) (range 0-20, 0 means no activity). DAS is composed of subscores for skin (DAS skin, 0-9) and muscle (DAS muscle, 0-11).
- Cumulative organ damage was measured by Myositis Damage Index (MDI, range 0-35/40) ( 58). MDI scoring does not distinguish between different causes of organ damage; a high score can also be secondary to disease activity, comorbidity or drug side-effects.
- To assess physical function the Health Assessment Questionnaire (HAQ) ( 82) (patients aged  $\geq 18$  years, n=39) and the Child HAQ ( 83) (<18 years, n=20) were used.
- Physical health was measured by the Short Form-36 (SF-36) physical component summary (PCS) ( 84).
- Inactive disease was defined by the proposed PRINTO (Paediatric Rheumatology International Trials Organization) criteria of 2012, at least 3 of the 4 following: manual muscle test (MMT-8)  $\geq 78$  (0-80) ( 85), physician global assessment of muscle activity (phyGloVAS)  $\leq 0.2$ . Childhood Myositis Assessment Scale (CMAS)  $\geq 48$  and creatine kinase (CK)  $\leq 150$  ( 86).

Retrospective disease data (onset, course, comorbidity) and DAS and MDI from the first year post-diagnosis were obtained from the medical records at OUS or, when necessary, from other hospitals. Disease onset was defined as time of the first muscle

or skin symptom clearly related to JDM and disease duration as the time from disease onset to the follow-up examination. History and use of medication was obtained both from study participants and by chart review.

## **Echocardiography**

### *Background*

Since its beginning in the 1960's, echocardiography as a method has developed and expanded dramatically. M-mode images of poor quality, reserved for a dedicated minority of enthusiasts were followed by Doppler and 2D images in the late 1970's. Along with colour coded- and tissue Doppler, transoesophageal echocardiography was also developed. Digitalization and advanced software led to further improved quality of the imaging ( 87). Even during the last decade, researchers have paid interest to several new echocardiographic modalities, such as speckle tracking- ( 88), strain- and 3D echocardiography ( 89). Although Magnetic Resonance Imaging of the heart (CMRI) has greatly improved and today contributes significantly, echocardiography is still by far the most important cardiac imaging technique in research as well as in clinical cardiology. Being affordable, robust, portable and fast, echocardiography has been the superior tool in most concerns. Still, echocardiography is faced by challenges such as standardization of measurements and limited acoustic window. Reduced visibility of LV walls makes reliable assessment impossible in some patients. Also, some techniques require highly specialized software or investigators. Another limitation in echocardiography can be high intra- and inter-observer variability.

In our study, Dr. Ivar Sjaastad performed all echocardiographic examinations, utilizing a Vivid 7 ultrasound scanner (GE - Vingmed Ultrasound, Horten, Norway) ( 17; 90). The

data were analysed by the candidate TS, blinded to clinical information and to patient/control identity. We investigated inter- and intra-observer variability (paper II) and both were found to be acceptable.

*Systolic function – assessed by long axis strain*

We assessed systolic function by mitral annulus plane (MA) displacement, rather than by LV ejection fraction (EF). Although more generally used, EF has limitations due to difficulties in data acquisition when the acoustic window is poor, and assumptions of LV geometry ( 17; 91). MA displacement has been an index of systolic LV function since the early era of echocardiography ( 92; 93) and has recently regained interest. It has proven to be an accurate, easy and sensitive way of assessing systolic function and predicting cardiovascular risk ( 94). During systole, the LV apex is relatively stationary; hence the MA descent from diastole to systole reflects LV movement in the longitudinal axis and LV contractility. Long axis strain is the relative estimate of MA displacement, normalized to LV end diastolic length (the length of LV increases with body size) ( 95); it is calculated as MA displacement expressed as percentage of LV end-diastolic length:

Calculation of long axis strain:

$$\text{Long axis strain} = 100\% \times \frac{\text{end diastolic LV length} - \text{end systolic LV length}}{\text{end diastolic LV length}}$$

Low long axis strain is associated with systolic dysfunction. In our cohort normalization of LV length was needed since our patients had large variation in age and body size.

We assessed long axis strain in the four-chamber apical view by M-mode echocardiography, but it can be measured with TDI and MRI modalities ( 95) as well.

This method's sensitivity to systolic dysfunction is probably higher than movement in the transverse/short axis (in M-mode, fraction shortening) and EF, and it is comparable to global longitudinal strain ( 96; 97).

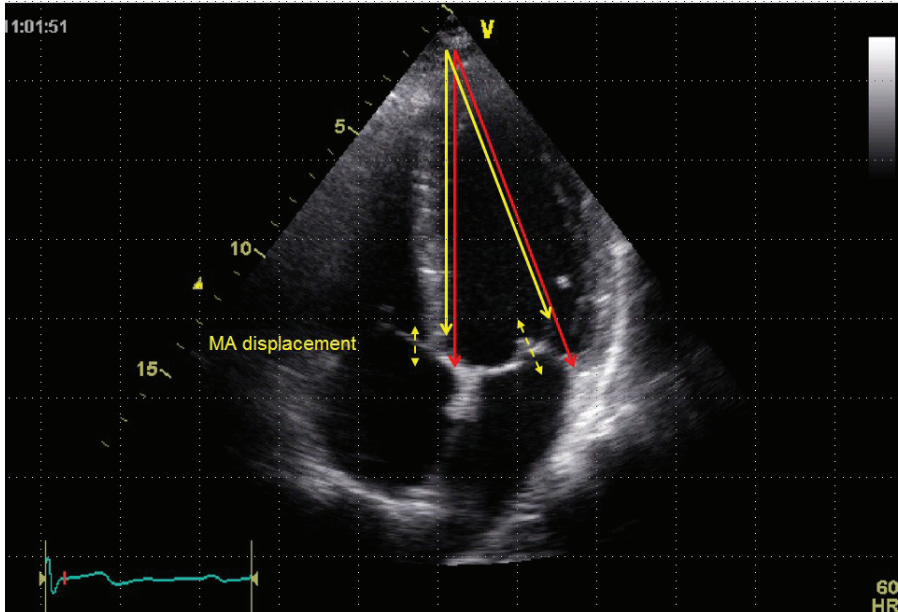


Figure: Mitral annulus displacement, assessed in 4-chamber view by 2D echocardiography.

#### *Diastolic function – assessed by E/e'-ratio and e'*

E/A-ratio (early- and late diastolic transmitral velocity of blood)  $<1$ , measured by conventional Doppler, has commonly been used as an indicator of impaired LV relaxation. In the progress of diastolic dysfunction, the phenomenon “pseudo-normalisation” (E/A-ratio  $>1$ ), occurs, which could make the diagnosis difficult. The last two decades it has become evident that TDI is more feasible than conventional Doppler to assess diastolic function ( 98). In our analyses, we used colour coded TDI to assess early diastolic tissue velocity (e') with sample volumes averaged from four positions (in

the MA - lateral and septal position in four-chamber view and two correspondingly positions in two-chamber view). When pulsed wave TDI is used, generally accepted cut-off's are; diastolic dysfunction:  $E/e' > 15$  and possible diastolic dysfunction:  $8 < E/e' < 15$  ( 18; 21). There are no generally accepted cut-off value value for colour coded TDI, but compared to pulsed wave, it is probably an underestimate of the pulsed wave TDI. Reports suggest that colour coded measures approximately 25% higher velocities than pulsed wave TDI ( 99); this corresponds well with our findings: in paper I, we defined "possible diastolic dysfunction" as  $E/e' > \text{mean} + 2\text{SD}$  of the matched control values, which was  $>9.5$ . In clinical settings however,  $e'$  alone has proven to be equally reliable to  $E/e'$  ( 100); being a single parameter, it is simpler and faster. Furthermore,  $e'$  is reported to be less load dependent than  $E/e'$  ( 98). Therefore, in paper II we utilized  $e'$  alone as the parameter of diastolic dysfunction.

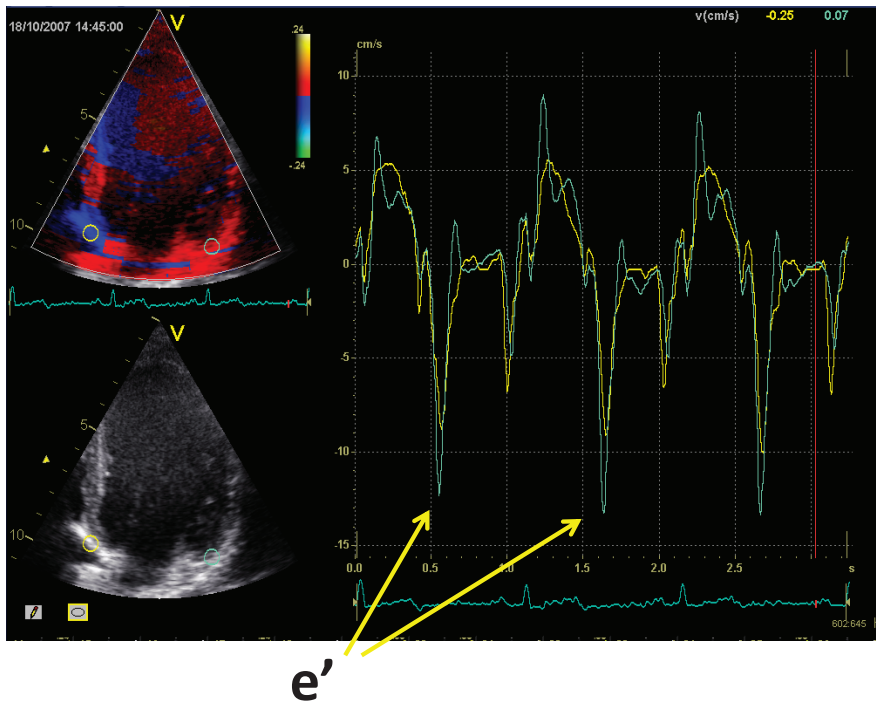


Figure:  $e'$  assessed by tissue velocity in the mitral annulus, by colour coded TDI.



## **ECG**

12-channel electrocardiography (ECG) was analysed (Paper I), blinded to clinical information and patient/control identity. Rhythm and ST segment were assessed, and PR-, corrected QT-interval (QTc) and QRS-duration were measured according to current practice ( 101). LV hypertrophy was assessed by Cornell voltage x QRS duration product which is probably more sensitive than the Sokalow criteria ( 102). Although no LV hypertrophy was detected in the patients by echocardiography, the patients did have a higher Cornell voltage x QRS duration product than the controls. The significance of this we have not discussed in depth, but it could suggest that this is a way to detect subtle changes in LV mass, not detectable on echocardiography.

## **Laboratory analyses**

### *Analyses of cytokines - Luminex and ELISA technology*

26 cytokine were analysed by Luminex technology; a multiplex protein quantification method. Capture antibodies are coated to microspheres, not wells as in ELISA, in sandwich immunoassays. With Luminex technology it is possible to quantify up to 100 different analytes simultaneously in a flow cytometer ( 61). This can be done with small sample volume, at a relatively low cost. The technology has been validated with good correlations against available ELISA kits ( 103). In our study, Luminex was used according to the manufacturer's protocol with minor modifications, described in paper III. Optimizing the method to the sample material and design of the standard curves were performed prior to analyses, even though, challenges in the quantification exist. Cytokines may either be degraded by proteases or become undetectable due to antibodies and soluble receptors in the sample ( 61).Also, even stored at -70 degrees Celcius, freezing, storage and defreezing may impair the quality of the samples.

For paper III, IL-18, TGF- $\beta$ 1) and IFN- $\alpha$  were analysed with enzyme-linked immunosorbent assay (ELISA) technique. For IFN- $\alpha$  (a type 1 IFN) - analyses, ELISA has been a challenge due to sensitivity and specificity ( 104). Typically, type 1 IFN signature (IFN-inducible gene- or protein expression in peripheral blood or affected tissue) has been easier show than increased type IFN- $\alpha$  itself ( 71).

There is no definite cut-off level for pathological levels of the CC chemokines eotaxin and MCP-1. As we did for the parameter of diastolic function  $E/e'$ , we therefore defined *high eotaxin* and *high MCP-1* levels as serum concentration  $>$ mean +2SD of the values in the matched controls.

#### *Other serum analyses*

In patients and controls, venous samples including basic hematologic parameters, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were collected. In JDM patients, creatine kinase (CK) and cardiac markers (Pro-brain natriuretic peptide (pro-BNP) and Troponin-T in 37 patients) were analysed.

In the preparation of Papers II and IV, lipid status was analysed. Since rheumatic diseases have lower cholesterol levels than the normal population and that cholesterol-levels alone might be of limited value in the assessment of atherosclerotic risk in these patients ( 75; 105). Therefore, high density lipoprotein/low density lipoprotein (HDL/LDL) - and total cholesterol/HDL (TC/HDL) – ratios were of particular interest to detect an unfavourable lipid profile in the JDM patients. The TC/HDL-ratio, although less known, was in a large meta-analysis of prospective observational studies, mostly from western Europe or North America, consisting of almost 900,000 patients shown to be superior to

other lipid parameters in predicting ischemic heart disease ( 106). Hence, this was one of the parameters we focused on in paper IV. As our samples were drawn on non-fasting subjects, we did not comment on triglyceride- or glucose levels in the patients.

### **Statistics**

The statistics used are described in the papers. Differences between scorers (paper II) and between patients and matched controls, were tested by the paired sample t-test. Two tailed tests were used for all calculations except for comparisons where a priori patients, based on the literature were unlikely to have lower values than controls; e.g. pathological ECG (paper I), ESR and CRP (paper III).

In the calculations of cytokines, five outliers were detected through Bonferroni corrections. These and their matched patients/controls were removed from the data set therefore results from the remaining 54 patients/control pairs are presented in paper III and IV.

## SUMMARY OF RESULTS

### **Paper I: Cardiac dysfunction in juvenile dermatomyositis: a case control study.**

- E/e' was elevated (>9.5) in 13 (22%) patients but in none of the controls ( $p<0.001$ ), suggesting a subclinical LV diastolic dysfunction in JDM.
- Ten patients presented with pathological ECG compared to 4 controls ( $p=0.054$ ). 6 of 13 patients with elevated E/e' vs. 4 of 44 with normal E/e', had pathological ECG ( $p=0.002$ );
- Previous or current hypertension was found in 12 (20%) patients vs. 0 controls ( $p<0.001$ ). Systolic BP was higher in patients with elevated E/e' than in those with normal E/e' (132mmHg  $\pm$ 24 vs. 112mmHg  $\pm$ 18,  $p=0.012$ ).
- Diastolic function (E/e') correlated with cumulative organ damage (MDI) assessed at follow-up ( $r_{sp}=0.41$ ,  $p=0.001$ ) and disease activity (DAS) at 1 year post-diagnosis ( $r_{sp}=0.56$ ,  $p<0.001$ ). DAS total 1 year post-diagnosis, also predicted pathological E/e' after controlling for age and gender (standardized  $\beta=0.49$ ,  $R^2=36\%$ ;  $p<0.001$ ).
- During disease course, 7 (12%) JDM patients developed pericarditis.

### **Paper II: In juvenile dermatomyositis cardiac systolic dysfunction is present after long-term follow-up, and is predicted by sustained early skin activity.**

- Systolic function (long axis strain) was impaired in patients compared to controls (16.6% (2.5) versus 17.7% (2.0),  $p=0.001$ ). Between patients with active and inactive disease, no difference was seen.

- Disease duration correlated with systolic and diastolic (e') function ( $r_{sp} = -0.50$  and  $r_{sp} = -0.73$ ,  $p's < 0.001$ ) and so did cumulative organ damage (MDI) 1 year post-diagnosis ( $r_{sp} = -0.36$  and  $r_{sp} = -0.46$ ) and MDI at follow-up ( $r_{sp} = -0.33$  and  $r_{sp} = -0.60$ ), all  $p's < 0.01$ .
- High early disease activity score (DAS) 1 year post-diagnosis in skin, but not in muscle, predicted systolic (standardized  $\beta = -0.28$ ,  $p = 0.01$ ,  $R^2 = 48\%$ ) and diastolic dysfunction ( $\beta = -0.36$ ,  $p < 0.001$ ,  $R^2 = 72\%$ ) at follow-up.
- A common pathway to two different cardiac manifestations may be present, perhaps with similar pathogenesis as skin affection.

**Paper III: Increased levels of eotaxin and MCP-1 in juvenile dermatomyositis median 16.8 years after disease onset; associations with disease activity, duration and organ damage.**

- Of 26 cytokines analysed by Luminex, only eotaxin, MCP-1 and IP-10 were elevated in patients compared to controls (31.5%, 37.2% and 43.2%,  $p's < 0.05$ ). IL-8, IL-13, TNF- $\alpha$  and IL-6, were numerically higher in the patients;  $p's = 0.06 - 0.08$ .
- Levels of eotaxin and MCP-1 correlated with disease duration ( $r_{sp} = 0.47$  and  $r_{sp} = 0.64$ ,  $p's < 0.001$ ).
- In a linear regression analysis, at follow-up MCP-1 was associated with cumulative organ damage (MDI) (standardized  $\beta = 0.43$ ,  $p = 0.002$ ) after adjusting for disease duration and gender. High MDI 1-year post-diagnosis predicted high levels of eotaxin and MCP-1 at follow-up (standardized  $\beta = 0.24$  and  $0.29$ ,  $p's < 0.05$ ).
- The findings highlight a role of eotaxin and MCP-1, possibly involved in a sustained inflammation in JDM.

**Paper IV: In active state of juvenile dermatomyositis: elevated eotaxin and MCP-1, and cholesterol level in the upper normal range are associated with cardiac dysfunction.**

- In patients, but not in controls, eotaxin and MCP-1 correlated with systolic (long axis strain) and diastolic function ( $e'$ ), particularly in those with active disease (systolic function,  $r_{sp}=-0.74$  and  $r_{sp}=-0.60$ ; diastolic function,  $r_{sp}=-0.69$  and  $r_{sp}=-0.80$ ).
- Total cholesterol level was lower in patients than controls (4.19 (0.82) vs. 4.60 (0.87) mmol/L  $p<0.01$ ).
- However, total cholesterol levels in the upper normal range were associated with systolic and diastolic dysfunction ( $r_{sp}=-0.56$ ,  $p<0.01$  and  $r_{sp}=-0.64$ ,  $p<0.001$ ) and with high eotaxin and MCP-1 ( $r_{sp}=0.56$ ,  $r_{sp}=0.50$ ,  $p$ 's $<0.01$ ) in patients with active disease, but not in those with inactive disease or in controls ( $r_{sp}$ 's  $<\pm 0.2$ ).
- It is possible that eotaxin and MCP-1 enhance susceptibility to cardiac dysfunction in patients with active disease through sustained inflammation.

## DISCUSSION

### Systolic and diastolic dysfunction in JDM

The main findings in paper I and II is the diastolic and systolic dysfunction seen in JDM patients examined after medium to long term follow-up. This has never been shown earlier in controlled studies neither in JDM nor, to our knowledge, in adult PM/DM. 13 patients fulfilled our criteria for possible diastolic dysfunction, however, the echocardiographic signs of cardiac dysfunction were subclinical and mainly detectable on a group level when compared with healthy controls. Our study design with sex- and age-matched controls has therefore been crucial for detection of these results.

In literature on diastolic dysfunction,  $E/e'$  has been more extensively documented than  $e'$  alone (21). Recently  $e'$  alone has been advocated as an easy and equally reliable parameter on diastolic dysfunction (100). In our analyses, we found  $E/e'$  and  $e'$  alone to be equally impaired in JDM patients compared to the controls (paper I).  $E/e'$  and  $e'$  were both associated with blood pressure, with stronger correlations for  $e'$ .

JDM patients had impaired long axis strain compared to the controls whereas no difference was seen in EF. In the analyses of systolic function we found long axis strain to be a more suitable parameter than EF: long-axis strain was successfully assessed in all, whereas EF was not obtainable due to acquisition challenges in 13 of 118 individuals. As already discussed, EF is a well established index of systolic function but has several limitations. In our analyses, EF correlated neither with diastolic function ( $e'$  or  $E/e'$ ), nor with blood pressure, whereas long axis strain correlated well with both. Patients with possible diastolic dysfunction had impaired long axis strain compared to the patients with normal diastolic function (14.7% (3.1) vs.

17.2% (2.1),  $p=0.001$ , unpublished data). This may suggest that diastolic and systolic dysfunction result from the same pathogenetic processes.

### **Cardiac dysfunction: a long term complication of JDM**

What is the significance of a subclinical cardiac dysfunction in JDM patients? Notably, only one of our patients had clinically detectable heart disease. In the acute phase of JDM, cardiac affection such as myocarditis has been reported ( 52; 55), but is uncommon. In our study, long disease duration was associated with pathological ECG and impaired parameters of systolic and diastolic function, particularly long axis strain and  $e'$ . Hence, we believe that cardiac dysfunction is a long term complication in JDM. However, some studies have suggested that cardiac affection in JDM is rare ( 5). But since JDM is a disease of childhood and adolescence and our patients had a median age of 21.5 years (range 6.7–55.4), clinically overt cardiac disease would hardly be expected until later in life. In adult PM/DM increased risk of cardiovascular death or clinically overt heart disease have been documented ( 48; 49). In these patients, cardiac end points are easier to show probably because this is an adult population. JDM share many disease mechanisms with adult PM/DM. Thus, it is likely that JDM patients, similar to PM/DM- and patients with other rheumatic diseases, are at risk of premature cardiac disease. This however, remains to be shown, and long term observational studies are needed to show hard cardiovascular end points. In our cohort, the patients that got their diagnosis before 1990 were treated less aggressively ( 4). This may also have contributed to the correlation seen between disease duration and cardiac function.

A history of pericarditis was found in a remarkable high number of the patients (7 of 59), 5 of these within 1-2 years after JDM diagnosis. However, pericarditis did not



correlate with cardiac function, ECG-findings or disease parameters. This can suggest that the underlying mechanisms of pericarditis are different from those responsible for cardiac dysfunction in JDM.

### **Cardiac dysfunction; associations with disease activity and organ damage**

Tools assessing disease activity and organ damage is crucial in the care of patients with myositis. JDM is a systemic disease ( 4; 14) and organ damage is scored according to severity and number of additional organ systems involved. Disease activity by DAS (used in the present thesis) on the other hand, is evaluated through the activity of skin- and muscle disease. We used MDI and DAS among several tools to assess organ damage and disease activity in myositis ( 58).

Both long axis strain,  $e'$  and  $E/e'$  were associated with disease activity and organ damage,  $e'$  stronger than  $E/e'$ . For disease activity the associations were present only for DAS 1 year post-diagnosis, not DAS follow-up, and more so for DAS skin than DAS muscle. The poor correlation with DAS muscle is interesting; one of the reasons why we hypothesized cardiac dysfunction in JDM was a presumed generalized affection of muscle and similarity between cardiac and skeletal muscle tissue. We assumed that what caused inflammation in skeletal muscle, could affect cardiac muscle as well. Instead, we found a stronger association between DAS skin and cardiac function; high DAS skin 1 year post-diagnosis also predicted both systolic and diastolic dysfunction. This supports a disease mechanism in the myocardium similar to that occurring in skin, rather than in skeletal muscle. JDM is a vasculopathy affecting small vessels; this is visualized by nailfold capillaroscopy (NFC). Studies have reported associations between pathological NFC and skin- but not muscle affection ( 107; 108). This

implicates that the small vessel disease is more prominent in skin than in muscle. A similar small vessel vasculopathy might be present in myocardium as well.

The PRINTO criteria for clinically inactive disease were published in 2012. We were not able to detect any difference between the inactive and the active patients in respect of cardiac parameters. This observation goes well with the lack of correlation between cardiac parameters and DAS at follow-up. Whereas high activity in skin early in the disease (DAS 1 year post-diagnosis) is associated with cardiac dysfunction, disease activity at a later stage of the disease (DAS at follow-up and active state JDM) does not seem to have an impact on cardiac function. It seems likely that high early disease activity induces permanent organ damage, detectable at a later stage.

Both e' and long axis strain were associated with cumulative organ damage at 1 year post-diagnosis and at follow-up; e' more so with MDI at follow-up. The cardiovascular components in MDI include: hypertension, LV dysfunction/cardiomyopathy, myocardial infarction and ischemic heart disease (IMACS form 08, Myositis damage index, 2001). It should be underlined that the MDI scoring of the patients were done prior to our echocardiographic analyses; our cardiac analyses are not included in the MDI scoring. Since LV dysfunction is a MDI criterion, the correlation between our echocardiographic findings and cumulative organ damage fits well with this.

### **Significance of elevated levels of CC chemokines in JDM**

As mentioned in the Introduction, a number of studies have addressed interferon signatures and cytokine activity/concentration, at gene or protein levels in JDM ( 69; 73; 74). Most of the studies have examined patients at a medium term follow-up with

disease duration 6 years or less.

We examined 29 cytokines median 17 years after disease onset and found MCP-1, eotaxin and IP-10 to be significantly increased in JDM patients. All the other cytokines analysed (except IFN- $\alpha$ ) were numerically elevated in the patients compared to controls. This tendency is remarkable keeping in mind that cytokines were analysed at a time point where half of the patients had inactive disease with few or no symptoms. Since only weak correlations were seen between MCP-1 and eotaxin and age in the controls, the correlations between MCP-1 and eotaxin and disease duration in the patients were not a result from aging per se. It is noteworthy that none of the cytokines showed a negative correlation with disease duration as one perhaps might expect, examined years after the acute phase of the disease. These findings suggest a sustained inflammation in JDM, ongoing also during the phase of remission. Even the data give indications; it is not obvious how this affects the clinical course of the disease.

At follow-up, MCP-1 and eotaxin correlated with cumulative organ damage (MDI). However, at this time point there were no correlations with disease activity (DAS); neither were there any differences in cytokine levels between patients when stratified to active and inactive state of the disease. Eotaxin and MCP-1 correlated weakly with both organ damage and disease activity, only in skin, not muscle, assessed early (1 year post-diagnosis) in the disease course. Similarly, for cardiac function, we also saw the strongest associations present were with DAS skin 1 year post-diagnosis and with MDI at follow-up. It seems like disease processes are initiated early, perhaps causing a sustained inflammation (elevation of MCP1 and eotaxin), resulting in myocardial

damage late in the disease course. This may, as already suggested, be caused by small vessel vasculitis in skin and myocardium.

Why should sustained inflammation manifest as late organ damage but not increased disease activity? If JDM “burns out”, it is hard to explain the observation that the pro-inflammatory chemokines MCP-1 and eotaxin increase along with increased disease duration. Eotaxin has been associated with fibrosis in different tissues ( 109; 110). It is possible that eotaxin induces similar tissue fibrosis in the heart, either by recruiting granulocytes that release pro-fibrotic substances, or by itself. None of our parameters were associated with DAS follow-up; it could be that this tool is suboptimal in assessing certain kinds of late disease activity. From our perspective, it is reasonable to assume an ongoing process of disease activity if a patient has elevated level of cytokines and cardiac dysfunction. Therefore, a low DAS may not be representative for all kinds of late disease activity in JDM.

Five cytokines were numerically elevated in JDM patients with borderline significant p-values ( $p < 0.08$ ). These were IL-13, IL-8, IL-1Ra and notably, IL-6 and TNF- $\alpha$ : the two best documented targets of receptor blockade treatment in rheumatic diseases ( 64; 65). We may have been underpowered to detect an increase in these cytokines.

In year 2000, in an often cited paper, Pachman et al. reported that presence of the TNF- $\alpha$  308A allele and increased production of TNF- $\alpha$ , was associated with a severe disease course ( 69). Another study showed increased type1 interferon activity in adult and juvenile DM; type 1 inducible proteins (including IL-6, IP-10 and MCP-1) were associated with disease activity ( 74). In our study, eotaxin and MCP-1 were the only cytokines that correlated with early disease activity, disease duration and organ

damage; none of the other cytokines did. Still, we are not able establish causality: eotaxin and MCP-1 might be responsible for impaired disease parameters through inflammation, but they might also be driven by other disease mechanisms.

In paper II, we have speculated in a biphasic cytokine response where an initial peak in cytokine levels is followed by a decrease and then a steady long term raise in the remission phase. Large, prospective studies are needed to show this. We are not aware of studies on cytokine with repetitive sample collections from the different phases (acute and chronic) of JDM. Due to the inflammatory nature of the disease, a general activation of cytokines in the period around disease onset and in the early phase of the disease seems likely. This was shown in a Swedish population based study where healthy persons who shortly after developed RA had increased levels of a number of cytokines (including MCP-1, eotaxin, TNF- $\alpha$ , IL-6 but not IP-10) compared to those who did not develop RA ( 111). Due to the rarity of the disease, such changes would be hard to show in JDM.

### **MCP-1 and eotaxin and cardiac function**

Studies done on cytokines and cardiac affection in rheumatic diseases are limited; in particular, we have not found any studies addressing this in myositis. In the literature, MCP-1 is perhaps the cytokine strongest associated with myocardial inflammation, atherosclerosis development and fibrosis; it also predicts cardiovascular events in patients with acute coronary syndrome ( 32; 36). Eotaxin is closely related to MCP-1 and also linked to cardiac fibrosis ( 112).

In our data, MCP-1 and eotaxin were associated with disease parameters (paper III) and with cardiac function (paper IV). Due to this concurrence, the findings in paper II are not surprising; in patients but not controls, both eotaxin and MCP-1 correlate with systolic and diastolic function. It appears that eotaxin is stronger linked to systolic function whereas MCP-1 to diastolic function: patients with high eotaxin tended to have systolic impairment and patients with high MCP-1 had diastolic impairment. For the other 27 cytokines analysed, including IP-10, TNF- $\alpha$  and IL 6; no associations with cardiac function were seen. This leaves MCP-1 and eotaxin in a unique position and is a main finding in our study: unlike all other cytokines, eotaxin and MCP-1 were elevated in JDM patients and correlated with disease parameters as well as cardiac function. The role of eotaxin and MCP-1 in the cardiac affection in JDM seems solid: there are associations with systolic and diastolic dysfunction and also with parameters of dyslipidemia and blood pressure. For MCP-1, this fits nicely with the overwhelming amount of documentation connecting MCP-1 to cardiac disease ( 29; 113; 32; 114; 115). Eotaxin is far less studied; but with 49% homology, 64% shared protein structure ( 116) and in our data, intercorrelation with MCP-1, it would not be surprising if eotaxin in the future will prove to be another important chemokine in the pathogenesis of cardiac diseases.

Similar to that discussed for disease parameters; whether there is causality in the correlation between the CC chemokines and cardiac dysfunction, is yet to be answered. We have investigated the patients at a single time point (though different time from patient to patient) of the disease course. It could well be that the CC chemokines are responsible for changes in cardiac function that are uncovered in the study and that

eotaxin and MCP-1 could be useful biomarkers for these. On the other hand, it could also be that eotaxin and MCP-1 are stimulated and increased by other unknown disease mechanisms. However, experimental studies with mouse models has shown reduced myocarditis both through CCR2 –receptor blockade (to which MCP-1 is a natural agonist) and in CCR2 knock out mice ( 117), while increased heart failure through myocarditis was seen in other mouse models with increased MCP-1 gen expression ( 118). Even if there is a way from mice to men with JDM, these results support that MCP-1 has a role in the development of cardiac dysfunction seen in JDM.

### **Unfavorable lipid profile in JDM**

The association between lipid metabolism and cardiac dysfunction in rheumatic diseases appears to be complex ( 105). In RA, increased susceptibility to atherosclerosis is present despite cholesterol levels in the normal range ( 38; 49; 75). Cardiovascular risk stratification of patients with rheumatic diseases must take this into consideration. JDM patients had lower cholesterol levels, but higher TC/HDL-ratio than the controls. Also, lipodystrophy was seen in 19% of the JDM patients; these indicate an unfavourable lipid-profile. As in other rheumatic and autoimmune diseases, increased atherosclerotic burden is present in adult DM ( 49); in JDM, this is not yet shown. Most of JDM patients in our cohort are probably too young to have developed detectable atherosclerotic heart disease. The cardiac dysfunction observed in our study, might reflect an early stage in the atherosclerotic development or the atherosclerosis may already be present as small vessel disease. It is possible that JDM patients are at risk of developing premature atherosclerotic heart disease.

In RA it has been shown that impaired cardiovascular parameters are associated with

active state of the disease. 82 Norwegian RA patients had higher pro-BNP and higher systolic blood pressure but lower cholesterol levels than 31 patients with inactive disease and 86 controls ( 76). We did not detect any differences in cytokine-levels, lipid-levels or cardiac function between those with inactive and those with active disease, but the following observations are interesting: correlations between the CC chemokines eotaxin and MCP-1, and cardiac function, blood pressure, lipodystrophy and lipid parameters (total cholesterol and TC/HDL-ratio) were only present in patients with active, not inactive disease and not in controls. Similarly, lipid parameters (total cholesterol and TC/HDL-ratio) only correlated with cardiac function, blood pressure and lipodystrophy in patients with active, not inactive disease and not in controls. In those with active disease state, eotaxin and MCP-1 may increase vulnerability to cholesterol and lowered the threshold of harmful cholesterol levels. These patients might be more sensitive to pathological myocardial remodelling due to chronic inflammation. Seemingly at risk of cardiovascular complications, it is possible that those with active state of JDM would benefit from more aggressive prophylactic treatment. On the other hand, one can not rule out that MCP-1 and eotaxin are confounding factors for other processes responsible.

It is somewhat intriguing: three of the PRINTO criteria are based upon disease activity in muscle (MMT-8, CMAS and CK) ( 86) whereas few of the parameters in our analyses were associated with disease activity in muscle. Yet, the PRINTO criteria seem suitable in stratifying JDM patients into groups with distinct cardiovascular and inflammatory profiles. These indications must be interpreted carefully though, especially since there were no differences in cytokine- or lipid-levels or cardiac function on a group level, between those with inactive and those with active disease.



Prospective follow-up studies could clarify whether targeted receptor blockers of the chemokines and use of statins can prevent cardiovascular complications and whether PRINTO criteria are useful in cardiovascular risk stratification in JDM.

### **Limitations and future plans**

A retrospective inception cohort like ours is suitable when assessing long term effects of the disease. Our median follow-up time is 16.8 years, with a large span of disease duration (2.0-38.1 years). However, the retrospective chart review on which some of the scores (early disease activity -DAS and MDI) are based upon, remains a possible source of error as it relies entirely on the quality of the documentation. The retrospective scoring and application of diagnostic criteria might have been based on incomplete charts, and hence represents a limitation of the study.

Also, it could be criticized that our study is only a glimpse into long disease process. At present, we are reexamining the patients in our JDM cohort, approximately 8 years after their first visit. This will give us a unique possibility to see development of disease parameters, cytokine profile and cardiac function in each and one patient over time.

## CONCLUSIONS

We have found systolic and diastolic dysfunction as long term consequences in the disease of JDM; both with associations to long disease duration, high initial disease activity in skin and high cumulative organ damage later in the disease course. By echocardiography, long axis strain and  $e'$  proved to be suitable indices of systolic and diastolic function.

The CC chemokines eotaxin and MCP-1 were increased in the JDM patients. High serum levels were associated with long disease duration, high cumulative organ damage and cardiac dysfunction.

Low cholesterol levels, yet an unfavorable lipid profile, were seen in JDM. Only present in those with active disease, associations between cholesterol in the upper normal range and increased eotaxin, increased MCP-1 and cardiac dysfunction, may indicate increased vulnerability to atherosclerotic disease in those with in active state of JDM.

High disease activity in skin 1 year post-diagnosis predicted both systolic and diastolic dysfunction, whereas high cumulative organ damage 1 year post-diagnosis predicted high eotaxin and MCP-1.

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# Increased Levels of Eotaxin and MCP-1 in Juvenile Dermatomyositis Median 16.8 Years after Disease Onset; Associations with Disease Activity, Duration and Organ Damage

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## Abstract

**Objective:** To compare cytokine profiles in patients with juvenile dermatomyositis (JDM) after medium to long-term follow-up with matched controls, and to examine associations between cytokine levels and disease activity, disease duration and organ damage.

**Methods:** Fifty-four JDM patients were examined median 16.8 years (2–38) after disease onset (follow-up) and compared with 54 sex- and age-matched controls. Cytokine concentrations in serum were quantified by Luminex technology. In patients, disease activity score (DAS), myositis damage index (MDI) and other disease parameters were collected by chart review (early parameters) and clinical examination (follow-up).

**Results:** Serum levels of eotaxin, monocyte chemoattractant protein-1 (MCP-1) and interferon-inducible protein 10 (IP-10) were elevated in JDM patients compared to controls (31.5%, 37.2% and 43.2% respectively, all  $p < 0.05$ ). Patients with active ( $n = 28$ ), but not inactive disease ( $n = 26$ ) had a higher level of MCP-1 than their respective controls. Levels of eotaxin and MCP-1 correlated with disease duration ( $r = 0.47$  and  $r = 0.64$ , both  $p < 0.001$ ) and age in patients, but not with age in controls. At follow-up, MDI was associated with MCP-1 (standardized  $\beta = 0.43$ ,  $p = 0.002$ ) after adjusting for disease duration and gender. High MDI 1 year post-diagnosis predicted high levels of eotaxin and MCP-1 at follow-up (standardized  $\beta = 0.24$  and  $0.29$ , both  $p < 0.05$ ) after adjusting for disease duration and gender.

**Conclusion:** Patients with JDM had higher eotaxin, MCP-1 and IP-10 than controls. High eotaxin and MCP-1 at follow-up was predicted by early disease parameters, and MCP-1 was associated with organ damage at follow-up, highlighting a role of these chemokines in JDM.

**Citation:** Sanner H, Schwartz T, Flatø B, Vistnes M, Christensen G, et al. (2014) Increased Levels of Eotaxin and MCP-1 in Juvenile Dermatomyositis Median 16.8 Years after Disease Onset; Associations with Disease Activity, Duration and Organ Damage. PLoS ONE 9(3): e92171. doi:10.1371/journal.pone.0092171

**Editor:** Paul Proost, University of Leuven, Rega Institute, Belgium

**Received:** October 8, 2013; **Accepted:** February 19, 2014; **Published:** March 19, 2014

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**Funding:** The authors have no support or funding to report.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Juvenile dermatomyositis (JDM) is a systemic autoimmune vasculopathy of childhood, involving proximal muscle weakness and characteristic skin lesions. While the mortality rate has decreased (now ~3%) [1], still 30–61% of patients have signs of sustained disease activity and 60–90% develop organ damage 7.2–16.8 years after disease onset [1–3]. Thus new therapeutic targets could improve patient care; however, the pathogenesis of JDM is not fully understood.

Cytokines are small signal molecules, produced by endothelial-immune- and muscle cells. They mediate and regulate innate and

adaptive immune responses and inflammatory reactions through a number of mechanisms including recruitment and activation of leukocytes [4]. During the last decade, the role of cytokines and chemokines (chemotactic cytokines) in the pathogenesis in myositis has been an area of interest [5,6]. However, most studies performed on cytokines consist of mixed patients groups with polymyositis (PM), adult dermatomyositis (DM) and juvenile DM and if controlled, the studies are small. Increased plasma levels of interleukin 18 (IL-18) [7] and IL-15 [8] are reported in patients with DM/PM early in the disease course (first year and median 1 year, respectively). IL-15 was also shown to correlate with disease activity [8]. In a controlled study on 37 DM and 19 JDM patients

(median disease duration 2 years), several chemokines including monocyte attractant protein-1 (MCP-1) and interferon-inducible protein 10 (IP-10) were increased [9]. Recently, criteria for clinically inactive disease state in JDM were proposed by the Paediatric Rheumatology International Trials Organization (PRINTO) [10]; however it is not clear whether disease state is associated with a specific signature of cytokines or inflammatory parameters.

Knowledge about cytokine abundance in myositis, in particular JDM, is limited. Specifically there is lack of studies with long-term follow up. Our sex- and age-matched patient-control pairs [11,12] provide a unique opportunity to compare the cytokine profile in JDM patients after medium to long-term follow-up and to explore how cytokine levels correlate with measures of disease activity and damage at follow-up and at 1 year post-diagnosis.

## Materials and Methods

### Patients and controls

Inclusion criteria were a probable or definitive diagnosis of DM according to the Bohan and Peter criteria [13], disease onset before 18 years, minimum 24 months disease duration and age  $\geq 6$  years at inclusion. We identified a retrospective inception cohort of 66 JDM patients diagnosed between January 1970 and June 2006 in Norway, previously described in detail [2,14,15]. Four of the patients were deceased; the remaining 62 could all be tracked through the Norwegian population register, and 59 (95%) participated in the study.

Sex- and age-matched controls were randomly drawn from the National Population Register. Exclusion criteria in the controls were: mobility problems, inflammatory rheumatic disease, other autoimmune conditions treated with immunosuppressive agents, and heart or lung disease (except for mild asthma). After cytokine analyses, statistical calculations to detect outliers were performed and 5 pairs were excluded (see statistical analysis). The data presented are based on the remaining 54 patients and 54 controls.

### Ethics statement

Written informed consent was obtained from all patients and controls (and their parents if aged below 16 years), according to the Declaration of Helsinki. The study was approved by the South-Eastern Regional Ethics Committee for Medical Research.

### Data collection and clinical measures

At Oslo University Hospital from September 2005 to May 2009, a single physician (HS) performed clinical examination of all patients, median 16.8 years (range 2–38 years) after disease onset (follow-up), and matched controls. In patients, disease activity was assessed by Disease Activity Score (DAS) for JDM [16] (range 0–20, 0 = no activity), which consists of DAS skin (0–9) and DAS muscle (0–11). Cumulative organ damage was measured by Myositis Damage Index (MDI, range 0–35/40) [17]. In addition, retrospective scoring of DAS and MDI from the first year post-diagnosis were performed, based on chart review [2]. From the criteria proposed by PRINTO (2012), inactive disease was defined as at least 3 of the following 4: manual muscle test (MMT-8)  $\geq 78$  (0–80), physician global assessment of muscle activity (phyGloVAS)  $\leq 0.2$ , Childhood Myositis Assessment Scale (CMAS)  $\geq 48$  and creatine kinase (CK)  $\leq 150$  [10,18]. JDM patients with inactive disease are referred to as JDM-inactive and the remaining patients are called JDM-active. Physical health was measured by the Short Form-36 (SF-36) physical component summary score (PCS) [19]. The Health Assessment Questionnaire (HAQ) [20] and the Childhood HAQ [21] were used to measure physical

function in patients aged  $\geq 18$  years ( $n = 35$ ) and  $< 18$  years ( $n = 19$ ), respectively. At time of follow-up, none of the study participants had clinical signs of infection. Disease onset was defined as the time of the first muscle or skin symptom clearly related to JDM (by chart review) and disease duration as the time from disease onset to follow-up examination. History of medication was obtained from study cases and by chart review.

### Laboratory analyses

At follow-up examination, venous blood samples were collected and serum concentrations of 29 cytokines analysed. IL-1 $\beta$ , IL-1 receptor antagonist (Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), granulocyte-colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon  $\gamma$  (IFN- $\gamma$ ), IP-10 (CXCL10), MCP-1 (CCL2), macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) (CCL3), MIP-1 $\beta$  (CCL4), eotaxin (CCL11), platelet-derived growth factor bb (PDGF), TNF- $\alpha$ , and vascular endothelial growth factor (VEGF) were quantified using Bio-Plex protein array systems (Bio-Rad, Hercules, CA), based on xMAP technology (Luminex, Austin, TX). The Luminex analyses were performed according to manufacturer's protocol, with minor modifications [22], including selection of high-sensitivity standard curve to optimize measurements of non-septic concentrations of cytokines. However, the high-sensitivity standard curve yielded physiological concentrations of Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES/CCL5) above detection limit. RANTES was therefore excluded for further analyses. An intra-assay variation with a coefficient of variation (CV) of  $7.49 \pm 0.81$  was calculated based on measurements of standards. To diminish the effect of the inter-assay variation, all samples were analyzed in a randomized fashion. Three of the 29 cytokines, IFN- $\alpha$ , IL-18 and transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ), were analysed with enzyme-linked immunosorbent assay (ELISA) technique.

Along with cytokine analyses, Th1/Th2 cell balance (ratio between CD4+ Th1 helper cells that produce IFN- $\gamma$  and IL-2 and CD4+ Th2 helper cells that produce IL-4, IL-5, IL-6, IL-10 and IL-13) was evaluated by calculating the ratio of IFN- $\gamma$ /IL-4 [23]. Erythrocyte sedimentation rates (ESR) were assessed and high-sensitive serum concentration of C-reactive protein (CRP) analysed.

### Statistical analysis

Differences between patients and matched controls were tested by the paired sample t-test for normally distributed continuous variables. Two tailed tests were used for all calculations except for comparisons where a priori patients, based on the literature were likely not to have lower values than controls (e.g. ESR and CRP). Bonferroni correction was performed when appropriate. Correlations were determined by Spearman correlation coefficient ( $r$ ). Association between eotaxin and MCP-1 (dependent variables) and MDI, DAS skin and DAS muscle measured 1 year post-diagnosis and at follow-up (independent variables) were tested in multivariate linear regression models with forward deletion of the variables after controlling for age and gender. Age was not included in the linear regression model due to high intercorrelation ( $r = 0.9$ ) with disease duration.  $p$  value  $< 0.05$  was considered significant. SPSS version 20.0 (SPSS, Chicago, IL) was used for statistical analyses.

To detect outlying individuals, we calculated the mean cytokine levels for all groups and found the Mahalanobis distance from the cytokine level of each individual to its respective group mean. Bonferroni corrected  $p$  values were obtained based on an

approximation of the Mahalanobis distance to a chi square distribution with the number of cytokines as degrees of freedom. One patient and four controls had samples with a p value <0.001 and were therefore considered to be outliers. These five and their matched control or patient were removed from the data set before the remaining statistical analyses, hence data from 54 pairs were analyzed and presented.

## Results

### Characteristics and serum cytokine levels in JDM patients and controls

Characteristics of the 54 JDM patients and 54 sex- and age-matched controls are shown in Table 1. Eotaxin-, MCP-1- and IP-10-levels were higher in patients than in controls (31.5%, 37.2% and 43.2% respectively, all  $p < 0.05$ , Table 2). No differences between patients and controls in levels of the other 26 cytokines, Th1/Th2 ratio (Table 2), CRP or ESR were found (Table 1).

### Cytokines and inflammatory parameters in JDM-active vs JDM-inactive and in JDM-active and JDM-inactive vs controls

According to PRINTO criteria, 26 (48%) of the patients had inactive disease. No differences were found between JDM-active and JDM-inactive in ESR (8.5 (6.1) vs 5.9 (4.7) mm,  $p = 0.09$ ),

CRP (2.7 (2.8) vs 1.8 (3.7) mg/L,  $p = 0.36$ ) or in the 29 cytokines studied (Table 2).

However, the 28 JDM-active had 47.9% higher level of MCP-1 (35.5 (19.9) vs 24.0 (10.7) pg/ml,  $p = 0.012$ ) than their matched controls; between JDM-inactive and their controls, no such difference in MCP-1 levels were seen (33.8 (24.2) vs 26.8 (12.2) pg/ml,  $p = 0.18$ ).

### Associations between cytokines, age and disease parameters at follow-up

Eotaxin and MCP-1 both correlated with disease duration ( $r = 0.64$  and  $r = 0.47$ ,  $p$ 's < 0.001, Table 3 and Figure 1) and age in patients. However, when exploring the association between age and serum cytokine levels in controls, no associations were found. IP-10 correlated neither with disease duration nor with age in patients or controls.

Both Eotaxin and MCP-1, but not IP-10, correlated with MDI at follow-up (Table 3). High MCP-1 was associated with high CRP, low SF-36 PCS, high CHAQ/HAQ and high cumulative prednisolone dose. Eotaxin and MCP-1 intercorrelated stronger in patients than in controls. No intercorrelation between IP-10 and IFN- $\alpha$  was seen neither in patients nor in controls ( $r = -0.12$  and  $r = 0.11$ ). No correlations were seen neither between eotaxin, MCP-1 nor IP-10 and disease activity (DAS) at follow-up. In a multivariate linear regression analysis, MDI at follow-up was associated with MCP-1 (standardized  $\beta = 0.43$ ,  $p = 0.002$ ,  $R^2$  final

**Table 1.** Characteristics and disease parameters in 54 patients with juvenile dermatomyositis and in 54 controls.

| Characteristics   | JDM patients     | Controls          |
|---|------------------|-------------------|
| Females   | 32 (59)          | 32 (59)           |
| Age at symptom onset (years)  | 7.7 (1.4–17.3)   | NA                |
| Age at diagnosis (years)  | 8.5 (2.1–19.3)   | NA                |
| <i>Variables assessed median 16.8 years after disease onset (follow-up)</i> |                  |                   |
| Age (years) at follow-up  | 22.0 (6.7–55.4)  | 22.1 (6.2–55.4)   |
| Duration from disease onset (years)   | 16.8 (2.0–38.1)  | NA                |
| CRP (<4 mg/L)   | 2.3 (3.3)        | 1.4 (3.1)         |
| ESR (<17 mm) <sup>†</sup>   | 7.0 (5.7)        | 5.7 (4.8)         |
| SF 36 PCS <sup>‡</sup> (0–100)  | 54.3 (26.9–60.9) | 56.9 (32.1–63.7)* |
| CHAQ/HAQ (0–3)  | 0 (0–1.38)       | NA                |
| MDI total (0–40)  | 3 (0–13)         | NA                |
| DAS skin (0–9)  | 4 (0–7)          | NA                |
| DAS muscle (0–11)   | 1 (0–8)          | NA                |
| DAS total (0–20)  | 5 (0–13)         | NA                |
| Prednisolone dosis, cumulative (g)  | 10.6 (12.3)      | NA                |
| Prednisolone or DMARDs  | 16 (30)          | NA                |
| <i>Variables assessed 1 year post- diagnosis</i>                            |                  |                   |
| MDI total (0–40)  | 1 (0–7)          | NA                |
| DAS skin (0–9)  | 4 (0–8)          | NA                |
| DAS muscle (0–11)   | 1 (0–7)          | NA                |
| DAS total (0–20)  | 5 (0–15)         | NA                |

Values are number (%), median (range) or mean (SD). JDM: juvenile dermatomyositis; NA: not applicable; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; SF-36 PCS: Short Form 36 physical component Summary; CHAQ: Childhood Health Assessment Questionnaire; DMARDs: disease modifying anti-rheumatic drugs; MDI: Myositis Damage Index; DAS: Disease Activity Score.

\* $p < 0.05$ .

<sup>‡</sup> $n = 46$  pairs, only assessed in those >13 years;

<sup>†</sup> $n = 50$  pairs.

doi:10.1371/journal.pone.0092171.t001

**Table 2.** Cytokine levels in patients with juvenile dermatomyositis assessed median 16.8 years after disease onset, and in controls.

|                | JDM active   | JDM inactive  | All JDM      | Controls     | p value |
|----------------|--------------|---------------|--------------|--------------|---------|
| MCP-1          | 35.5 (19.9)  | 33.8 (24.2)   | 34.7 (21.9)  | 25.3 (11.4)  | 0.006   |
| IP-10          | 1598 (1631)  | 1361 (877)    | 1484 (1316)  | 1036 (475)   | 0.026   |
| Eotaxin        | 150 (118)    | 133 (90)      | 142 (105)    | 108 (63.6)   | 0.039   |
| IL-6           | 8.4 (14.1)   | 4.9 (4.0)     | 6.7 (10.6)   | 4.0 (2.0)    | 0.060   |
| TNF- $\alpha$  | 23.3 (25.7)  | 21.4 (18.0)   | 22.4 (22.2)  | 16.3 (7.2)   | 0.065   |
| IL-13          | 2.7 (4.7)    | 2.8 (4.6)     | 2.8 (4.6)    | 1.6 (0.9)    | 0.078   |
| IL-8           | 11.3 (3.3)   | 10.6 (2.0)    | 10.9 (2.7)   | 10.2 (2.2)   | 0.080   |
| IL-1Ra         | 204 (396)    | 134 (140)     | 170 (301)    | 98.7 (61.5)  | 0.084   |
| IFN- $\gamma$  | 62.0 (86.8)  | 49.1 (43.5)   | 54.7 (70.5)  | 40.2 (20.9)  | 0.134   |
| IL-10          | 6.1 (23.2)   | 3.5 (6.8)     | 4.8 (17.3)   | 1.7 (1.6)    | 0.183   |
| IL-15          | 2.3 (3.1)    | 2.4 (3.0)     | 2.4 (3.0)    | 1.7 (1.7)    | 0.210   |
| IL-18          | 422 (152)    | 415 (213)     | 419 (182)    | 391 (154)    | 0.336   |
| TGF- $\beta$ 1 | 28000 (6770) | 29900 (10700) | 28900 (8860) | 29500 (7360) | 0.703   |
| IL-4           | 2.1 (0.57)   | 2.1 (0.55)    | 2.1 (0.55)   | 2.0 (0.57)   | 0.794   |
| IL-1 $\beta$   | 0.96 (0.90)  | 1.1 (1.1)     | 1.0 (1.0)    | 1.0 (0.9)    | 0.946   |
| Th1/Th2        | 27.6 (35.3)  | 23.8 (22.6)   | 25.8 (29.6)  | 19.7 (9.4)   | 0.780   |
| IFN- $\alpha$  | 11.9 (1.8)   | 11.1 (0.8)    | 11.5 (1.5)   | 12.0 (1.4)   | 0.07    |

Values for cytokine levels are mean (SD) pg/ml; n: all JDM = 54, controls = 54, JDM active = 28, JDM inactive = 26. p value when comparing cytokine levels in all JDM and controls; for the comparison active vs inactive JDM, no differences were detected. The cytokines shown were selected based on associations seen in the present and/or previous studies on dermatomyositis or other rheumatic diseases. JDM: juvenile dermatomyositis; MCP: monocyte chemoattractant protein; IP: interferon-inducible protein; IL: interleukine; TNF: tumor necrosis factor; Ra: receptor antagonist; TGF: transforming growth factor; Th1/Th2, IFN- $\gamma$ /IL-4.  
doi:10.1371/journal.pone.0092171.t002

model = 40%), none of the control measures (age and gender) were significant. A borderline significant association was seen between MDI and eotaxin (standardized  $\beta = 0.25$ ,  $p = 0.054$ ) in a similar linear regression analysis.

#### Early predictors of elevated eotaxin and MCP-1 levels

MDI and DAS total assessed 1 year post-diagnosis, correlated both with eotaxin and MCP-1 (Table 4). DAS skin, but not DAS muscle correlated with eotaxin and borderline with MCP-1.

In a linear regression analysis, MDI 1 year post-diagnosis predicted high MCP-1 (standardized  $\beta = 0.29$ ,  $p = 0.025$ ). Of the control measures, disease duration contributed significantly (standardized  $\beta = 0.32$ ,  $p = 0.014$ ), but not gender ( $R^2$  final model = 34%).

Accordingly, MDI 1 year post-diagnosis also predicted high eotaxin (standardized  $\beta = 0.24$ ,  $p = 0.049$ ), both of the control measures were significant (gender, standardized  $\beta = 0.23$ ,  $p = 0.045$ ; disease duration, standardized  $\beta = 0.41$ ,  $p = 0.001$ ;  $R^2$  final model = 41%).

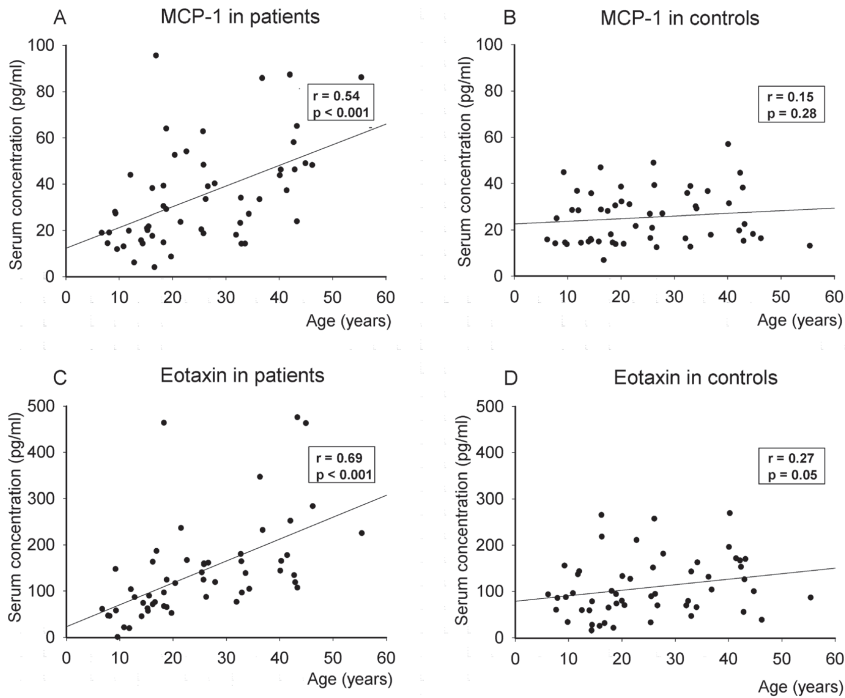
#### Discussion

In our study we have investigated cytokine abundance in JDM patients and found, median 16.8 years after disease onset, increased serum levels of eotaxin, MCP-1 and IP-10, compared to matched controls. When stratified in JDM-active and JDM-inactive, MCP-1 was elevated in JDM-active in comparison to their respective controls; not in JDM-inactive compared to controls. Eotaxin and MCP-1 both correlated with disease duration, and increased levels were predicted by high score of organ damage early in the disease course. MCP-1 was associated with cumulative organ damage at follow-up. To our knowledge,

no other controlled study has investigated circulating cytokine profile in an unselected JDM cohort after long-term follow up.

We have previously described the representativeness of our cohort [2], which we believe contains the vast majority of Norwegian JDM patients diagnosed between 1970 and 2006. Our cohort is comparable with other hospital or registry based cohorts with regards to female predominance, age at diagnosis, medication and muscle weakness at disease onset [24,25]. The representativeness of the patients and the sex- and age matching with controls drawn randomly from the National Population Register, represent strengths of our study.

We aimed at detecting differences in circulating levels of cytokines in JDM patients compared to controls, and found a significant increase in 3 and a numeric increase with p values of 0.06–0.08 for 5 of 28 cytokines. Eotaxin and MCP-1 correlated with disease duration and therefore, necessarily with age. For patients, age was substantially stronger correlated with eotaxin and MCP-1 than for controls, indicating that the correlation between disease duration and CC chemokines is not driven by aging per se. In previous studies on cytokines, DM and JDM patients have been investigated at time of diagnosis or early in disease course [8,9]. Increased serum levels of eotaxin, MCP-1 and IP-10, were found in a study of 9 JDM patients with clinically active disease [26]. The association between MCP-1 and active disease is supported by our findings: when stratified according to the recently (2012) proposed PRINTO criteria [10], differences in MCP-1 levels compared to controls were seen in JDM-active but not in JDM-inactive. Although we should be careful with our conclusions; we may have been underpowered to detect differences between JDM-active and JDM-inactive. However, the association with disease duration in all patients suggests that eotaxin and MCP-1 may contribute to a sustained inflammation and continue to play a role in JDM throughout the disease course as well.



**Figure 1. Correlations between monocyte chemoattractant protein-1 (MCP-1) (A and B) and eotaxin (C and D) and age, in 54 patients with juvenile dermatomyositis and sex- and age-matched controls.** *r*, Spearman correlation coefficient. doi:10.1371/journal.pone.0092171.g001

**Table 3. Correlations between MCP-1, eotaxin and clinical and disease variables in patients with juvenile dermatomyositis assessed median 16.8 years after disease onset, and in controls.**

| Clinical variables | MCP-1    |          | Eotaxin  |          |
|--------------------|----------|----------|----------|----------|
|                    | Patients | Controls | Patients | Controls |
| Male gender        | 0.37*    | 0.21     | 0.33*    | 0.39*    |
| Age                | 0.54**   | 0.15     | 0.69**   | 0.27*    |
| Disease duration   | 0.47**   | NA       | 0.64**   | NA       |
| ESR                | -0.05    | -0.08    | -0.10    | -0.20    |
| CRP                | 0.27*    | 0.15     | 0.09     | 0.01     |
| Eotaxin            | 0.70**   | 0.56**   | NA       | NA       |
| MDI                | 0.52**   | NA       | 0.52**   | NA       |
| DAS total          | 0.25     | NA       | 0.18     | NA       |
| DAS skin           | 0.17     | NA       | 0.20     | NA       |
| DAS muscle         | 0.20     | NA       | 0.09     | NA       |
| Prednisolone       | 0.28*    | NA       | 0.22     | NA       |
| SF 36 PCS          | -0.36*   | 0.09     | -0.24    | 0.11     |
| CHAQ/HAQ           | 0.32*    | NA       | 0.21     | NA       |

Values are  $r$  = Spearman correlation coefficient. MCP: monocyte chemoattractant protein; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; MDI: Myositis Damage Index; DAS: Disease Activity Score; Prednisolone: cumulative prednisolone dose during disease course; CHAQ: Childhood Health Assessment Questionnaire; SF-36 PCS: Short Form 36 physical component Summary. \* $p < 0.05$ ; \*\* $p < 0.001$ . doi:10.1371/journal.pone.0092171.t003

**Table 4.** Correlations between MCP-1, eotaxin and disease variables in patients with juvenile dermatomyositis 1 year post - diagnosis.

|            | MCP-1 |         | Eotaxin |         |
|------------|-------|---------|---------|---------|
|            | r     | p value | r       | p value |
| MDI        | 0.35  | 0.01    | 0.40    | 0.003   |
| DAS total  | 0.28  | 0.039   | 0.36    | 0.007   |
| DAS skin   | 0.27  | 0.053   | 0.36    | 0.008   |
| DAS muscle | 0.18  | 0.20    | 0.18    | 0.21    |

MCP: monocyte chemoattractant protein; r: Spearman correlation coefficient; MDI: Myositis Damage Index; DAS: Disease Activity Score.  
doi:10.1371/journal.pone.0092171.t004

Several studies suggest a role of IFN- $\alpha$  activity in adult and juvenile dermatomyositis [27,28]. Since IFN- $\alpha$  was comparable in patients and controls, we did not analyze correlations with disease parameters.

Our observation that cotaxin correlated with early DAS skin, indicates a link between cotaxin and skin affection, in JDM. Some studies associate cotaxin to fibrosis in different tissues as heart, liver and lungs [29–31]. In JDM, cotaxin might induce similar tissue fibrosis, either by recruiting granulocytes that release pro-fibrotic substances, or by itself. Furthermore, we found a correlation between cotaxin and organ damage (MDI) at follow-up, and in this context a pro-fibrotic effect could be relevant. The increased cotaxin and MCP-1 levels in patients could support a hypothesis of low-grade sustained inflammation in JDM, contributing to accumulate organ damage as suggested in juvenile idiopathic arthritis [32]. Furthermore, correlation between cotaxin at follow-up, and disease activity and organ damage at 1 year post-diagnosis could indicate that this is a process initiated early in the course of the disease.

It is reasonable to believe that JDM patients have a more widespread and pronounced inflammation at the time of diagnosis than at long-term follow-up. A large study from Sweden in 2010 showed that in rheumatoid arthritis (RA), many cytokines, including cotaxin and MCP-1, were increased even before disease onset, with further increase at the time of diagnosis [33]. In JDM, a small study showed initial inflammation by measuring increased serum level of IL-18 at time of diagnosis; the level then decreased through the first year of the disease [23]. In our study, extensive information about disease course was obtained through data from patients with disease duration ranging from 2 to 38 years. It is noteworthy that none of the cytokines showed a negative correlation with disease duration as one perhaps might expect. Whether there is a continuous increase in cotaxin abundance after the initial active disease, remains unknown. Given the cross-sectional nature of the study, we did not have data on the cytokine levels from the initial years of the disease. One could speculate in a biphasic response: a high initial level of cotaxin, then a decline until, again, a steady climb after 2 years and onwards based on the data in our study. This could be pursued by comparing our long-term results with a prospective study with serial cytokine samples, during the early phase of the disease.

MCP-1 is an attractor and activator of monocytes and T-lymphocytes and is more studied than cotaxin. Besides being an important actor in the immune response, MCP-1 is involved in inflammation, angiogenesis and formation of atherosclerosis [34]. The angiogenic effect is especially interesting since JDM is a vasculopathy, this could be evaluated by capillaroscopy.

Homology between cotaxin and MCP-1 is 49% and they share 64% of the protein structure [35]; our data also show intercor-

relation between the two. Eotaxin is the natural agonist of CC chemokine receptor 3 (CCR3), thus elevated circulating levels of this chemokine may potentially increase the recruitment of CCR3-expressing cells, thereby maintaining chronic inflammation. However, cotaxin has also been shown to be a partial agonist of the receptor CCR2 for which MCP-1 is a full agonist [36]. Thus, cotaxin can partially block MCP-1 effects and could for instance modulate monocyte recruitment in inflammatory condition which is a main effect of MCP-1. Such interactions may well be present in JDM, although this has not yet been studied.

MCP-1 correlated consistently with organ damage and early disease activity and, as well as with other inflammatory parameters such as CRP. Also, the association with cumulative prednisolone dosis is interesting, possibly reflecting longstanding active disease. In the DM/JDM patients studied by Bilgic et al [9], correlation between MCP-1, IP-10, IL-6 and global disease activity was also found the first two years of the disease. In our study, high early organ damage predicted elevated levels of both cotaxin and MCP-1. This suggests that cotaxin and MCP-1 measured at follow-up could be useful biomarkers of disease outcome in JDM; particularly since they are both associated with long-term cumulative organ damage. Also since cotaxin and MCP-1 are up regulated in the acute phase of JDM [26] one could speculate that these cytokines could be early biomarkers of organ damage late in disease course.

Eotaxin and MCP-1 may represent targets for biological treatment in JDM. Anti-CCL2/MCP-1 [37], anti-CCL11/eotaxin (bertilimumab) and CCR3 antagonist [38] are available and potential treatment options. However, effects of cytokines are diverse and complex. For example: in the literature, IP-10 is considered as a type1 interferon (IFN- $\alpha$ ) regulated cytokine [27], despite this, we saw no correlations between IP-10 and IFN- $\alpha$  in our study. Furthermore, one study showed no clinical improvement in RA by blocking CCR2 [37], whereas another study on a mouse model of RA surprisingly showed exacerbation of arthritis when CCR2 was knocked out [39]. Thus, it is not obvious whether modulation MCP-1 or cotaxin targets will have beneficial effects in JDM, and interactions at receptor level between cotaxin and MCP-1 can obscure interpretation of the results.

In conclusion; in 54 JDM patients seen median 16.8 years after symptom onset, we have shown higher levels of cotaxin, MCP-1 and IP-10, compared to controls. On a subgroup level, increased MCP-1 compared to controls was seen only in JDM-active, not in JDM-inactive. Both cotaxin and MCP-1 correlated with disease duration and organ damage; for IP-10, such correlations were not seen. It is not clear whether cotaxin and MCP-1 per se cause sustained inflammation and represent possible therapeutic targets. They might also be markers for disease damage as a result of disease activity caused by other unknown mechanisms. Either way,



the novel knowledge on these substances can improve insight and treatment modalities of JDM.

## Acknowledgments

We thank Ståle Nygård for helpful statistical advices and Hilde Dishington for laboratory assistance.

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## Author Contributions

Conceived and designed the experiments: HS TS BF IS. Performed the experiments: HS MV. Analyzed the data: HS TS BF MV GC IS. Contributed reagents/materials/analysis tools: HS MV. Wrote the paper: HS TS IS. Made critical review and approved the final version of the manuscript: HS TS BF MV GC IS. Had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis: HS TS BF MV GC IS.

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