Nicotinamide Adenine Dinucleotide biosynthesis enzymes in rheumatoid arthritis

Thesis submitted in accordance with the requirement of Cardiff University for the degree of Doctor in Medicine by

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October 2016

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Acknowledgements

First and foremost, I would like to thank my supervisors Mari Nowell, Anwen Williams and Simon Jones for their continued guidance, support and above all patience. I have truly appreciated your guidance, dependable advice and consistent contributions.

I would also like to thank my colleagues Laura Evans, Louise Osgood, Gareth Jones, Christopher Thomas and Valerie O'Donnell for their continued support and advice.

I would also like to thank my dear wife, Finnu Naduthopil, for her perseverance, patience and support. You are truly amazing.

Summary

Introduction: Synovial fibroblasts (SF) display a 'hyperactive' phenotype in patients with rheumatoid arthritis (RA). Nicotinamide adenine dinucleotide (NAD^+) plays a role in cell metabolism, but may also be a key molecule in maintaining this 'activated' phenotype. NAD^+ can be synthesised from precursor vitamin molecules, nicotinamide (Nam), nicotinic acid (NA) and Tryptophan (TRP); with their respective phosphoribosyl transferases (NAMPT, NAPRT, QAPRT) and Indoleamine (IDO) being the rate limiting enzymes involved in these pathways. NAMPT and IDO are known to be elevated in RA synovial tissue (ST). However, the expression and regulation of other NAD^+ biosynthesis enzymes are unknown.

Methods: RA, OA and normal ST were obtained from joints of patients undergoing surgery and expression of NAD^+ biosynthesis enzymes were quantified using qPCR. Synovial fibroblasts were cultured and stimulated with 10ng/ml of TNF-α, IL-1β, OSM & IFN-γ and the expression of NAD^+ biosynthesis enzymes were quantified using qPCR.

Results: qPCR analyses showed that all NAD^+ biosynthesis enzymes tested were constitutively expressed in synovial tissue *ex vivo* and *in vitro*, with the exception of NMN adenyltransferase (NMNAT)-3. NAMPT, IDO, QAPRT, NADSYN and NMNAT-2 were all upregulated in RA ST compared to normal tissue, however only NAMPT was significantly upregulated in RA compared to OA and normal, (NAMPT reached statistical significance when patients on anti-TNF therapy were excluded). Moreover, NAMPT was found to be upregulated in ST of young actively developing individuals, decreasing with age. Expression of NAD salvage enzymes, NAMPT and NMNAT-2 in ST correlated with each other and *de novo* NAD enzymes, IDO, QAPRT, NADSYN and NMNAT-2 were also correlated with each other in ST. NAMPT and IDO were both significantly upregulated *in vitro* following stimulation with OSM & IFN-γ but only NAMPT and NMNAT-2 were upregulated following stimulation with TNF-α & IL-1β. NAPRT expression was found to be low in RA ST and there was no upregulation following stimulation by OSM, IFN-γ, TNF-α & IL-1β.

Conclusion: The data presented in this thesis emphasises NAMPT and IDO as a potential therapeutic target in rheumatoid arthritis.

List of Abbreviations

1-MT – 1-methyl-tryptophan

ACTB – Actin β

AIA – Antigen induced arthritis

CCL – Chemokine (C-C motif) ligand

CXCL – Chemokine (C-X-C motif) ligand

CIA – Collagen induced arthritis

CRP – C reactive protein

DMARD – Disease modifying anti-rheumatoid drug

DMEM – Dulbecco's Modified Eagle Medium

ESR – Erythrocyte sedimentation rate

FCS – Foetal calf serum

GM-CSF – Granulocyte-monocyte colony stimulating factor

HLA – Human leukocyte antigen

ICAM – Intercellular adhesion molecule

IDO – Indoleamine 2, 3 Dioxygenase

IFN γ – Interferon Gamma

IL – Interleukin

KYN – Kynurenine

LC/MS/MS – Liquid chromatography/tandem mass spectrometry

MAPK – Mitogen activated protein kinase

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

NA – Nicotinic Acid

NAD – Nicotinamide Adenine Dinucleotide

NADSYN – NAD Synthetase.

Nam – Nicotinamide

NaMN – Nicotinamide mononucleotide

NAMPT – Nicotinamide Phosphoribosyl Transferase

NAPRT – Nicotinic Acid Phosphoribosyl Transferase

NF-κB – Nuclear factor kappa B

NMN – Nicotinamide Mononucleotide

NMNAT 1, 2 & 3 – Nicotinamide mononucleotide adenyltransferase 1, 2 & 3

OPG – Osteoprotegrin

OSM – Oncostatin M

PBEF – Pre-B cell colony enhancing factor

PBS – Phosphate bufferd saline

PRL – Prolactin

QAPRT – Quinolinic Acid Phosphoribosyl Transferase

Quin – Quinolinic Acid

RA – Rheumatoid arthritis

RANKL – Receptor activator of nuclear factor K ligand

STAT – Signal transducer and activator of transcription

TNF-α – Tumour necrosis factor alpha

Trp – Tryptophan

UBC – Ubiquitin C

VCAM – Vascular cell adhesion molecule

VEGF – Vascular endothelial growth factor

Table of Contents

Chapter 1 – General Introduction

1.1. Rheumatoid Arthritis

RA is a systemic inflammatory disease which affects about 1% of world population (Gabriel 2001). It is an autoimmune condition affecting multiple joints and the inflammatory process particularly affects the synovial membrane. Joint pain, swelling and redness are common symptoms leading to erosion of bone and cartilage (Fig 1.1) resulting in joint deformity (Fig 1.2). Extra-articular manifestations can affect multiple organs such as lung (Colby 1998), heart (Gabriel 2010), kidneys (Lawson and Maclean 1966) and skin (Sibbitt and Williams 1982). RA can begin at any age and is associated with fatigue, weight loss and prolonged stiffness after rest. There is substantial economic burden for patients and health services with a third of affected patients losing their job by five years despite treatment (Barrett et al. 2000; Young et al. 2002). Total direct cost for patients receiving biologic therapy was about €19,308 (£16,784) and those not receiving biologic agents was €2666 (£2317) per year (Cimmino et al. 2011).

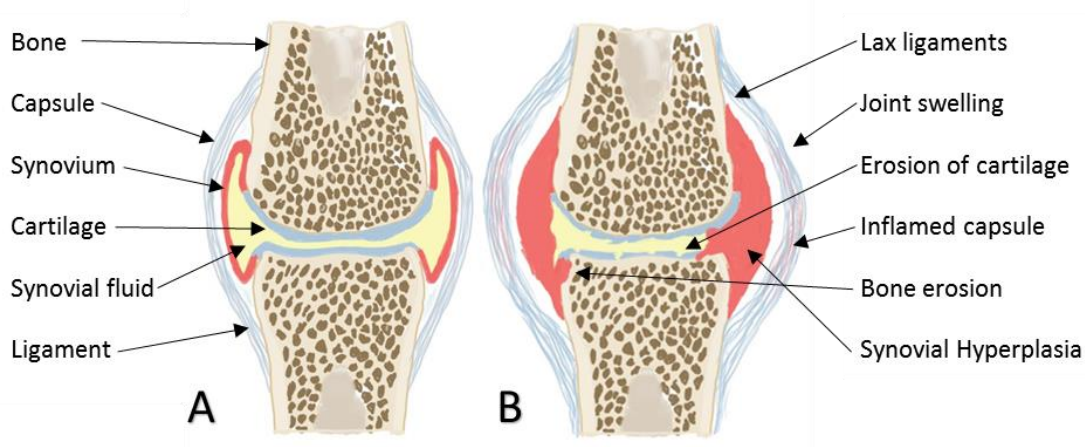


Fig 1.1. Diagramatic representation of a synovial joint. Comparison of normal joint (A) to joint in RA (B).

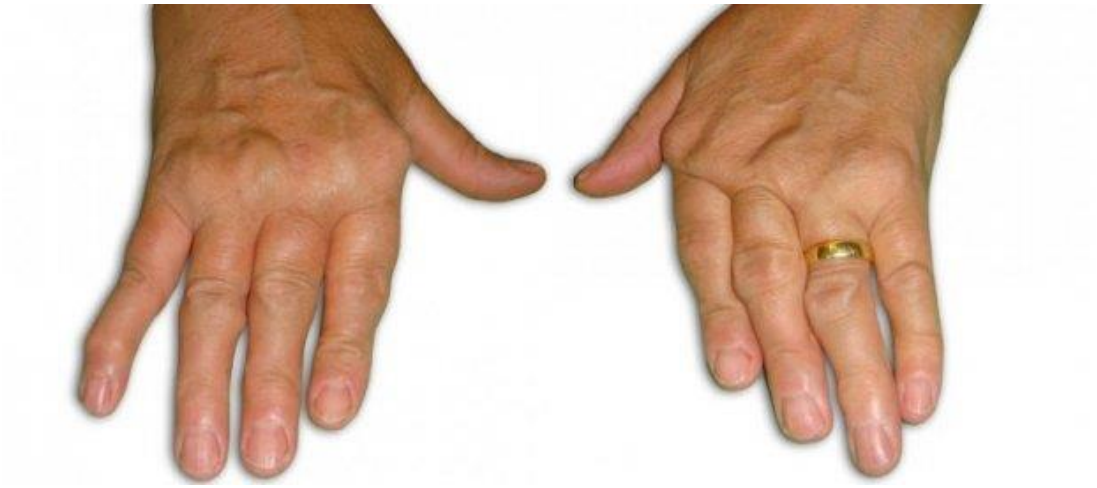


Fig 1.2. Clinical photography of bilateral rheumatoid hands demonstrating significant deformity; multiple swollen joints, radial deviation of wrist and ulnar deviation of fingers.

1.2. Criteria for diagnosis

According to the 2010 Rheumatoid Arthritis Classification Criteria (Aletaha et al. 2010) (American College of Rheumatology / European League against Rheumatism collaboration) 'definite RA' is based on the confirmed presence of synovitis (inflammation of synovial membrane) in at least 1 joint, absence of an alternate diagnosis that better explains the synovitis, and achievement of a total score of 6 or greater (of a possible 10) from individual scores in 4 domains; these domains include [a] The number and site of involved joints (score range 0 – 5), [b] Any serologic abnormalities (score range 0 – 3), [c] An elevated acute phase response (score range 0 – 1) and finally, [d] the symptom duration (2 levels: range 0 – 1) (table 1.1). Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

Criteria		Score
a	**Joint involvement[§]**	
	1 large joint[¶]	0
	2 – 10 large joints	1
	1 – 3 small joints (with or without involvement of large joints)[#]	2
	4 – 10 small joints (with or without involvement of large joints)	3
	>10 joints (at least 1 small joint)[**]	5
b	**Serology (at least 1 test result is needed for classification)[††]**	
	Negative RF *and* negative ACPA	0
	Low-positive RF *or* low-positive ACPA	2
	High-positive RF *or* high-positive ACPA	3
c	**Acute-phase reactants (at least 1 test result is needed for classification)[‡‡]**	
	Normal CRP *and* normal ESR	0
	Abnormal CRP *or* abnormal ESR	1
d	**Duration of symptoms[§]**	
	<6 weeks	0
	>6 weeks	1

Table 1.1. The 2010 American College of Rheumatology/European League against Rheumatism classification criteria for rheumatoid arthritis (Aletaha et al. 2010).

§ Joint involvement refers to any *swollen* or *tender* joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are *excluded from assessment*. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement.

¶ "Large joints" refers to shoulders, elbows, hips, knees, and ankles.

"Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

** In this category, at least 1 of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular, etc.).

†† Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF. ACPA = anti-citrullinated protein antibody.

‡‡ Normal/abnormal is determined by local laboratory standards. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

§§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

1.3. Aetiology

The exact aetiology for the development of RA is unclear and various factors have been hypothesised to trigger the onset of the disease. Discovery of rheumatoid factor in the serum of patients with RA in 1957 led to the belief that RA might be an autoimmune disease caused by self-reactive autoantibodies (Franklin et al. 1957), but not all patients of RA have positive autoantibodies. RA has also been known to run in families leading to the belief that there might be a genetic link; Human leukocyte antigen (HLA)-DR genes which reside in the major histocompatibility complex (MHC) and participate in antigen presentation have been strongly associated with the disease (Stastny 1976; Nepom et al. 1989). Environmental factors are also believed to have an effect on the induction, severity and rate of progression of the disease; recent studies have strongly implicated smoking as an important risk factor for the development of the disease in HLA-DR4 positive individuals (Heliovaara et al. 1993; Klareskog et al. 2007; Sugiyama et al. 2010). Finally, infective organisms such as Epstein Barr virus have been implicated in the onset of RA, but no single organism have been proven to be responsible (Ferrell et al. 1981; Saal et al. 1999; Carty et al. 2004).

1.4. Clinical manifestation

RA primarily affects synovial joints, although extra-articular manifestations can involve the skin, lung, kidneys, heart and eyes. Joints become swollen, tender, warm and stiff with synovitis leading to erosion of bone and cartilage causing deformity and loss of function. Rheumatoid nodules are the most characteristic extra-articular feature - a typical rheumatoid nodule may be a few millimetres to a few centimetres in diameter and is usually found over bony prominences such as elbow, heel and knuckles. Other skin manifestations include pyoderma gangrenosum, erythema nodosum and palmar erythema. Interstitial lung disease, fibrosis and pleural effusions are recognised complications of rheumatoid disease. Renal amyloidosis can occur as a consequence of chronic inflammation and RA can affect glomerulus of kidney directly through development of vasculopathy. Patients are prone to develop atherosclerosis and myocardial infarction, indeed cardiovascular mortality in patients with RA is up to 50% higher than the general population (Meune et al. 2009). Other cardiac complications include pericarditis, endocarditis, valvulitis and fibrosis. Patients with RA can also present with inflammation of the sclera leading to episcleritis. Patients are often anaemic but may also have neutropaenia and

thrombocytosis. Fatigue, fever, malaise, morning stiffness, loss of appetite and loss of weight are also common symptoms.

1.5. Histopathology

Key histological features of RA include synovial lining layer hypertrophy, sublining infiltration with mononuclear cells, increased vascularity and fibrin deposition (Fig 1.3 A-C). The surface of the lining layer is often covered with fibrin deposits generated from the activation of fibrinolytic system by synovial fluid (Fig 1.3D). The lining layer may be completely replaced by a fibrin cap and in highly inflamed tissue, this fibrin may extend deep into the sublining layers. In the early stage of the disease, the sublining infiltrate may be minimal or modest (Willemze et al. 2008) which may include macrophages (Kraan et al. 1998) and natural killer cells (Tak et al. 1994). Diffuse mononuclear infiltrates and small lymphoid aggregates are seen in both early and late RA; however, aggregates resembling lymphoid follicles with germinal centres are typically seen only in well-established cases (Fig 1.3A). Pannus, the highly destructive tissue present at the interface between synovium, cartilage and bone is a characteristic feature of erosive RA and contains large numbers of macrophages, fibroblasts and osteoclasts that express high levels of proteases (Fig 1.3E) (Hitchon and El-Gabalawy 2011). These cells cause resorption of bone leading to bone erosions and in turn leads to invasion of cells from synovial membrane.

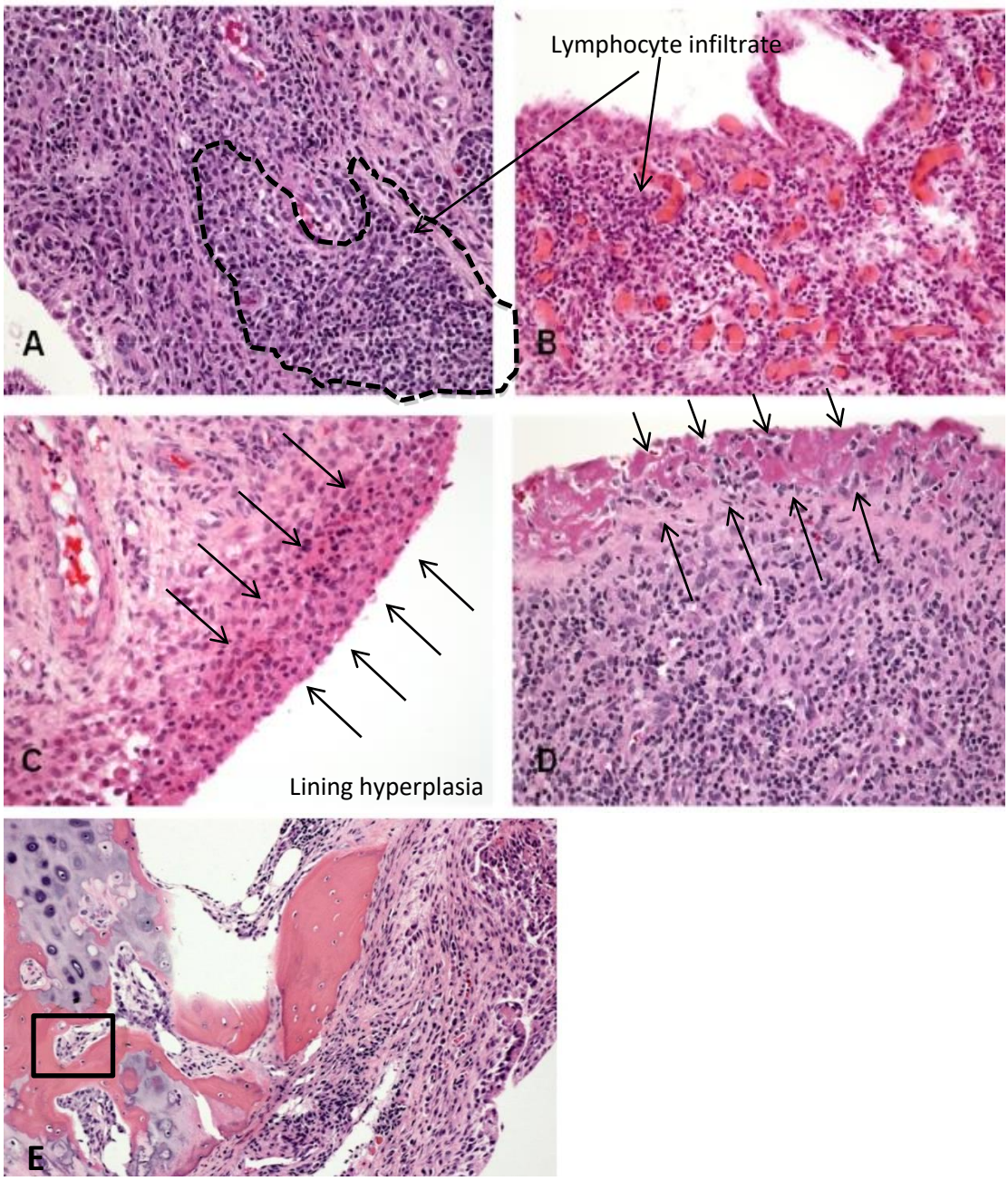


Figure 1.3. Histopathology of RA synovitis. (**A**) lymphoid aggregate; (**B**) Diffuse lymphocytes infiltrate; (**C**) Hyperplasia of the lining layer; (**D**) Fibrin cap replacing a denuded lining layer (arrows). (**E**) Interface between pannus tissue and bone in a patient with RA, showing the synovial lesion invading the adjacent bone (rectangle). Adapted from Hitchon and El-Gabalawy (2011) (Hitchon and El-Gabalawy 2011).

1.6. Cytokines in synovial inflammation

Cytokines are proteins secreted by specific cells which carry signals locally from one cell to another. Studies have shown that in RA there is increased production of pro and anti-inflammatory cytokines with high expression found in synovial fluid (Fontana et al. 1982; Di Giovine et al. 1988; Hopkins et al. 1988; Hopkins and Meager 1988; Houssiau et al. 1988) and tissues (Buchan et al. 1988; Firestein et al. 1990; Chu et al. 1991a; Field et al. 1991). The chronicity of RA could be due to an imbalance between these pro and anti-inflammatory cytokines. Cytokines are implicated in each phase of pathogenesis of RA by promoting autoimmunity, maintaining chronic synovitis and by causing the destruction of adjacent joint tissues (McInnes and Schett 2007). With the onset of RA, T helper 1 (T_H1) and T_H17 cells produce inflammatory cytokines and chemokines such as interferon-γ (IFN-γ), interleukin-1 (IL-1) and interleukin–17 (IL-17) which leads to the stimulation of macrophages, synovial fibroblasts (Schulze-Koops and Kalden 2001) and chondrocytes (Liacini et al. 2002) (Fig 1.4).

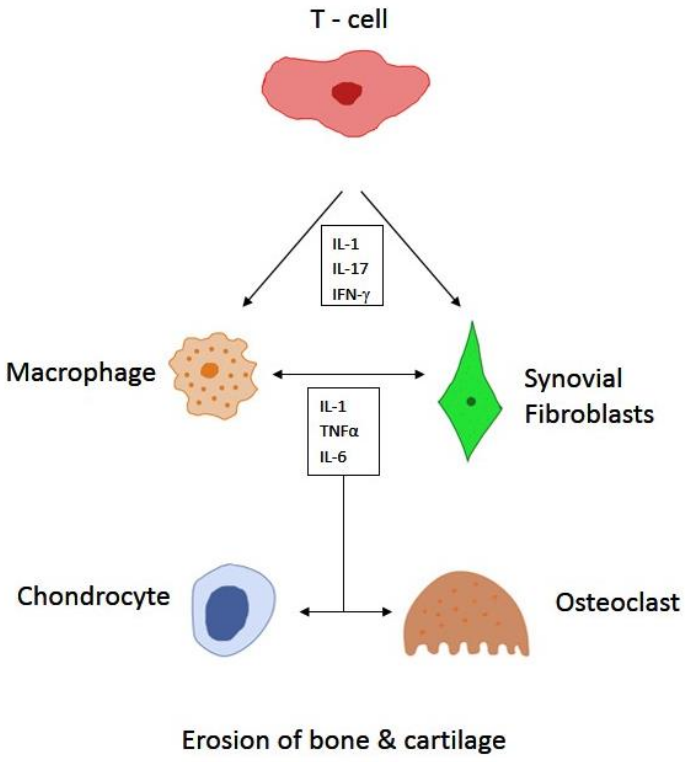


Fig 1.4. Cell types and cytokine pathway involved in the destruction of bone and cartilage in RA.

B-cells are also stimulated to produce immunoglobulin such as rheumatoid factor and anti-cyclic citrullinated peptide which leads to further activation of macrophages and synovial fibroblasts (Nardella et al. 1983; Lu et al. 2010). Whether the T-cell activates the B-cell or vice-versa has been a matter of much debate over many years (Takemura et al. 2001; Vita et al. 2002; Firestein 2003; Raza et al. 2005). Thus stimulated fibroblasts and macrophages release a number of cytokines, most notably, tumour necrosis factor-α (TNF-α), IL-1, interleukin-6 (IL-6) and oncostatin M (OSM) which ultimately leads to continuing synovial inflammation, bone erosions, cartilage damage, and endothelial cell proliferation.

1.6.1 TNF- α

TNF-α is a potent cytokine that stimulates a variety of cells and is produced mainly by activated macrophages and also by fibroblasts, B cells and T cells. TNF-α is highly expressed in serum (Tetta et al. 1990), synovial fluid (Tetta et al. 1990) and synovial tissues (Chu et al. 1991b) in patients with RA. It has both autocrine effect as well as a paracrine inducer of other inflammatory cytokines such as IL-1, IL-6, IL-8 and granulocyte-monocyte colony stimulating factors (Haworth et al. 1991; Butler et al. 1995). TNF-α contributes to joint destruction and synovitis in RA by inducing resorption of bone and cartilage (Saklatvala et al. 1984; Bertolini et al. 1986), stimulating growth of fibroblast (Vilcek et al. 1986) and inducing prostaglandin E2 and collagenase secretion from synovial cells (Dayer et al. 1985). TNF is also a potent inducer of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin (Nakada et al. 1998) which leads to endothelial cell migration and inflammation.

1.6.2 IL-1β

IL-1β was one of the first cytokine which was isolated from synovial fluid in RA and its level in serum correlated with clinical disease activity (Eastgate et al. 1988). It is produced mainly by macrophages and monocytes. It is a potent cytokine which mediates the destruction of bone and cartilage (Joosten et al. 1999) and impairs their repair (van de Loo and van den Berg 1990). Injection of murine recombinant IL-1 into mice knee joints led to enhanced loss of proteoglycan and also inhibition of proteoglycan synthesis (van de Loo and van den Berg 1990). IL-1 is more potent than TNF-α in causing cartilage destruction in vivo. There appears to be synergism in the action of TNF and IL-1. In rats with collagen induced arthritis (CIA), a combination therapy with IL-1 receptor antagonist (IL-1Ra) and PEGylated soluble tumour necrosis factor receptor type I (PEG sTNFRI) resulted in additive effect on clinical and

histologic parameters than when treated with either agents alone (Bendele et al. 2000).

1.6.3 IL-6

IL-6 is a 26kDa protein that was discovered in 1986 which features pleiotropic activity (Kishimoto 2006). IL-6 levels were found to be elevated in synovial fluid and synovial tissues in RA (Hirano et al. 1988) and serum levels correlated with disease activity (Madhok et al. 1993). In mice with CIA, excess serum production of IL-6 was observed within 24hrs of type II collagen immunisation with the development of arthritis subsequently. However when these mice were treated with anti-IL-6R monoclonal antibody it inhibited the development of arthritis in a dose dependant manner (Takagi et al. 1998). IL-6 has been shown to induce T cell growth and differentiation by augmenting IL-2 receptor expression and IL-2 production (Kishimoto 2006). IL-6 plays an important role in inflammation by increasing the expression of ICAM-1 and by inducing the production of chemokines such as MCP-1 and IL-8 in inflamed joints (Suzuki et al. 2010). IL-6 along with soluble IL-6R stimulates osteoclasts and regulates receptor activator of nuclear factor kappa -B ligand (RANKL) and osteoprotegrin (OPG) leading to bone resorption and joint destruction (Kotake et al. 1996; Palmqvist et al. 2002).

1.6.4 IL-17

Interleukin-17 is a T cell derived pro-inflammatory cytokine. In mice with CIA, the incidence and severity of the disease was marked in IL-$17^{+/+}$ mice when compared with IL-$17^{-/-}$ (Nakae et al. 2003). IL-17 expression is increased in RA synovium and RA synovial fluid (Ziolkowska et al. 2000; Kehlen et al. 2002). IL-17 induces the production of IL-6 and IL-8 from RASF and is mediated via NF-κB and phosphatidylinositol 3-kinase (Hwang et al. 2004). IL-17 induces the inhibition of proteoglycans from cartilage and also type I collagen from synovium and bone (Chabaud et al. 2000). Patients with RA who did not respond to anti-TNF therapy were found to have increased levels of serum IL-17 when compared to responders suggesting a possible role for targeting therapy (Chen et al. 2011a).

1.6.5 Oncostatin M

Oncostatin M (OSM), an IL-6 family cytokine, is significantly expressed in the synovial fluid of patients with RA (Hui et al. 1997; Manicourt et al. 2000). It is produced by activated monocytes, T-lymphocytes (Zarling et al. 1986) and also by synovial tissue

macrophages (Okamoto et al. 1997; Cawston et al. 1998). OSM mRNA is also highly expressed in synovial tissues of RA patients (Okamoto et al. 1997). Injecting human OSM into goat joints causes cartilage re-sorption and inhibition of proteoglycan synthesis, suggesting its role in the pathogenesis of RA (Bell et al. 1999). Mice with CIA had increased expression of OSM mRNA and treatment with anti-OSM antibody led to amelioration of clinical severity and number of affected paws (Plater-Zyberk et al. 2001). Furthermore, OSM promotes angiogenesis, endothelial cell migration (Fearon et al. 2006) and synergises with IL-1β to promote extracellular matrix turnover and cartilage degradation (Li et al. 2001; Fearon et al. 2006).

1.6.6 IFN-γ

IFN-γ is a naturally occurring cytokine with antiviral, anti-proliferative and immune-modulatory properties. They are produced by T helper 1 cells in RA which then act on macrophages to release other cytokines. Levels of IFN-γ mRNA are found to be high in the synovial fluid (Bucht et al. 1996) and synovial tissue (Cañete et al. 2000) in RA. Initially IFN-γ was felt to be an anti-inflammatory cytokine. A study by Page *et al* have shown that IFN-γ down regulated the IL-1β driven production of MMP-1 & -3 in vitro and also reduced the expression of IL-1β in arthritic joint and prevented cartilage damage in antigen induced arthritis (AIA) mice (Page et al. 2010). IFN-γ receptor knockout mice had accelerated onset of CIA than the wild types (Vermeire et al. 1997). Similar accelerated onset of CIA was also seen in wild type mice treated with monoclonal antibodies against IFN-γ (Vermeire et al. 1997). However, controversy exists with regards to the exact function of IFN-γ in inflammation. IFN-γ has been found to stimulate the production of TNFα (Yocum et al. 1989) and IL-1 (Donnelly et al. 1990) by the synovial mononuclear cells. It induces the expression of chemokine CX3CLI by RA osteoblast in synergistic fashion with TNF-α (Isozaki et al. 2008).

1.7. Signalling pathways

Pathogenesis of RA is clearly very complex, with T-lymphocytes, macrophages, synovial fibroblasts and chondrocytes being involved in releasing and responding to a large variety of cytokines. These cytokines transmit signal from the cell membrane to the nucleus via various complex signalling pathways such as Janus kinases (JAK), signal transducer and activator of transcription (STAT), nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK) and phosphoinositide 3' kinases (Fig 1.5).

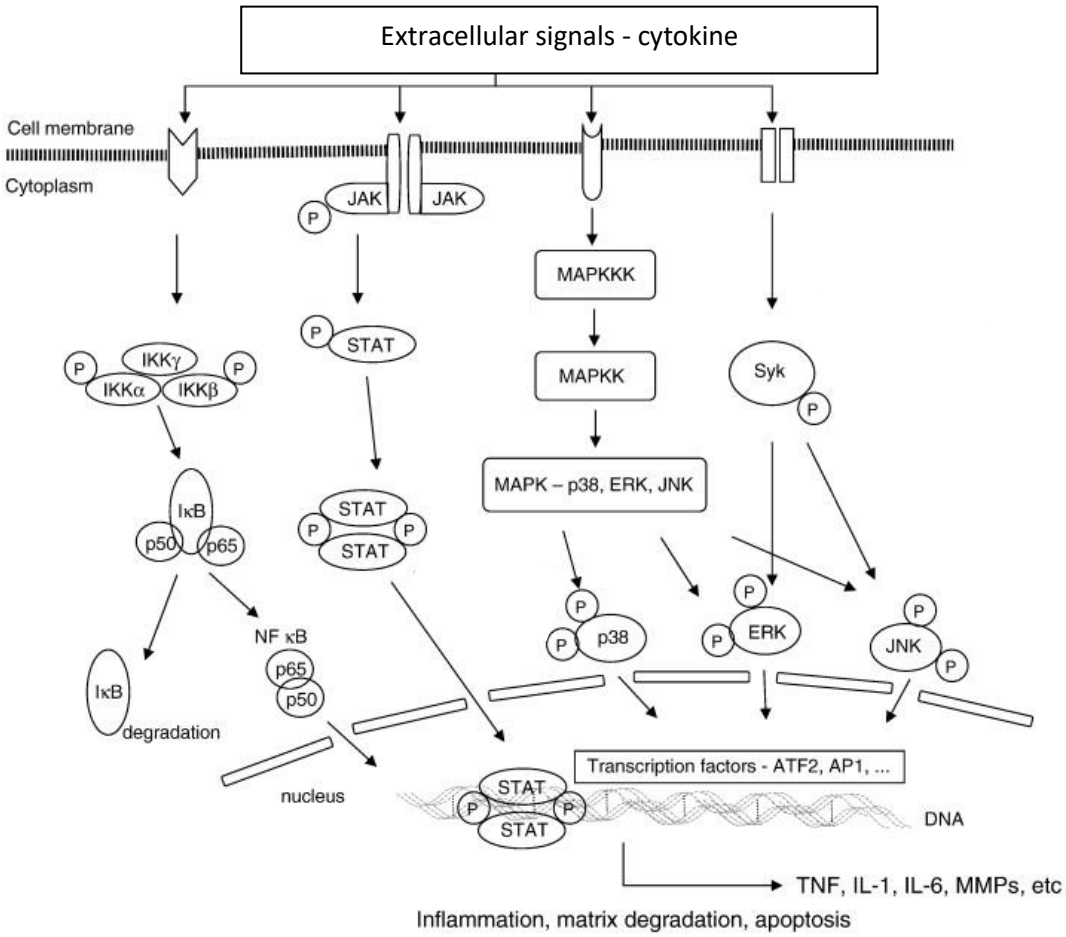


Fig 1.5. Schematic drawing of signal transduction pathways and transcription. Upon exposure of a cell to a proinflammatory environment, several regulatory enzymes are phosphorylated and activated. As a result, an intracellular signalling cascade is activated to transmit the signal from a receptor, which affect the expression of genes for cytokines, matrix metalloproteinases, apoptosis regulating molecules, proliferation, etc. (Adapted from Senolt *et al* (2009))

1.7.1. JAK-STAT signalling

In mammals four different kinds of JAKs; JAK1, JAK2, JAK3 and Tyk2 (Wilks 1989; Firmbach-Kraft et al. 1990; Partanen et al. 1990; Cance et al. 1993) and seven STATs; STAT1-4, STAT5a & 5b and STAT6 have so far been identified (Fu 1992; Fu et al. 1992; Schindler et al. 1992; Veals et al. 1992). STAT1, STAT4 and Jak3 expression has been found to be significantly expressed in RA synovium in the lining and sublining layers (Walker et al. 2006). The binding of a ligand (e.g. IFN, IL-6) to the extracellular domain of its receptor complex activates associated JAKs, leading to the tyrosine phosphorylation of receptors, which generate docking site(s) for STATs through the STAT's SH2 domain. This leads to phosphorylation and dimerization of the STAT transcription factors, which then dissociate from the receptor cytoplasmic domain and translocate to nucleus where they modulate the expression of target

genes (Fig. 1.5). Different JAKs and STATs have been linked to different cytokines, and these are summarised in Table 1.2 below.

Signalling molecule	Known activating cytokines
JAK 1	**INF**, **IL-6**, **OSM**, IL-2, IL-4, IL-7, IL-9, IL-10, IL-15 and IL-11
JAK2	**IFN-γ**, IL-3, GM-CSF, prolactin, erythropoietin and thrombopoietin
JAK3	IL-2, IL-4, IL-7, IL-9 and IL-15
Tyk2	**IL-6, OSM**, IL-11, IL-12, IFN-α/β, IL-10, IL-13
STAT1	**IFN, IL-6,** IL-10
STAT2	IFN-α/β & λ
STAT3	**IL-6,** IL-2, IL-10, **OSM**
STAT4	IL-12, IL-23
STAT5	IL-2, prolactin, **OSM**
STAT6	IL-4, IL-13

Table 1.2. Jak-STAT signalling molecules linked to different cytokines (Imada and Leonard 2000; Ortmann et al. 2000). Cytokines which have been used in this study have been highlighted in **bold**.

1.7.2. Nuclear Factor κB (NF-κB) Signalling

NF-κB is present in the cytoplasm of a cell in its inactive form as it is bound by an inhibitory protein, IκB (Fig. 1.5). In response to extracellular signalling by various cytokines (including **TNF-α/β, IL-1 α/β*,** IL-2, M-CSF and GM-CSF), IκB gets phosphorylated and degraded, releasing the NF-κB which enters the nucleus and activates NF-κB regulated target genes. This process is eventually terminated by the NF-κB induced synthesis of IκB and consequently, cytoplasmic sequestration of this transcription factor (Li and Verma 2002). Immunohistochemical studies have shown NF-κB to be present within the synovial sublining region and also in endothelium in patients with RA with negligible staining in normal synovial samples (Handel et al. 1995).

1.7.3. Mitogen Activated Protein (MAP) Kinases

MAP kinases consist of three kinase families – extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Schett et al. 2000; Schmitz et al. 2002; Radziwill 2007). All three kinases are abundantly seen in RA with ERK activation predominantly seen in cells of sublining regions and around synovial microvessels, while p38 activation is predominantly seen in the synovial lining and in endothelial cells of synovial microvessels. JNK activation is mainly seen in mononuclear cell infiltrates of the sublining regions of the synovium (Schett et al.

* Text in bold are the cytokines of interest in this study

2000). Following activation of MAP kinases (e.g., by TNF-α, IL-1 & IL-6), MAPK are phosphorylated and enter the nucleus to bind to the promoter regions of several genes.

1.8. Action of Cytokines

Following activation by cytokines via various signalling pathways, cells such as monocytes, macrophages, T-lymphocytes and synovial fibroblasts are induced to produce more cytokines such as TNF-α, IL-1, IL-6, IFN and granulocyte-monocyte colony stimulating factor (GM-CSF). However, there are a number of other soluble factors that are also produced, that have significance in RA.

1.8.1. Leucocyte Recruitment

Cytokines result in leucocyte recruitment by increasing the expression of extracellular adhesion molecules such as ICAM-1 and by release of chemo-attractant proteins (chemokines) such as CCL2, CCL5, CCL8, CCL15 and CXCL8 (Haringman et al. 2006). Chemokines activate integrins (molecules on leucocytes) which mediates adhesion of leucocytes to vascular endothelium through ICAM or VCAM. Once adherent, chemokines induces changes in leucocytes leading to its migration across the endothelium into the inflammatory sites (Adams and Rlloyd 1997).

1.8.2. Tissue Degradation

Cytokines such as IL-1 and TNF activate NF-κB in synovial fibroblasts inducing matrix metalloproteinase (MMP) -1, -3 and -13 (Liacini et al. 2002; Brentano et al. 2007; Evans et al. 2011). MMPs initially breakdown type II collagen and the resultant proteoglycans released is further broken down by various proteases. In addition to collagen degradation, aggrecan is also degraded resulting in degradation of cartilage (Rannou et al. 2006). RANKL is expressed by a variety of cells in RA including T-lymphocytes and synovial fibroblast. Stimulation of these cells by cytokines such as TNF-α and M-CSF leads to activation and maturation of osteoclast which results in bone erosions and destruction (Takayanagi et al. 2000).

1.8.3. Angiogenesis

Cytokines such as TNF-α, IL-1, IL-15, and IL-18 play major roles in regulating angiogenesis in RA synovium (DeBusk et al. ; Angiolillo et al. 1997; Park et al. 2001; Voronov et al. 2003; Kim et al. 2009). Angiogenic properties of these cytokines may be mediated through secondary angiogenic mediators such as vascular endothelial

growth factor (VEGF), platelet derived growth factor (PDGF), insulin-like growth factor, transforming growth factor-β and fibroblast growth factor (FGF) (Koch 1998; Szekanecz et al. 1998; Szekanecz and Koch 2001; Rosengren et al. 2010). These angiogenic factors lead to endothelial cell activation, increased vascular permeability, breakdown of basement membrane, endothelial cell migration and proliferation at the site of stimulus and remodelling to form new capillaries (Clavel et al. 2003). Hyperplasia of the synovial cells leads to an increase in the distance between the proliferating synovial cells and blood vessels leading to hypoperfusion and local tissue hypoxia which also stimulates angiogenesis (Paleolog 2002). Angiogenesis leads to further infiltration of inflammatory cells and production of inflammatory mediators perpetuating synovitis and pannus formation.

1.9. Nicotinamide Adenine Dinucleotide (NAD^+)

NAD^+ is a metabolite that is an important cofactor and secondary messenger for a number of cellular processes which are essential for cell survival. NAD^+ is involved in energy production, genomic stability, calcium metabolism and apoptosis. In eukaryotes, NAD^+ is synthesised from vitamins such as nicotinamide, tryptophan and nicotinic acid via salvage, *de novo* and Preiss-Handler pathway respectively with NAMPT, IDO, QAPRT and NAPRT as the rate limiting enzymes (Fig 1.6). Little is known about two of the enzymes (NAMPT and IDO) in RA, but very little about other NAD^+ biosynthesis enzymes and this thesis hopes to address this gap in our knowledge.

1.10. NAMPT

Nicotinamide phosphoribosyl transferase (NAMPT) is a 52kD protein which was initially discovered as a factor enhancing the effect of stem cell factor and IL-7 on pre-B-cell colony formation and was therefore initially termed as pre-B-cell colony enhancing factor (PBEF) (Samal et al. 1994). In 2005, Fukuhara *et al* reported that an adipokine or visceral fat derived hormone mimicked insulin like function and termed it visfatin (Fukuhara et al. 2005). Two independent reports, Rongvaux *et al* (2002) and Revollo *et al* (2004) since demonstrated that PBEF was mammalian NAMPT, with both reports reporting similar K_m values (substrate concentration at which the rate of reaction reaches 50% the maximum values of [X]). Therefore, three different nomenclatures – NAMPT, PBEF and visfatin have so far been given to this protein. However, NAMPT has been approved as the official nomenclature of the gene and the protein by both the HUGO Gene Nomenclature Committee (HGNC) and the Mouse Genomic Nomenclature Committee (MGNC).

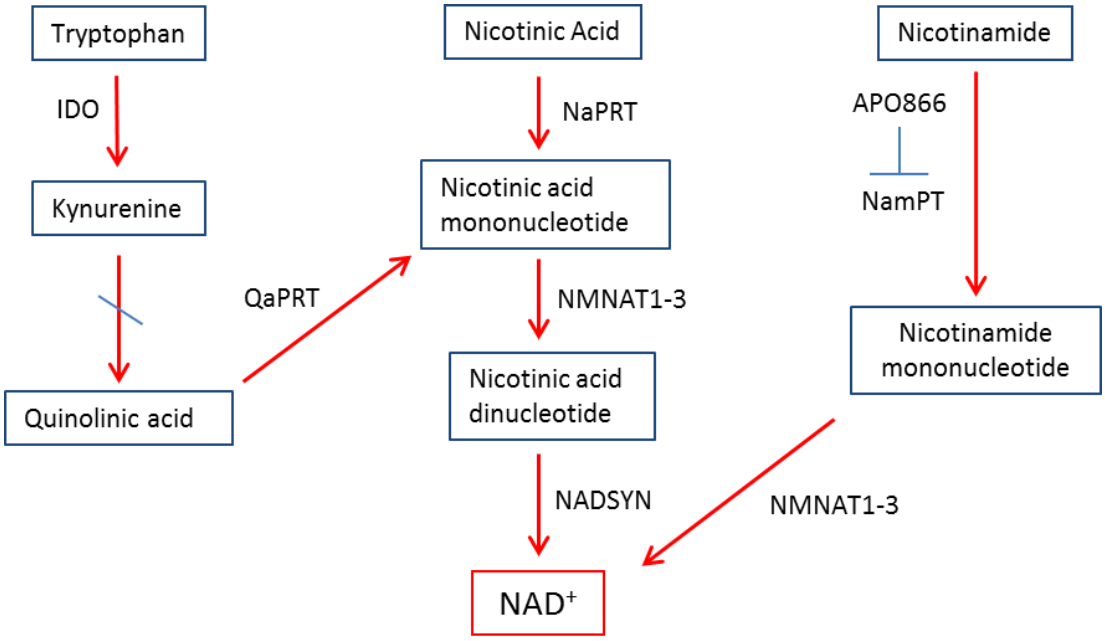


Fig 1.6. Pathway of NAD^+ biosynthesis.

IDO – indoleamine, QaPRT – quinolinic acid phosphoribosyl transferase, NaPRT – nicotinic acid PRT, NMNAT – nicotinamide mononucleotide adenyl transferase, NADSYN – NAD synthetase, NamPT – nicotinamide PRT.

In mammals, NAMPT has two forms – intracellular and extracellular (iNAMPT and eNAMPT respectively) (Revollo et al. 2007a). While the function of iNAMPT has been firmly established as an essential nicotinamide adenine dinucleotide (NAD^+) biosynthesis enzyme (Rongvaux et al. 2002; Revollo et al. 2004), the physiological role of eNAMPT remains controversial.

1.9.1. iNAMPT

iNAMPT is the rate limiting enzyme in the synthesis of NAD^+ from nicotinamide. NAD^+ is also produced by different vitamins such as tryptophan, nicotinic acid and nicotinamide riboside. Please refer to section 3.1 for detailed information regarding NAD^+ biosynthesis.

1.9.2. eNAMPT

There is debate in the literature as to whether NAMPT is exclusively an enzyme or a dual protein with both enzymatic and cytokine-like activity. Moschen *et al* (2007) demonstrated that eNAMPT induces the production of IL-1β, TNF-α and IL-6 from $CD14^+$ monocytes. It also increased the surface expression of co-stimulatory molecules CD54, CD40 and CD80 (Moschen et al. 2007). NAMPT has been shown to induce the above effect involving p38, MEK1 and NF-κB pathways, and this effect can be inhibited by MAKP inhibitors (Moschen et al. 2007). Li *et al* (2008) have also demonstrated that eNAMPT protects macrophages from endoplasmic reticulum (ER) stress-induced apoptosis by activating an IL-6/STAT3 signalling pathway via a nonenzymatic mechanism.

eNAMPT is positively secreted through a non-classical secretory pathway by fully differentiated mouse and human adipocytes (Revollo et al. 2007b), and also by human and rat primary hepatocytes (Imai and Kiess 2009). It has been shown that fully differentiated adipocytes are a natural producer of eNAMPT, which is capable of converting circulating nicotinamide to nicotinamide mononucleotide (NMN) (see section 3.1) (Revollo et al. 2007b). Some believe that NMN can be carried by the blood to distant tissues or organs, to be internalised in the cell and then converted to NAD^+ by NMN acetyl transferase (NMNAT) inside cells (Imai 2009b).

1.9.3. Relation of NAMPT to arthritis & inflammation

NAMPT functions as an essential enzyme in the biosynthesis of NAD^+ that enhances cellular resistance to genotoxic stress and may confer to cells of the immune system the ability to survive during stressful situations such as inflammation (Rongvaux et al.

2008). NAMPT expression is upregulated on activation of immune cells such as monocytes, macrophages, dendritic cells, T cells, and B cells when stimulated with lipopolysaccharide, TNF-α, IL-1β, or IL-6 (Rongvaux et al. 2002; Iqbal and Zaidi 2006; Busso et al. 2008). Abnormal NAMPT expression has been demonstrated in cells and tissues in a number of diseases with NAMPT showing high expression in chronically inflamed tissues such as inflammatory bowel disease (Moschen et al. 2007), rheumatoid tissue (Nowell et al. 2006), atherosclerotic plaques (Dahl et al. 2007) and has potential implications in the pathogenesis of acute lung injury (Shui et al. 2005).

Elevated levels of NAMPT have been reported in serum and synovial fluids of patients with RA (Nowell et al. 2006; Otero et al. 2006; Brentano et al. 2007; Meier et al. 2012) and its level correlate with C-reactive protein levels (Otero et al. 2006; Brentano et al. 2007) and clinical disease activity in patients with RA (Brentano et al. 2007). NAMPT gene expression is significantly increased in synovial tissues, peripheral blood mononuclear cells (PBMC) and peripheral blood granulocytes in patients with RA (Nowell et al. 2006; Brentano et al. 2007; Matsui et al. 2008; Meier et al. 2012). Immunohistochemical study of synovial tissue in RA has shown NAMPT to be present within the cytoplasm and nucleus of synovial apical cells which are predominantly fibroblast like and macrophage like cells and also within the endothelial cells lining the capillaries (Nowell et al. 2006).

In mouse models of arthritis, synovial expression of NAMPT is increased by four fold in antigen induced arthritis (AIA) (Nowell et al. 2006) with increased levels also seen in serum and paws of mice with CIA (Busso et al. 2008; Evans et al. 2011). Inhibiting NAMPT using a small molecule inhibitor, APO866, effectively halts the progression of CIA and also improves the clinical score in diseased mice (Busso et al. 2008; Evans et al. 2011).

NAMPT promotes the expression of MMP-3, CCL2 and CXCL8 by activated human fibroblasts *in vitro* and this effect is inhibited by APO866 (Evans et al. 2011). In RA, NAMPT has been shown to activate human leucocytes to induce proinflammatory cytokines including IL-1β, IL-6 and TNF-α (Brentano et al. 2007; Moschen et al. 2007; Neumann et al. 2011).

A study by Jia *et al* (2004) has shown that NAMPT is expressed in neutrophils and monocytes in response to activation by lipopolysaccharide, TNF-α and IL-1β. Preventing NAMPT translation with an antisense oligonucleotide blocks the inhibition of apoptosis of activated neutrophils, suggesting a requisite role for NAMPT in delaying neutrophil apoptosis (Jia et al. 2004). These factors within the synovial fluid

of RA are believed to prolong the neutrophil lifespan and activity, which then contributes to inflammatory process.

These evidence suggests that aberrant NAMPT activity is likely to have an effect on how inflammatory cells respond to chronic inflammation and clearly has a part in the development of synovitis and joint destruction.

1.10. Current Treatment

According to NICE guidelines (NICE 2009) when patients are diagnosed with RA, the first line of treatment is a combination of disease modifying anti-rheumatoid drugs (DMARDs) including methotrexate and at least one other DMARD, plus short term glucocorticoids. When patients fail more than two different DMARD treatments, then biologics are initiated.

1.10.1 Disease Modifying Anti-Rheumatoid Drugs (DMARDs)

Methotrexate was originally developed as a folate antagonist for the treatment of cancer. The first reported use of methotrexate in RA was in 1950s (Gubner et al. 1951). In 1980s, four well designed, blinded, placebo controlled studies established the use of methotrexate in RA (Thompson et al. 1984; Andersen et al. 1985; Weinblatt et al. 1985; Williams et al. 1985). Sulfasalazine was developed in the 1930s, specifically for RA, by combining an antibacterial agent (sulfapyridine) and an anti-inflammatory agent (5-aminosalicylic acid) (Svartz 1942). The exact mechanism of action of sulfasalazine is unknown but it is believed to have anti-inflammatory or immunomodulatory properties (Smedegård and Björk 1995; Krakauer 2015). Leflunomide was approved by FDA in 1988 as an oral therapy for treatment of active RA. Leflunomide inhibits *de novo* pyrimidine synthesis, resulting in inhibition of T-cell proliferation and suppression of TNF-induced cellular responses, as well as inhibition of matrix metalloproteinases and osteoclasts (Breedveld and Dayer 2000). Hydroxychloroquine sulphate and chloroquine phosphate are Quinine based drugs used in the treatment of RA. The exact mechanism of action is unknown but it is believed to alkalinise macrophage lysosomes and also stabilise lysosomal membranes, thereby inhibiting the release of lysosomal enzymes. It has also been postulated that hydroxychloroquine inhibits stimulation of toll-like receptors which are responsible for inducing inflammatory responses through activation of the innate immune system (Kyburz et al. 2006).

1.10.2 Biologics

According to the National Cancer Institute, a biological drug is defined as *'a substance that is made from a living organism or its products and is used in the prevention, diagnosis, or treatment of cancer and other diseases'* (NCI). Biological drugs include antibodies, interleukins and vaccines. Since the late 1990s, advances in molecular biology led to the development of biologics which has led to new treatment opportunities in RA. Anti-TNF biologic therapy has revolutionised the way we treat patients with RA (table 1.3), though anti-TNF medications are very expensive, costing upto £10,000 per person annually (NICE 2007).

1.10.2.1. Side Effects

Biologics interfere with the immune system, hence, should be avoided in patients with severe infections. Patients should be tested for prior TB infections as biologics may cause reactivation of latent disease (Dixon et al. 2010). Anti-TNF should be avoided in patients with symptomatic congestive heart failure (Chung et al. 2003). However a Cochrane review by Singh *et al* (2011) didn't show any statistically significant difference of experiencing heart failure in patients taking biologics to placebo. Anti-TNF therapies are also not recommended for patients with multiple sclerosis (Mohan et al. 2001) and current or previous lymphoma. Long term use of anti-TNF increases the risk of developing cancer such as lymphoma (Geborek et al. 2005), however recent evidence suggest that the risk of developing cancer amongst patients on biologics is low when compared to patients on DMARDs (Ramiro et al. 2014).

Patients on anti-TNF have increased risk of developing infections and hence they need to be stopped prior to any surgery. However, this could lead to increase pain from inflamed joints and therefore strategies need to be employed involving the pain team and rheumatologist in minimising the effects of cessation of these medications.

Biologic Name	Target	Structure	Administration
Anti-TNF			
Etanercept	TNF	Dimeric fusion protein: extracellular portion of human p75 TNF receptor linked to the Fc region of IgG_1	Subcutaneous injection: Once or twice weekly
Infliximab	TNF	Chimeric human-murine monoclonal antibody: Fc portion of human IgG1 and murine Fab fragment to TNF	Intravenous infusion: once every six weeks
Adalimumab	TNF	Recombinant human monoclonal antibody specific to TNF	Intravenous infusion: once every two weeks
Certolizumab pegol	TNF	PEGylated Fab' fragment of humanised antibody to TNF	Subcutaneous injection: Every two weeks
Golimumab	TNF	Human IgG1 monoclonal antibody to TNF	Subcutaneous injection: once a month
IL-1 Inhibitor			
Anakinra	IL-1	Recombinant IL-1 receptor antagonist	Subcutaneous dose: daily
IL-6 receptor antagonist			
Tocilizumab	IL-6R	Human antibody to IL-6 receptor	Intravenously: every four weeks
B-cell antibody			
Rituximab	CD20	Human/mouse chimeric antibody to CD20 (found on the surface of B-cells)	Intravenously: two doses given two weeks apart every six months
B-Cell T-cell co-stimulation inhibitor			
Abatacept	CD28	Recombinant human fusion protein of CTLA-4 and the Fc domain of IgG1	Intravenously: every four weeks

Table 1.3. Biologics used in the treatment of RA

1.10.3. Small Molecule Inhibitors

Small molecules are low molecular weight compounds (<1 kDa) that have a biological effect (Stanczyk et al. 2008). The advantages of small molecules are that, due to their small size, they are able to get inside the cell. Many small molecules can also be manufactured at relatively short duration and are thus cost effective, and finally, many can be manufactured to be orally available.

Small molecules act by targeting intracellular signalling proteins, surface receptors or enzymes and modulate their function. An inhibitor of MAP kinase p38 is currently in phase II clinical trials for RA, however in a randomised controlled trial, pamapimod (a p38 MAP kinase inhibitor) was less effective than methotrexate (Cohen et al. 2009). Inhibitors of Syk (spleen tyrosine kinase) kinase have proved more successful; Fostamatinib, a Syk kinase inhibitor, in a randomised controlled trial, achieved remission in half of the patients after 12 weeks of oral administration (Weinblatt et al. 2008). Inhibitors of JAK/STAT pathways have also proved effective; a JAK3 inhibitor (tofacitinib or CP-690,550) in phase II clinical trials, has shown to have achieved remission in a third of the subjects after 24 weeks of administration (Kremer et al. 2012). A small molecule inhibitor against the cell surface A3 adenosine receptor (CF101) has shown to improve signs and symptoms in patients with RA but did not achieve statistical significance in a phase II clinical trial (Silverman et al. 2008).

Biological heterogeneity between patients necessitates that one drug doesn't fit all, combinations of biological agents may be required and treatment needs to be individualized. Identification of new and more cost-effective therapies is a priority and small molecule inhibitors and anti-NAMPT strategy may well benefit several, if not all individuals affected by arthritis. Novel targets are therefore actively being pursued.

1.10.4. NAMPT inhibitors

NAMPT can be inhibited by a small molecule inhibitor, FK866 or APO866 (Hasmann and Schemainda 2003), inducing cell death in a number of cancer cell lines (Hasmann and Schemainda 2003; Nahimana et al. 2009; Olesen et al. 2010). APO866 has shown some therapeutic pre-clinical success in treating proliferative disease by blocking tumour growth (Muruganandham et al. 2005) and collagen-induced arthritis (Evans et al. 2011). APO866 is specific to NAMPT and shows no specificity to other phosphoribosyl transferases. APO866 is currently undergoing Phase II clinical trials for treatment of advanced melanoma, B-chronic lymphocytic leukaemia and cutaneous T-cell lymphomas.

Although APO866 has been investigated mostly as an anticancer drug, some studies have shown that APO866 has considerable efficacy in preclinical studies in models of experimental arthritis (Busso et al. 2008; Evans et al. 2011), experimental autoimmune encephalitis (Bruzzone et al. 2009) and hepatitis (Moschen et al. 2011). In an experimental model of RA and CIA, APO866 reduced the mean arthritis severity score of animals and decreased pro-inflammatory cytokine secretion in affected joints and its activity was comparable to Etanercept (Busso et al. 2008). Evans *et al* (2011) showed that treatment with APO866 in mice with established disease significantly reduced the mean arthritis severity score, reduced synovial inflammation, cartilage destruction and halted bone erosion. In addition, APO866 reduced the activity of MMP-3, CCL2, and RANKL in vivo, and inhibited the production of CCL2 and RANKL in synovial explants (Evans et al. 2011). The fact that APO866 does not impair autoantibody production in this experimental model suggests that the beneficial effects of APO866 is due to impaired production of pro-inflammatory (TNF-α, IL-1β) and pro-degradatory (MMP-3, RANKL) factors locally within joint tissues (Busso et al. 2008; Evans et al. 2011; Nowell et al. 2012).

Although APO866 is a potent inhibitor of NAMPT, it has low bioavailability, short half-life, dose limiting thrombocytopaenia and had a tendency to bind to plasma proteins (Holen et al. 2008). Alternatives are available such as CB30865, which is a subnanomolar cytotoxic compound (Skelton et al. 1998; Skelton et al. 1999), and CHS-828 / GMX1778, a pyridyl cyanoguanidine (Schou et al. 1997). CHs-828 has showed potent anti-tumour activity in a number of tumour cell lines (Hjarnaa et al. 1999) and has led to phase I clinical trial for solid tumours (Ravaud et al. 2005).

1.11. Summary and Objectives of thesis.

NAMPT is a novel protein which is an enzyme required for NAD^+ biosynthesis. Whether NAMPT is purely an enzyme or enzyme with cytokine like activity has been a matter of debate. NAMPT is also elevated in serum and synovial fluid of patients with RA and significantly, in mice with CIA. NAMPT concentration steadily increases in paws over time and following treatment with NAMPT inhibitor, APO866, only 28% of the animals showed signs of arthritis compared to 100% of animals who were not treated (Evans et al. 2011).

As NAD^+ is important for cell metabolism, it is possible that NAMPT is regulated inappropriately in chronic diseases such as RA to maintain a hyperactive and protective phenotype by enhanced NAD^+ bioavailability. It may be possible that manipulating NAMPT activity via small molecule inhibition may be a viable and cost

effective treatment strategy in RA, with normal tissues continuing to produce NAD^+ via alternate pathways e.g. the nicotinic acid pathway.

However, aside from IFN-induction of IDO (Indoleamine 2, 3 Dioxygenase), and the known effect of a number of cytokines on NAMPT expression, it is not known whether any other NAD^+ biosynthesis enzymes are [1] cytokine-inducible *in vitro*, or are [2] upregulated *in* vivo in diseased (arthritic) tissue.

The working hypothesis for this thesis is that IDO and NAMPT are inducible in inflammatory arthritis tissue and therefore ideal of targeted therapies.

Therefore, the aim of chapter 3 is to characterise NAD^+ biosynthesis enzyme expression in synovial tissue derived from patients with RA, OA and non-arthritic individuals. Chapter 4 explores the effect of pro-inflammatory cytokines on NAD^+ biosynthesis enzyme expression *in vitro* in RA synovium.

Chapter 2 – Materials & Methods

2.1 Materials

RNAlater® solution for transporting and storing tissues was obtained from Life technologies™. General reagents for processing RNA e.g. NAOH and Ethanol etc were obtained from Sigma-Aldrich (Dorset, UK) unless stated otherwise. TRIzol® was obtained from Invitrogen™ (Massachusetts, USA). RNeasy® Mini kit was obtained from Qiagen Ltd (Manchester, UK).

All reagents for cDNA synthesis and qPCR analysis were obtained from PrimerDesign Ltd (Southampton, UK).

Primers for Nicotinamide, Quinolinic acid and Nicotinic acid phosphoribosyl transferase (NAMPT, NAPRT, QAPRT respectively), Indoleamine 2, 3 dioxygenase (IDO), Nicotinamide mononucleotide adenyltransferase (NMNAT1, 2 & 3), NAD synthetase (NADSYN) were custom designed and verified by PrimerDesign Ltd (Southampton, UK). Primer sequences (forward primer, reverse primer) and estimated melting temperature Tm are outlined in table 2.4. SYBR® green reference gene assays (Ubiquitin C (UBC), Actin β (ACTB) and 18s) were purchased from PrimerDesign Ltd (Southampton, UK).

Dulbecco's Modified Eagle Medium (DMEM) / F-12 (1:1 mix of DMEM and Ham's F-12), Fetal calf serum and penicillin/streptomycin were obtained from Invitrogen™ (Massachusetts, USA).

2.2 Methods

2.2.1 Ethical Approval and sample collection

Ethical approval was obtained from Research Ethics Committee (REC) for Wales (REC reference – 10/MRE09/28, patient information sheet and consent enclosed in appendix). Synovial tissue samples were obtained from patients who were undergoing surgery on their joints such as total knee or hip replacements, trochleaplasty, arthroscopic ACL reconstruction, surgeries on foot and ankle. All RA patients fulfilled the Rheumatoid Arthritis classification criteria (Table 1.1). OA was diagnosed according to clinical features. The characteristics of the RA, OA and normal healthy patients are shown in table 2.1.

	RA (n = 17)	OA (n = 20)	Normal (n = 16)
Age, mean (range), years	61.61 (31 – 77)	72.75 (47 – 91)	21.06 (1 – 47)
Sex (F/M)	14/2	10/7	8/7
Synovial sample			
Knee	7	18	12
Hip	4	-	1
Ankle	1	-	-
Foot	3	-	-
Shoulder	-	-	2
Elbow	1	-	-
Medication			
NSAIDs	5	-	-
DMARDs	8	-	-
Plus steroids	2	-	-
Plus anti-TNF	4	-	-

Table 2.1. Characteristics of patients in the study. N = number of patients in each group, their gender, the joint the sample was taken from and if they were on any medications.

2.2.2 Synovial tissue processing

Synovial tissues obtained were collected in RNAlater® solution (Life Technologies), which stabilises and protects cellular RNA, and stored at -80^{0}C prior to RNA processing and analysis.

2.2.3 RNA Extraction using the 'Hybrid' Method'

The hybrid method combines the Invitrogen TRIzol® method with Qiagen RNeasy® spin columns to get high-quality, high yield RNA. Plasticware and distilled water (dH_2O) used for RNA extraction were rendered RNase free by autoclave. The mortar and pestle were cleaned sequentially with 3M NaOH, 70% ethanol and distilled water respectively.

Synovial tissue was disrupted under liquid nitrogen using a mortar and pestle and transferred to 1ml of TriZol® (Invitrogen™). Samples were vortexed and incubated at room temperature for 5 minutes and mixed with 200µl chloroform. After 5min the sample was centrifuged at 10,000 rpm at 4^{0}C for 15min. The aqueous phase, containing the RNA, was transferred carefully to a new micro-centrifuge tube (i.e.

without disturbing the aqueous/organic interface) to avoid DNA contamination. An equal volume of 70% ethanol was added and mixed by pipetting (ethanol promotes selective binding of RNA to the RNeasy membrane). 700µl of the sample was transferred to an RNeasy® (Qiagen) spin column and centrifuged at 10,000 rpm for 15 seconds. 350µl of buffer RWI (Qiagen), containing a guanidine salt and ethanol, was added to the column and centrifuged at 10,000 rpm for 15 seconds. 80µl of DNase I incubation solution (10µl DNase I stock solution in 70µl buffer RDD from the Qiagen RNase-Free DNase Set) was added directly to the column membrane at 20-30^{0}C for 15min to digest any residual DNA that may be present. 350µl of buffer RWI was added and the column centrifuged for 15 seconds at 10,000 rpm to remove any DNase I. The column was transferred to a new collection tube, and 500µl of buffer RPE (Qiagen) was added and spun at 10,000 rpm for 30 seconds to wash spin column membrane of any contaminants, followed by 500µl buffer RPE (Qiagen) and spun for 2min at 10,000 rpm to wash spin column membrane (long centrifugation dries the spin column membrane ensuring that no ethanol is carried over during RNA elution). 30µl of RNase free water was added directly onto column membrane and allowed to sit at room temperature for 10min before spinning for 1min at 10,000 rpm to elute RNA. It is then stored at -80^{0}C to prevent the loss of RNA at room temperature.

2.2.4 RNA Quantification using Nanodrop

1µl of RNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific). The volume of RNA solution required for 0.1µg RNA was calculated in preparation for reverse transcription.

2.2.5 Reverse Transcription (RT) of mRNA to cDNA

Reverse Transcription was performed using nanoScript reverse transcription kit from PrimerDesign Ltd, Southampton, UK. 1µl RT primer mix (0.5µl of Oligo-dT + 0.5µl of random nonamer) was added to 0.1µg of each sample of RNA, and the total volume made up to 10µl by adding RNase/DNase free water (random primers will bind anywhere in the genome and allow reverse transcriptase to fill up the gaps leading to higher yields whereas oligo-dT will bind to poly-A tail of the RNA before transcribing RNA. As poly-A tail is located at the extremity of the gene it will lead to full transcripts. The combination of oligo-dT primers and random primers will provide the highest yields and longest transcripts). Samples were heated to 65^{0}C for 5min (to denature RNA secondary structure) and immediately cooled on ice to let the primer anneal to RNA. A master mix containing RT reaction, 10mM dNTPs, 100mM DTT,

PrimerDesign Ltd RT enzyme (enzyme activity not disclosed) and RNase/DNase free water was made (Table 2.2) and 10µl added to each sample of RNA mix. One RNA sample was also made without the RT enzyme (-RT) to account for genomic DNA contamination.

Components	1 Sample	-RT
nanoScript 10X Buffer	2.0µl	2.0µl
dNTP mix 10mM	1.0µl	1.0µl
DTT 100mM	2.0µl	2.0µl
RNase/DNase free water	4.0µl	5.0µl
nanoScript Reverse Transcriptase enzyme	1.0µl	0µl
Total Volume	10.0µl	10.0µl

Table 2.2. Protocol for DNA synthesis.

Samples were incubated at 25^0C for 5min (to maximise annealing efficiency as oligo dT have a lower Tm) and 55^0C for 20min for reverse transcription of RNA to cDNA. The reaction was heat inactivated by incubating at 75^0C for 15min. Samples were diluted 1:10 with RNase free water and stored at -20^0C.

2.2.6 Quantitative Real Time Polymerase Chain Reaction (qPCR)

qPCR was carried out using an ABI 7900HT RT-PCR machine and associated SDS 2.4 software. All primers were designed and synthesised by PrimerDesign Ltd, Southampton, UK. qPCR was used to quantify the expression of genes accurately. During PCR the DNA was amplified exponentially during each cycle and quantified in real time. Each cycle consists of denaturation, annealing and extension steps (table 2.5). During denaturation process the temperature was raised to 95^0C to melt double stranded DNA to single strand. Temperature was lowered to 60^0C for the primer specific to gene of interest to bind to that gene. Temperature was raised to 72^0C, the optimum temperature for the polymerase, to allow the enzyme to bind and copy the DNA strand.

2.2.6.1 Normalisation

Although qPCR is quite accurate in quantification of gene expression, a number of errors might occur due to sample to sample variation of mRNA, variation in RNA integrity, variation in amount of starting material, different reverse transcription and PCR efficiencies (Bustin and Nolan 2004). To control for errors of qPCR, one of the most simple and popular method is normalisation to a reference gene (Huggett et al.

2005). Reference genes (also referred to as 'housekeeping' genes) are measured alongside the gene of interest.

2.2.6.2 geNorm

Historically, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ACTB (actin β), B2M (beta-2-microglobulin) and HPRT1 (hypoxanthine-guanidine phosphoribosyl transferase) were commonly used as reference genes for normalisation during PCR. However, variability in these reference genes have been noted due to response to experimental treatment and due to innate and natural variability between tissues and individuals (Schmittgen and Zakrajsek 2000; Dheda et al. 2004). Therefore, the use of all reference genes used for any particular experiment needs to be validated at the outset. geNorm is a popular algorithm used to determine the most stable reference gene among a panel of tested reference gene in a given sample (Vandesompele et al. 2002). geNorm uses the geometric mean of the expression of a reference gene based on principles and formula described by Vandesompele *et al* (2002) and the reference genes are ranked according to their stability - measure '*M'* - i.e., the average pairwise variation of a particular gene with all other control genes (Vandesompele et al. 2002). Genes with lowest *M* values have the most stable expression. The authors' also determined pairwise variation '*V'* for every control gene which is the standard deviation of the logarithmically transformed expression ratios with all other control genes (Vandesompele et al. 2002). geNorm program identifies the least stable (i.e., highest *M* value) reference gene and recalculates a new *M* value for the remaining reference genes. The algorithm first selects a pair of two candidate reference gene that have the smallest variability in ratios amongst all possible pairs of genes. Then the next stable reference gene is identified which has the highest agreement with the rest of the candidate genes and with geometric mean of the first two selected genes (n/n+1) until the addition of n+1 gene makes no significant effect on normalising factor. A value of 0.15 is taken, below which inclusion of additional genes is not required.

2.2.6.3 Reference gene determination using geNorm with PerfectProbe™ detection

15µl mix containing qPCR reagents (1µl of gene specific primer with Taqman® probe mix, 10µl of 2x master mix and 4µl of RNase free water) were added to each well of a 96 well qPCR plate. 5µl of either of cDNA sample, -RT or RNase free water was added to each well. Samples were analysed in duplicates. Reference genes investigated were human GAPDH, CYC1, YWHAZ, ACTB, UBC and 18s with Taqman® fluorophore using PerfectProbe™ technology

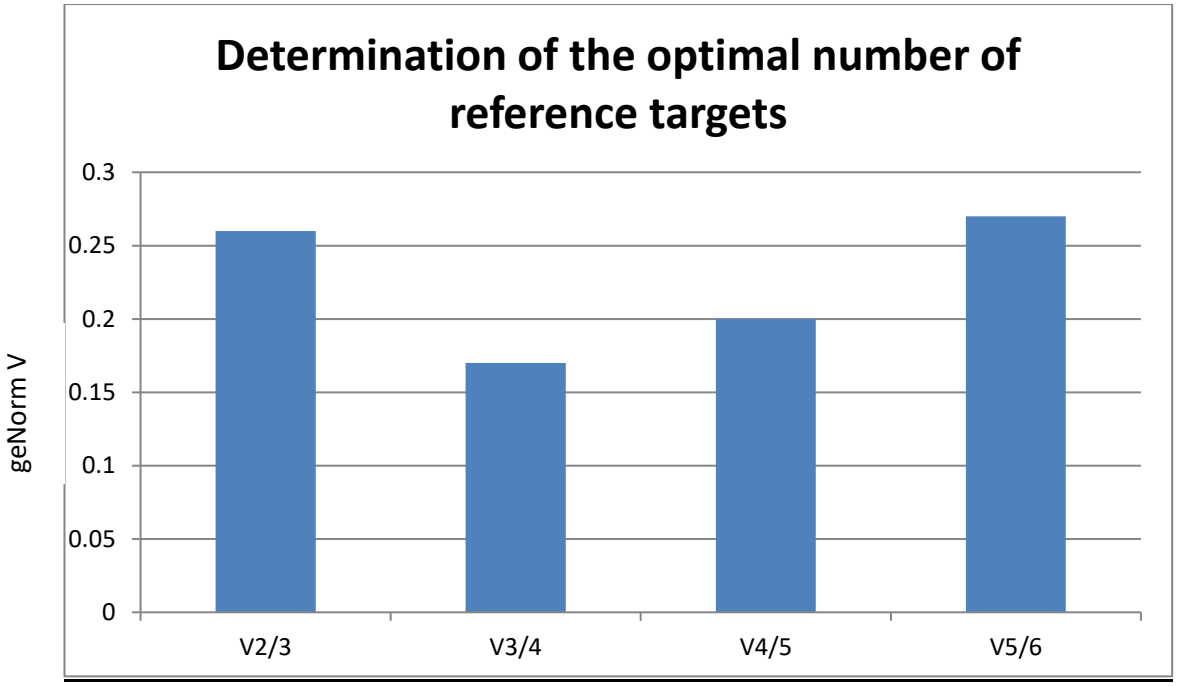
(http://www.primerdesign.co.uk/perfect_probe.html). qPCR was performed as outlined in table 2.3. Data was collected using FAM channel. geNorm analysis was carried out using qbasePLUS software (Biogazelle) (Fig 2.1).

Stage	Number of cycles	Time	Temperature	Data Collection
Enzyme activation	1	10 minutes	95^0C	No
Denaturation	50	15 seconds	95^0C	No
Annealing		30 seconds	50^0C	Yes
Extension		15 seconds	72^0C	Yes

Table 2.3. Two step qPCR protocol used for PerfectProbe™ qPCR

Official gene symbol	**Accession number**	**Anchor Nucleotide**	**Context length sequence (bp)**
UBC	NM_021009	452	192
18S	M10098	235	99
ACTB	NM_001101	1195	106
YWHAZ	NM_003406	2585	150
CYC1	NM_001916	929	207
GAPDH	NM_002046	1087	142

Table 2.4. Details of reference genes with Taqman® fluorophore attached. (Primer sequence details not provided)



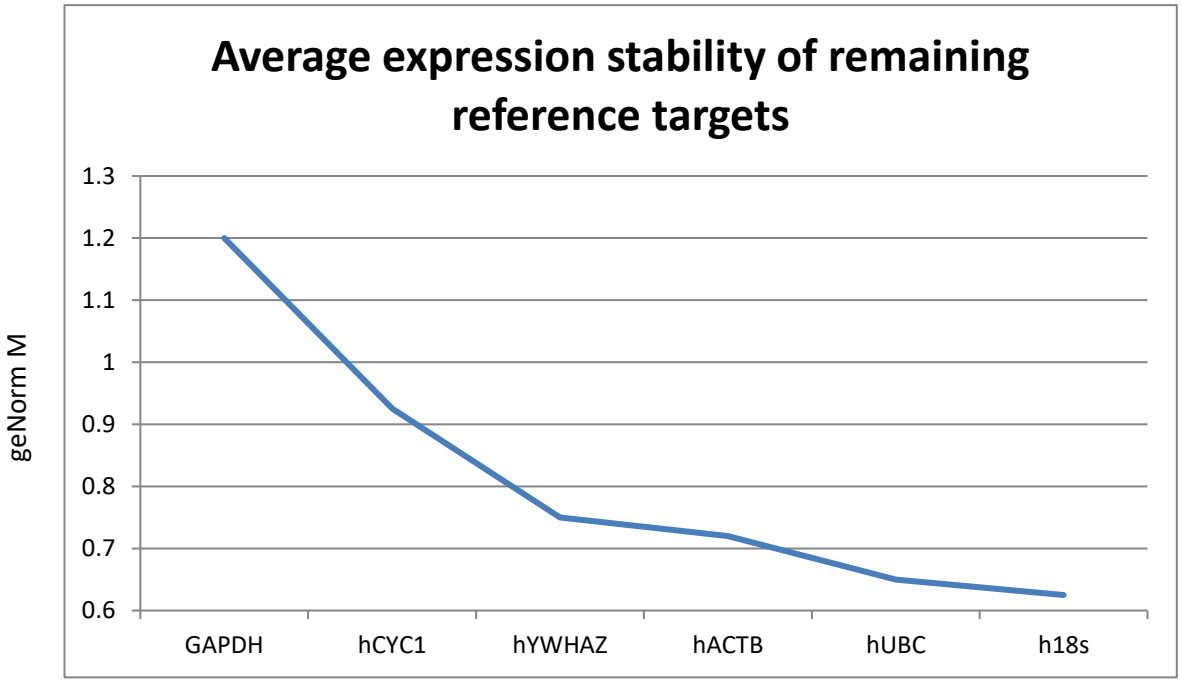


Fig 2.1. **Determination of optimal reference gene.** No optimal number of reference targets could be determined, as variability between sequential normalisation factors (based on the n and n+1 least variable reference target) is relatively high (geNorm V > 0.15). We therefore used 3 reference targets with lowest M values (h18s, hUBC and hACTB), as the use of multiple reference targets results in more accurate normalisation compared to the use of a single non-validated reference target.

2.2.6.4 Gene of interest qPCR with SYBR® green detection

A plate layout was prepared to avoid any doubt during pipetting. All samples for qPCR were performed in duplicates to avoid any pipetting errors. All pipetting was performed under a laminar hood using filter tips and pipettes designated for qPCR use only to avoid DNA contamination. A 15µl mix containing qPCR reagents (1µl of gene specific primer with SYBR® green probe, 10µl of 2x master mix [Precision Plus mastermix from PrimerDesign, Southampton, UK containing Taq Polymerase enzyme and magnesium chloride based buffer] and 4µl of RNase free water) was pipetted to each well of a 96 well qPCR plate and 5µl of cDNA was added to each well. Negative controls (RNase free water; to detect primer dimers and contamination and –RT sample (mix containing sample that has not been reverse transcribed); to detect genomic DNA contamination) were included in each analysis. The qPCR plate was sealed with a clean optical film to avoid evaporation. The plate was spun to ensure components were thoroughly mixed, collected to the bottom of the plate with air bubbles removal. qPCR was performed as outlined in table 2.6.

Primer Name	Sense Primer (Tm)	Antisense Primer (Tm)	Product size	Suggested Tm	Dissociation curve	Actual Tm
NAMPT	TTCCCACTACTCC AGCCTAAG (56.8)	TTTGTGTAAAGGGCA GGTTAATAAA (56.5)	94	69.7	Appendix 1a	80
NAPRT	GTGGTGCTGTCCG AGAGG (57.2)	GGAAAAGTGAGTGAT TCGTGTTG (57.2)	111	78.9	Appendix 1b	88
QAPRT	CCCCAGCCCTTGA TTTCTCC (58.4)	GGTGTCATCCTCTTC CGGTTTA (58.3)	93	74.7	Appendix 1c	83
NMNAT1	AGTCCTTTGCTGT TCCCAATT (56.3)	AGCACATCCGATTCA TAGATAAAC (55.9)	127	73	Appendix 1d	82
NMNAT2	ATTGCTGTCTTGT GCTTTGTG (56.2)	CGTAGCTGGTACTAG ATTTTGATAAA (56.5)	115	71.1	Appendix 1e	80
NADSYN	CCAAAAACAGAGG AGCAAGATAC (56.4)	GGTGTCCGACTCGTA ATAATGAT (56.9)	89	72.6	Appendix 1f	82
IDO	CAGTCCGTGAGTT TGTCCTTT (56.7)	CAGGAATCAGGATGT ACTTAGTCA (56.5)	129	75.6	Appendix 1g	84

Table 2.5. Characteristics of primers obtained from PrimerDesign Ltd showing forward and reverse sequence, product size, suggested melting temperature(Tm), actual Tm during experiment. NAMPT – nicotinamide phosphoribosyl transferase, NAPRT – nicotinic acid PRT, QAPRT – quinolinic acid PRT, NMNAT1 & 2 – nicotinamide mononitrate adenyl transferase, NADSYN – nicotinamide adenine dinucleotide synthetase, IDO – indoleamine acetic acid.

Stage	Number of Cycles	Time	Temperature	Data collection	Ramp rate
Enzyme activation	1	10 min	95^0C	No	100%
Denaturation	50	15 sec	95^0C	No	100%
Annealing/Extension		60 sec	60^0C	Yes	100%
Dissociation curve	1	15 sec	95^0C	No	100%
		15 sec	60^0C	No	100%
		15 sec	95^0C	Yes	2%

Table 2.6. Two step qPCR protocol used for SYBR® green qPCR.

SYBR® green will bind to any amplified double stranded DNA and bias could be introduced in the reaction in the presence of primer dimers or unspecified products; primer dimer arises when there is low concentration of target DNA or complimentary bases within or between primers (3'-3' or 5'-5' dimer). To check for artefacts during amplification, a dissociation curve step was added to every qPCR run as a quality control measure to check the specificity of qPCR (Fig 2.2). The principle is that every product has a different dissociation temperature depending on size and base content. As temperature slowly raises from 60^0C to 95^0C, the whole amplified product dissociates at certain temperatures, resulting in drop of fluorescence. Primer dimers or unspecified products melt at the different temperatures, resulting in a separate peak(s) to the specific product (example of a dissociation curve with primer dimers is shown in Fig 2.3). Only samples which had similar melting temperature, i.e., with single peaks were used for analyses (example shown in Fig 2.2). qPCR which showed anomalies in the dissociation were excluded from analyses.

2.2.6.5 ΔΔCt method

The relative quantity of cDNA in a sample was calculated according to ΔΔCt method. The **Baseline** is calculated in a sample as the average background and is calculated according to the noise level in the early cycles, when there is no detectable increase in fluorescence, due to qPCR products. The **Threshold** is calculated as the level of fluorescence above baseline, at which the signal can be considered not to be background. The **Ct** value is defined as the cycle in which there is significant increase in reporter signal, above the threshold i.e., the cycle in which the amplification curve crosses the threshold.

Once a Ct value is obtained for a specific product in a sample following qPCR, ΔCt is calculated as the difference between the Ct values of target gene and reference gene for each sample.

$$\Delta Ct = Ct_{target} - Ct_{reference\ gene}$$

Then the difference between the ΔCt of a sample and the ΔCt of the calibrator is calculated to obtain ΔΔCt value. One of the sample of a normal patient was used as a calibrator throughout our study.

$$\Delta\Delta Ct = \Delta Ct_{calibrator} - \Delta Ct_{sample}$$

Relative quantity (RQ) was calculated for each sample using the formula $RQ = 2^{-\Delta\Delta Ct}$ (First described by Livak K in Applied Biosystems user bulletin No.2 in 1997 and subsequently published as a journal article (Livak and Schmittgen 2001)).

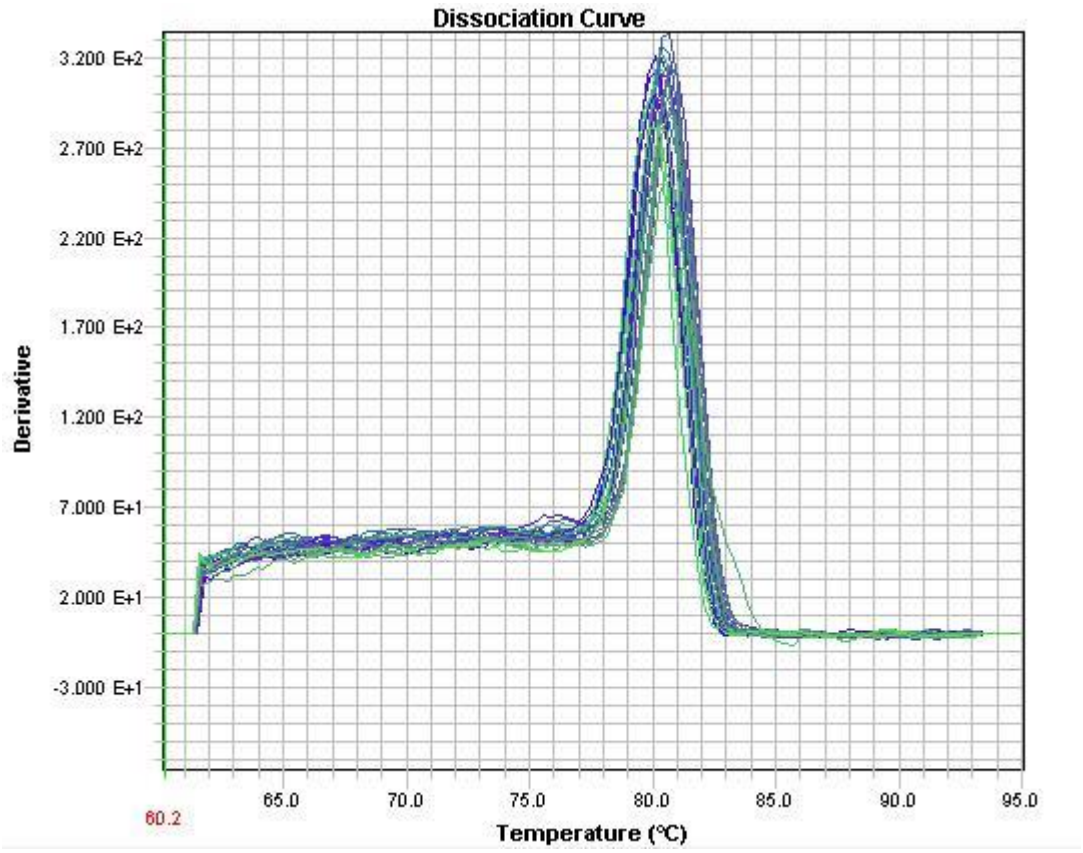


Fig 2.2. Dissociation curve of NAMPT showing single peaks following qPCR analysis. X axis corresponds to the temperature of the cycle and y axis corresponds to fluorescence.

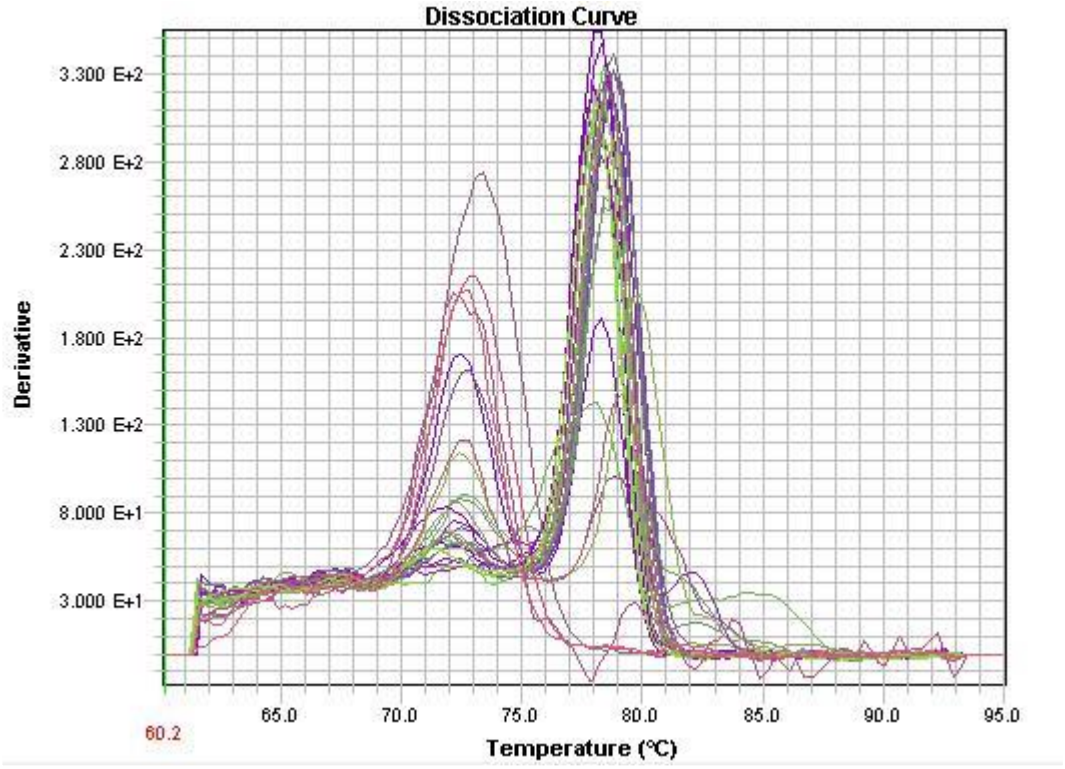


Fig 2.3. Dissociation curve showing primer dimer which peaks early around 72^{0}C followed by the peak of the specific product during qPCR.

2.2.7 Tissue collection

Human synovial tissue was collected at the time of surgery (total knee or hip replacements, trochleaplasty, arthroscopic ACL reconstruction, surgeries on foot and ankle) and stored in synovial fibroblast culture medium (SFCM); DMEM: F12 culture medium containing 10% foetal calf serum (FCS) and 100μg/ml Penicillin/streptomycin (All media purchased from Invitrogen™) and stored at 4^{0}C in preparation for tissue culture.

2.2.8 Tissue culture

Synovial tissue was digested in culture medium containing 1mg/ml collagenase and spun slowly for an hour at 37^{0}C to release the cells. The sample was centrifuged at 1000 rpm for 10 minutes and supernatant aspirated. 5ml SFCM was added to terminate digestion, mixed well and the cell suspension plated out in one T25 flask. Cells were incubated at 37^{0}C in 5% carbon dioxide (CO_2) environment. The culture media was changed every 72hrs until cells were more than 70% confluent. To passage the cells, the culture media was aspirated and cells washed twice with phosphate buffer saline solution (PBS) prior to the addition of 1ml of trypsin, incubated for 2-3 minutes in the incubator. Cells were observed under microscope to confirm they were free from anchoring to the base of the flask. 5ml of SFCM was added to it

and the sample transferred to a universal container and spun at 1000 rpm for 5 minutes. Supernatant was aspirated and 5ml of SFCM was added, mixed well and plated out into two T75 flask, another 10ml of SFCM was added and incubated at 37^0C in 5% carbon dioxide (CO_2) environment. The procedure was repeated until the cells had undergone three passages to obtain cells of same phenotype before being utilised for experiments. Cells not being utilised were stored in 10% dimethyl sulfoxide (DMSO) and 90% FCS and cryopreserved in liquid nitrogen.

2.2.9 Stimulation with Cytokines for mRNA analysis.

For cytokine stimulation experiments, synovial fibroblasts (SF) from patients with rheumatoid arthritis were cultured in SFCM in 6 well plates and incubated at 37^0C in 5% carbon dioxide (CO_2) environment until 70% confluent when they were serum starved for 48 hours. SF were stimulated with cytokines for 0, 2, 4, 6, 8 and 10 hours in triplicate. At the end of time course experiment, 1ml of Tri-reagent® was added and incubated at room temperature for 5 min. Cells were transferred to an eppendorf and RNA was extracted using hybrid method as described in section 2.2.3. RNA thus obtained was quantified using Nanodrop method (sec 2.2.4) and reverse transcripted into cDNA (sec 2.2.5) and qPCR analysis performed as described in section 2.2.6.4.

2.2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6. D'Agostino & Pearson omnibus normality test was performed to check if the data was normally distributed. One way ANOVA with bonferroni's correction was performed to compare the expression of NAD^+ biosynthesis enzymes between RA, OA and normal synovial tissues. Pearson's correlation was performed to look at the strength of relationship between the expressions of NAD^+ biosynthesis enzymes.

Chapter 3 – Expression of NAD^+ biosynthesis enzyme in rheumatoid synovium

3.1 Nicotinamide Adenine Dinucleotide (NAD^+)

Nicotinamide adenine dinucleotide (NAD^+) plays an integral role in cell survival. For instance, it takes part in a number of reduction-oxidation (redox) reactions involving it in cellular processes from breakdown and release of energy from proteins, carbohydrates and fats to fatty acid synthesis. NAD^+ is also used as a substrate for NAD^+-consuming enzymes such as sirtuins, poly (ADP-ribose) polymerases (PARP) and ADP-ribosyl cyclases, which are involved in gene expression, apoptosis and Ca^{2+} mobilisation (Belenky et al. 2007; Nowell et al. 2012).

3.1.1 NAD^+ Biosynthesis

NAD^+ is synthesised from various vitamins such as nicotinamide, tryptophan, nicotinamide riboside and nicotinic acid (Bogan and Brenner 2008) and from three main pathways; namely *de novo,* salvage and Preiss-Handler (Preiss and Handler 1957; Bogan and Brenner 2008) pathways (Fig 3.1).

There are a number of important enzymes involved in NAD^+ biosynthesis; Quinolinic acid PRT (QAPRT) is the rate limiting enzyme for the *de novo* NAD^+ synthesis pathway, using tryptophan as the precursor vitamin (Bogan and Brenner 2008); Nicotinic acid PRT (NAPRT) is the rate limiting enzyme in the Preiss-Handler pathway, with nicotinic acid as the precursor vitamin (Bogan and Brenner 2008). Nicotinamide is also generated during NAD^+ consuming reactions and can be recycled to NAD^+ via salvage pathway with NAMPT being a rate limiting enzyme (Fig 3.1).

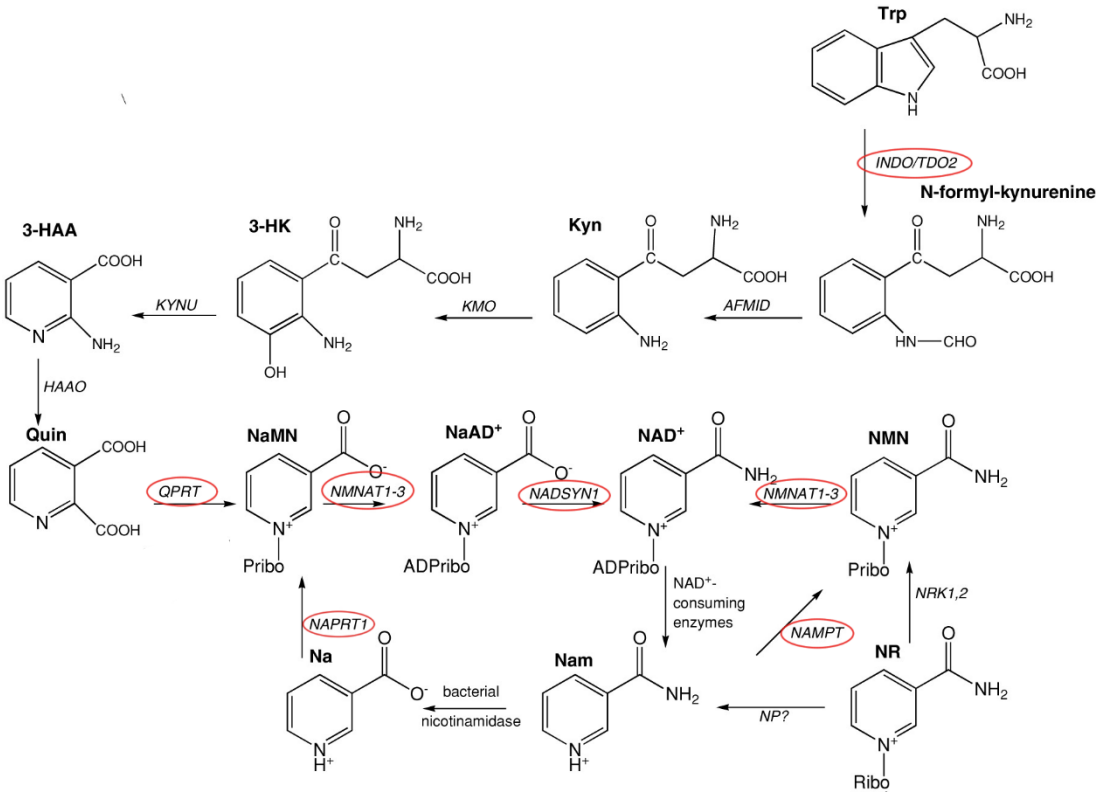


Fig 3.1. NAD^+ biosynthesis pathway. Taken from Bogan *et al* (2008)
Na – nicotinic acid, Nam – nicotinamide, NR – nicotinamide riboside, NMN – nicotinamide mononucleotide, NAD^+ - nicotinamide adenine dinucleotide, Quin – quinolinic acid, Trp – Tryptophan, QPRT – quinolinic acid phosphoribosyl transferase (PRT), NMNAT – nicotinamide mononucleotide adenyl transferase, NADSyn – NAD synthetase, NAMPT – nicotinamide PRT, NAPRT – nicotinic acid PRT. The enzymes which will be investigated in this chapter are circled in red.

3.1.2 Expression and distribution of NAD^+ biosynthesis enzymes

3.1.2.1 NAMPT

As discussed in section 1.9 in chapter 1, NAMPT is present in both intracellular and extracellular regions. NAMPT has the highest expression in human liver, bone marrow, peripheral blood leucocytes and muscle, whilst kidney and heart show intermediate levels of intracellular NAMPT (Samal et al. 1994; Friebe et al. 2011). Adipose tissue has been shown to abundantly express extracellular NAMPT (Fukuhara et al. 2005) (Fig 3.2) and NAMPT is also found in lung, placenta, intestines, pancreas and synovial tissue (Samal et al. 1994; Nowell et al. 2006; Nowell et al. 2012).

3.1.2.2 IDO

IDO is expressed in humans in brain, kidney, liver, adipose tissue, placenta and various immune cells throughout the body such as dendritic cells, monocytes, macrophages and microglial cells (Manuelpillai et al. 2005; Mándi and Vécsei 2012; Favennec et al. 2015)

3.1.2.3 Other NAD enzymes

QAPRT, NMNAT 1-3 (nicotinamide mononucleotide adenyl transferase) and NADSYN (NAD synthetase) are found in high concentration in human liver and brain where these convert tryptophan to NAD^+ (Feldblum et al. 1988; Okuno et al. 1988).

NAPRT is highly expressed in liver, kidney, heart and small intestine with moderate expression in lungs (Hara et al. 2007).

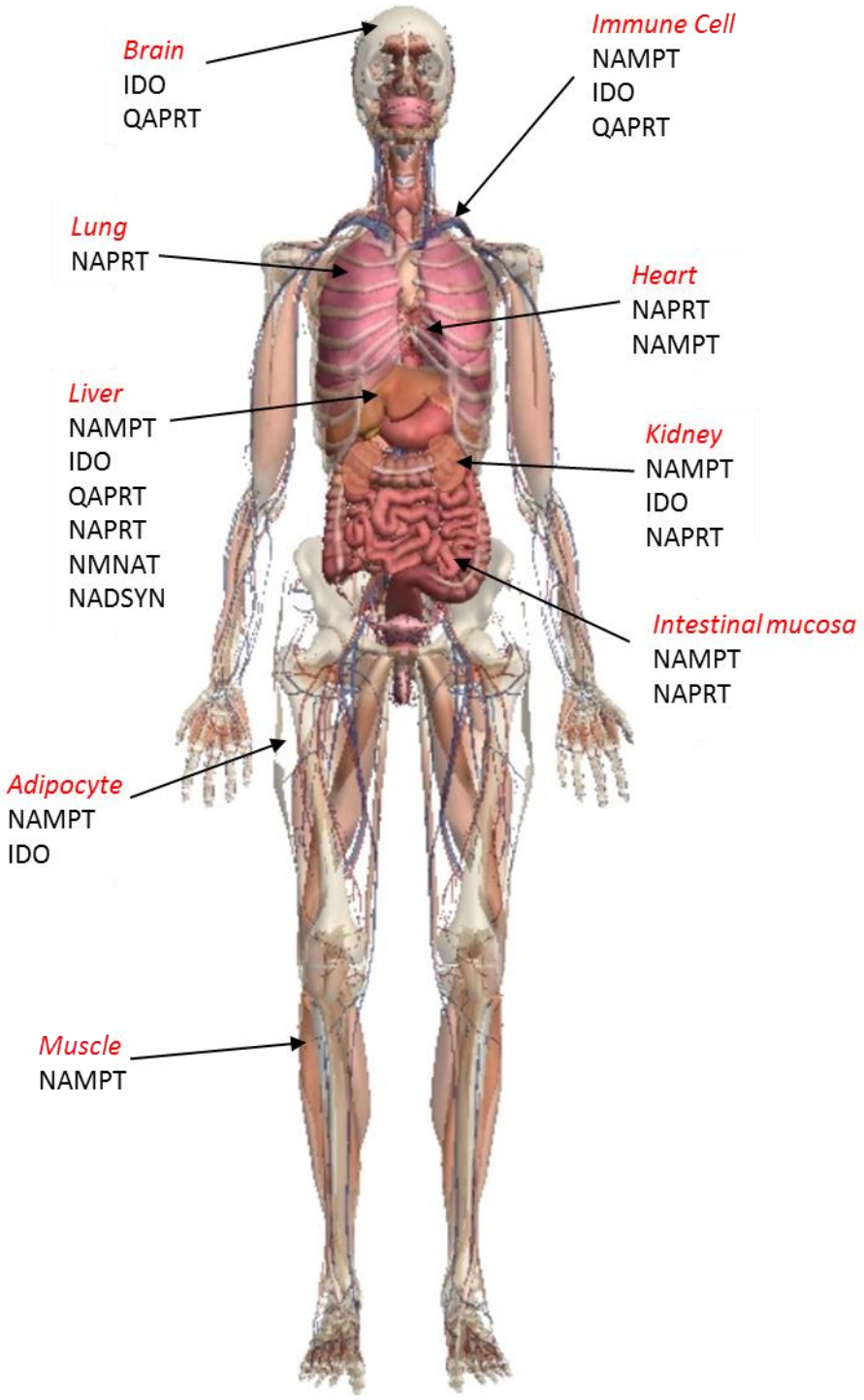


Fig 3.2. Diagram to highlight areas in normal, healthy human tissue that naturally express high levels of the different NAD^+ biosynthesis enzymes investigated in this study (Feldblum et al. 1988; Okuno et al. 1988; Samal et al. 1994; Nowell et al. 2006; Friebe et al. 2011; Mándi and Vécsei 2012; Nowell et al. 2012; Favennec et al. 2015).

3.1.3 NAD^+ biosynthesis and role in inflammation

Increased intracellular NAD^+ levels protect cells such as lymphocytes, human embryonic kidney cells, smooth muscle cells, cardiac and skeletal myocytes from genotoxic stress induced apoptotic cell death caused by DNA alkylating and PARP activating agents and reactive oxygen species (Pillai et al. 2005; van der Veer et al. 2005; Yang et al. 2007; Fulco et al. 2008; Rongvaux et al. 2008). Synthesis of TNF-α and IL-6 in monocytes (when stimulated by lipopolysaccharide *in vitro*) relies on intracellular NAD^+ levels produced via the NAD salvage pathway (Van Gool et al. 2009). Interestingly, intracellular NAD^+ levels and TNF-α production can be restored by adding nicotinamide mononucleotide or nicotinic acid by diverting synthesis of NAD^+ via the Preiss-Handler pathway using NAPRT as the rate limiting enzyme (Van Gool et al. 2009).

It is believed that NAMPT is the main NAD^+ biosynthesis enzyme up regulated following increased demand for NAD^+ in the proliferating cells (Bruzzone et al. 2009). Thus it is hypothesised that inhibiting NAMPT could deprive proliferating cells of NAD^+ leading to its death (Bruzzone et al. 2009). This property could be used as a possible targeted therapy in RA where cells like synovial fibroblasts are highly proliferative (reviewed by Nowell et al. 2012). As APO866 exclusively inhibits NAMPT activity, nicotinic acid has been shown to counteract APO866 toxicity in normal healthy tissue by producing NAD^+ via Preiss-Handler pathway (Olesen et al. 2010). As the NAD^+ synthesis via Preiss-Handler pathway is unable to meet its increased demand in highly proliferative cells, addition of nicotinic acid would only rescue normal healthy tissue (Olesen et al. 2010).

3.2 Aims of chapter 3

A number of studies have confirmed elevated levels of NAMPT in RA synovial tissue and fluid (Nowell et al. 2006; Brentano et al. 2007; Matsui et al. 2008; Meier et al. 2012). However, the expression and relationship between other NAD^+ biosynthesis enzymes in synovial tissue has yet to be defined. This chapter aims to characterise the expression of the principle enzymes involved in NAD^+ biosynthesis in RA, OA and normal 'healthy' synovial tissues to gain a better understanding of the significance of any elevated NAMPT in the RA synovium.

3.3 Methods

3.3.1 Synovial tissue sample and patient characteristics

Synovial samples were obtained during surgery as outlined in methods chapter section 2.2.1. Samples were taken from patients who had RA, OA or from normal healthy individuals. The number of patients, age, gender, joint and medications are as outlined in table 2.1.

3.3.2 qPCR analysis of NAD^+ biosynthesis enzymes in synovial tissue

mRNA was extracted from synovial samples and reverse transcribed to cDNA as described in chapter 2 section 2.2.3 and 2.2.5 respectively. qPCR analysis was performed as outlined in chapter 2 section 2.2.6.4 and expression of NAMPT, NAPRT, QAPRT, NMNAT 1, 2 & 3, NADSYN and IDO were determined. Genorm analysis was performed as outlined in section 2.2.6.3 to determine optimum reference gene. Analysis identified that optimal number of targets for synovial tissues were 3 (Fig 2.1) with Ubiquitin C (UBC), Actin β (ACTB) and 18s as the most stably expressed genes in this tissue to be used as reference genes for these analyses (Fig 2.1).

3.4 Statistical analysis of NAD^+ biosynthesis enzyme expression in synovial tissue sample

Statistical analysis was performed using GraphPad Prism 6 software. One way ANOVA with bonferroni's correction was performed to compare the expression of NAD^+ biosynthesis enzymes between two groups (i.e. RA vs OA, RA vs Normal and OA vs Normal synovial tissues). Graphs were presented in the form of vertical scatter plot with mean. Correlation between the expressions of individual NAD^+ biosynthesis enzymes was performed using Pearson correlation. P value of <0.05 was considered significant.

3.5 Results

Analyses of the enzymes involved in NAD^+ biosynthesis showed that NAMPT, NAPRT, QAPRT, NMNAT 1 & 2, NADSYN and IDO were all detected and constitutively expressed in all synovial tissues with the exception of NMNAT3.

3.5.1 NAD^+ biosynthesis enzyme expression in normal healthy synovial tissue

During assay of NAD^+ biosynthesis enzymes it was noted that some of the synovial tissues from normal, healthy tissues displayed unusually high expression of NAMPT (Fig 3.3a). On closer inspection, this trend was noticed only in tissues from patients under the age of 16 years.

When tissue enzyme expression was plotted against age in years of donor, NAMPT was highly expressed (RQ value = 15) in normal pre-pubertal subjects and gradually decreased over time by adulthood (i.e. >16yrs, Figure 3.3a). In contrast, the other NAD^+ biosynthesis enzymes analysed (NAPRT, IDO, QAPRT and NADSYN) showed no significant pattern of gene expression relative to age (Figure. 3.3); NMNAT- 1 &-2 were highly variable between subjects, with no significant trend in expression apparent over age (Figure 3.3 d & e).

The results of NAD^+ biosynthesis enzymes expression from patients under the age of 16 was therefore excluded from subsequent comparative analyses.

One patient who had a very recent trauma (fracture), showed abnormally high expression of all the enzymes. It was felt trauma could have contributed to this and was therefore excluded from the analysis.

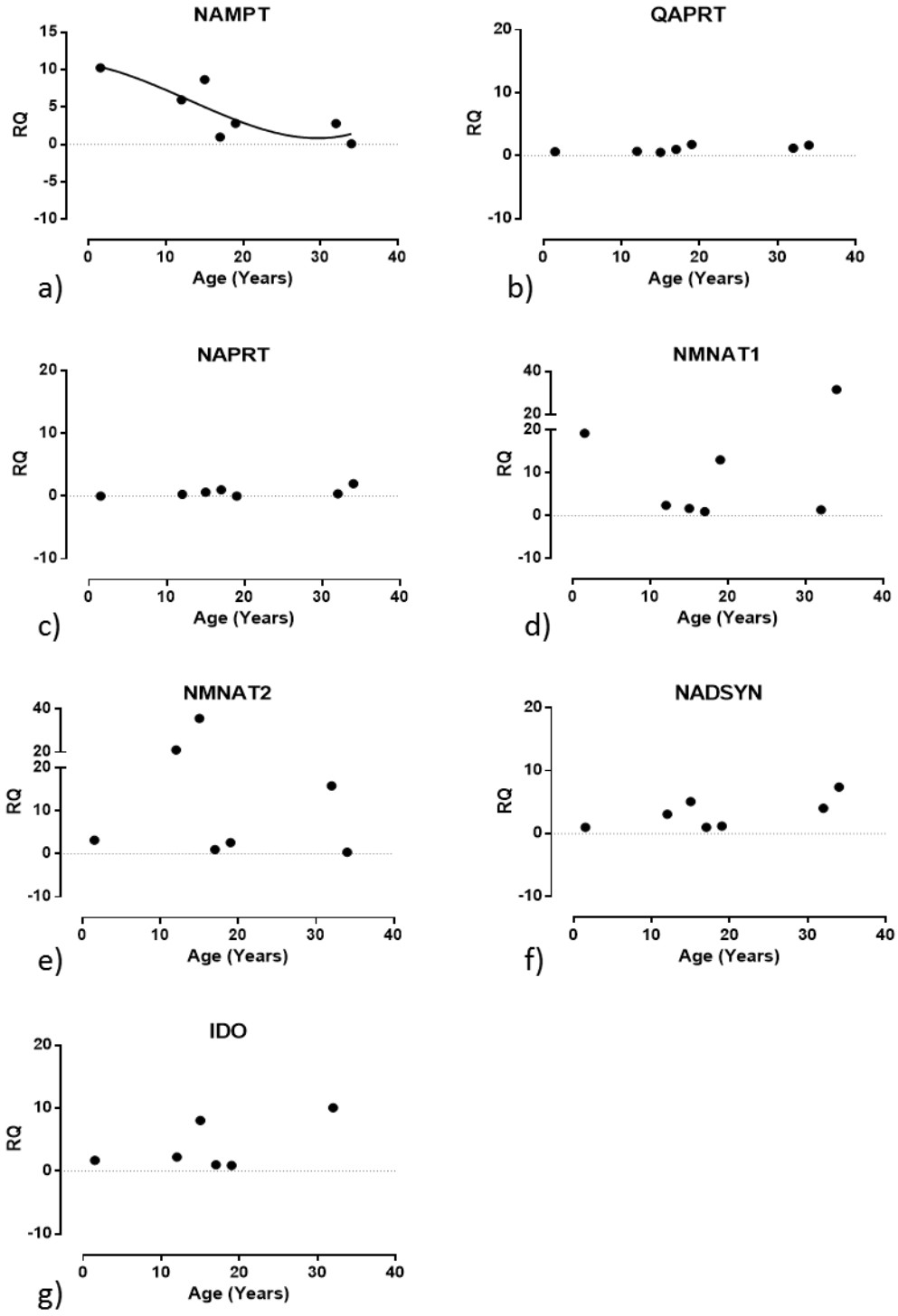


Fig 3.3. Relative quantity (RQ) of NAD^+ biosynthesis enzymes in synovial tissues from normal healthy patients in relation to their age. RQ was calculated using 18s, UBC and ACTB as the house keeping genes. Graph was plotted using non-linear regression and third order polynomial equation

3.5.2 NAD^+ biosynthesis enzyme expression in RA synovial tissue

3.5.2.1 Comparison of NAD^+ biosynthesis enzyme expression in synovial tissue between RA and normal healthy patients

When compared to expression in normal, adult healthy synovial tissue, all NAD^+ biosynthesis enzymes investigated displayed elevated expression in RA with the exception of NAPRT (Fig 3.4), but none of them were statistically significantly elevated. Some of the patients were on anti-TNF medication. When they were excluded from analysis there was statistically significant ($p = 0.0106$) increased relative expression of NAMPT in RA synovial tissue compared to synovium from healthy donors (Fig 3.5). There was 40-fold increased expression of IDO in RA synovial tissue when compared to normal however this was not statistically significant ($p = 0.0676$) (Fig 3.4). Although the expression of QAPRT, NMNAT2 and NADSYN were also elevated in RA synovial tissue their expression levels were not statistically significant ($p = 0.2370$, 0.0706 & 0.0878 respectively) when compared to OA synovial tissue (Fig 3.4 & 3.5).

3.5.2.2 Comparison of NAD^+ biosynthesis enzyme expression in synovial tissue between RA and OA patients

Expression of NAD^+ biosynthesis enzymes were found to show increased expression in RA synovial tissue when compared to OA synovial tissue with the exception of NAPRT (Fig 3.4). Only IDO showed nearly statistically significantly enhanced expression in RA synovial tissue when compared to OA synovial tissue ($p = 0.0504$) (Fig 3.4f). However, when patients who were on anti-TNF medication were excluded from analysis, the expression of NAMPT was found to be statistically significant in RA synovial tissue when compared to OA synovial tissues ($p = 0.0251$) (Fig 3.5a) with no change in the expression levels of other NAD^+ enzymes. Although the expression of QAPRT, NMNAT2 and NADSYN were also elevated in RA synovial tissue their expression levels were not statistically significant ($p = 0.1883$, 0.2225 & 0.2910 respectively) when compared to OA synovial tissue (Fig 3.4 & 3.5).

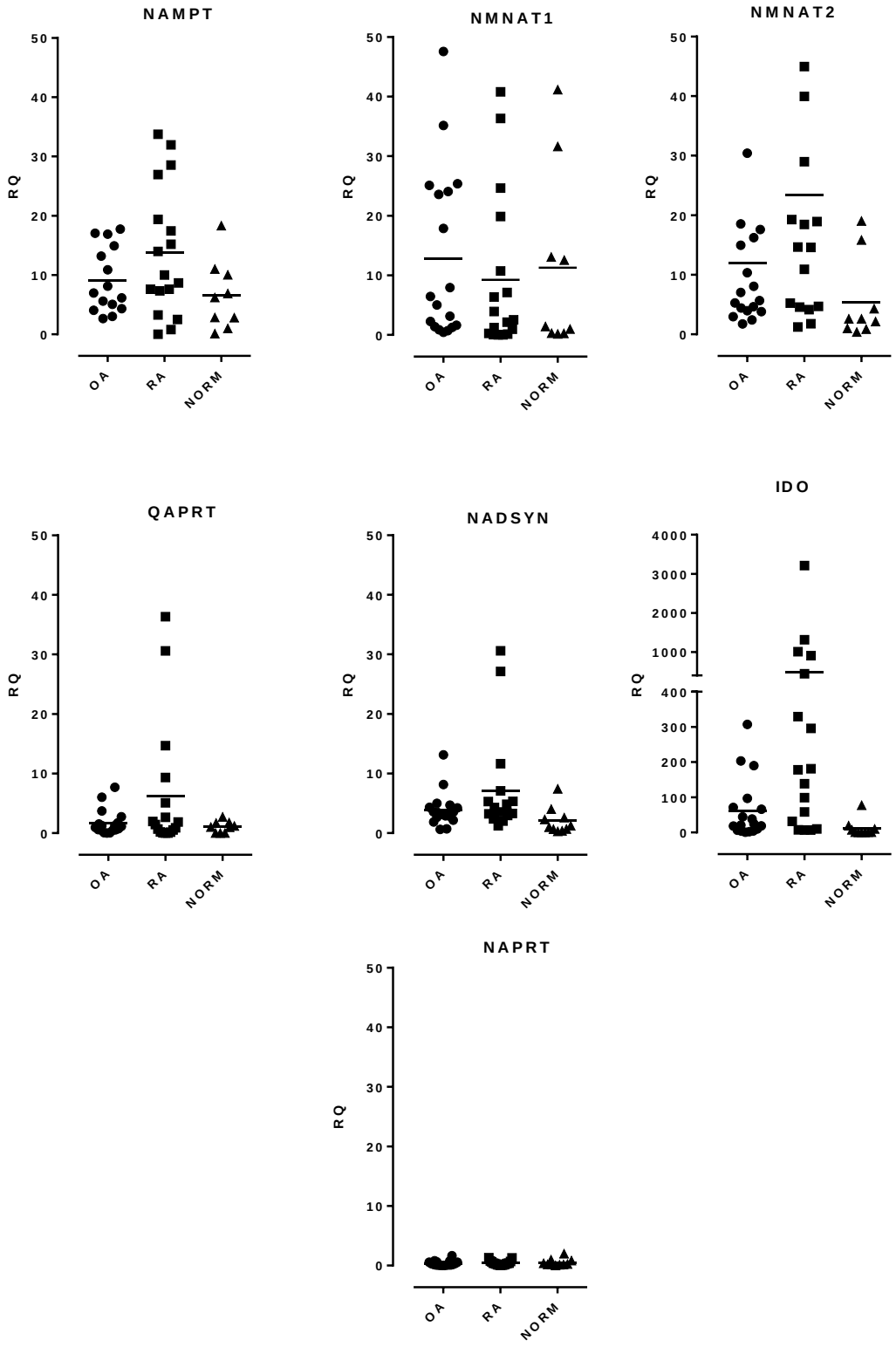


Fig 3.4. Relative quantity (RQ) (mean) of NAD+ biosynthesis enzymes investigated in synovial tissues taken from patients with Rheumatoid Arthritis (RA), Osteoarthritis (OA) and normal healthy subjects (NORM). 18s, UBC & ACTB were used as reference genes. One-way ANOVA test with bonferroni's test was used to check for statistical significance. * - P<0.05

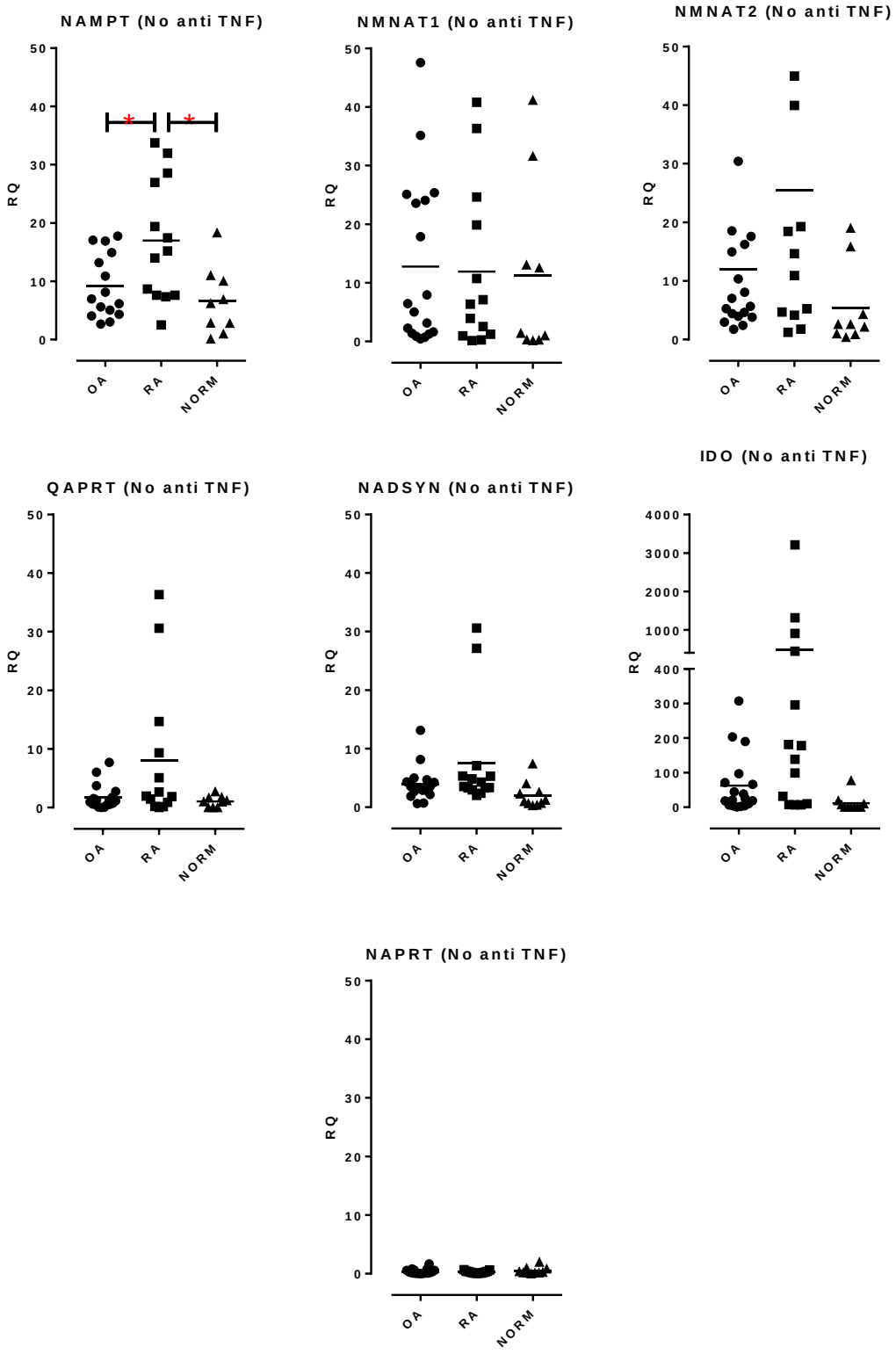


Fig 3.5. Relative quantities (RQ) (mean) of NAD^+ biosynthesis enzymes in synovial tissues taken from OA, normal healthy subjects and anti-TNF naïve RA patients. 18s, UBC & ACTB were used as reference genes. Unpaired *t* test was used to check for statistical significance. * - P<0.05

3.5.3 Correlation between the expression of NAD^+ biosynthesis enzymes

Dependencies of each of the NAD^+ enzymes investigated were carried out by correlation analysis. It was found that there was significant relationship between NAMPT and QAPRT ($p < 0.0001$), NMNAT2 ($p < 0.0001$), NADSYN ($p = 0.0002$) and IDO ($p = 0.0059$) (Fig 3.6a). There was also significant correlation between QAPRT and NMNAT2 ($p = 0.0099$), NADSYN ($p < 0.0001$) and IDO ($p < 0.0001$) (Fig 3.6b) and between the expression of NMNAT2 and NADSYN ($p = 0.0226$) and IDO ($p = 0.0269$) and between NADSYN and IDO ($p < 0.0001$) (Fig 3.6 c). In this study there was no significant correlation between the expressions of NAPRT and NMNAT1 with the rest of the NAD^+ biosynthesis enzymes

Enzyme	NAMPT	QPRT	NMNAT1	NMNAT2	NADSYN	IDO	NAPRT
NAMPT							
QPRT	***						
NMANT1	n.s.	n.s.					
NMNAT2	***	**	n.s.				
NADSYN	***	***	n.s.	*			
IDO	**	***	n.s.	*	***		
NAPRT	n.s.	n.s	n.s.	n.s	n.s	n.s	

Table 3.1. Correlation between expressions of NAD^+ biosynthesis enzymes. *=p<0.05, **-p<0.01, ***=p<0.001

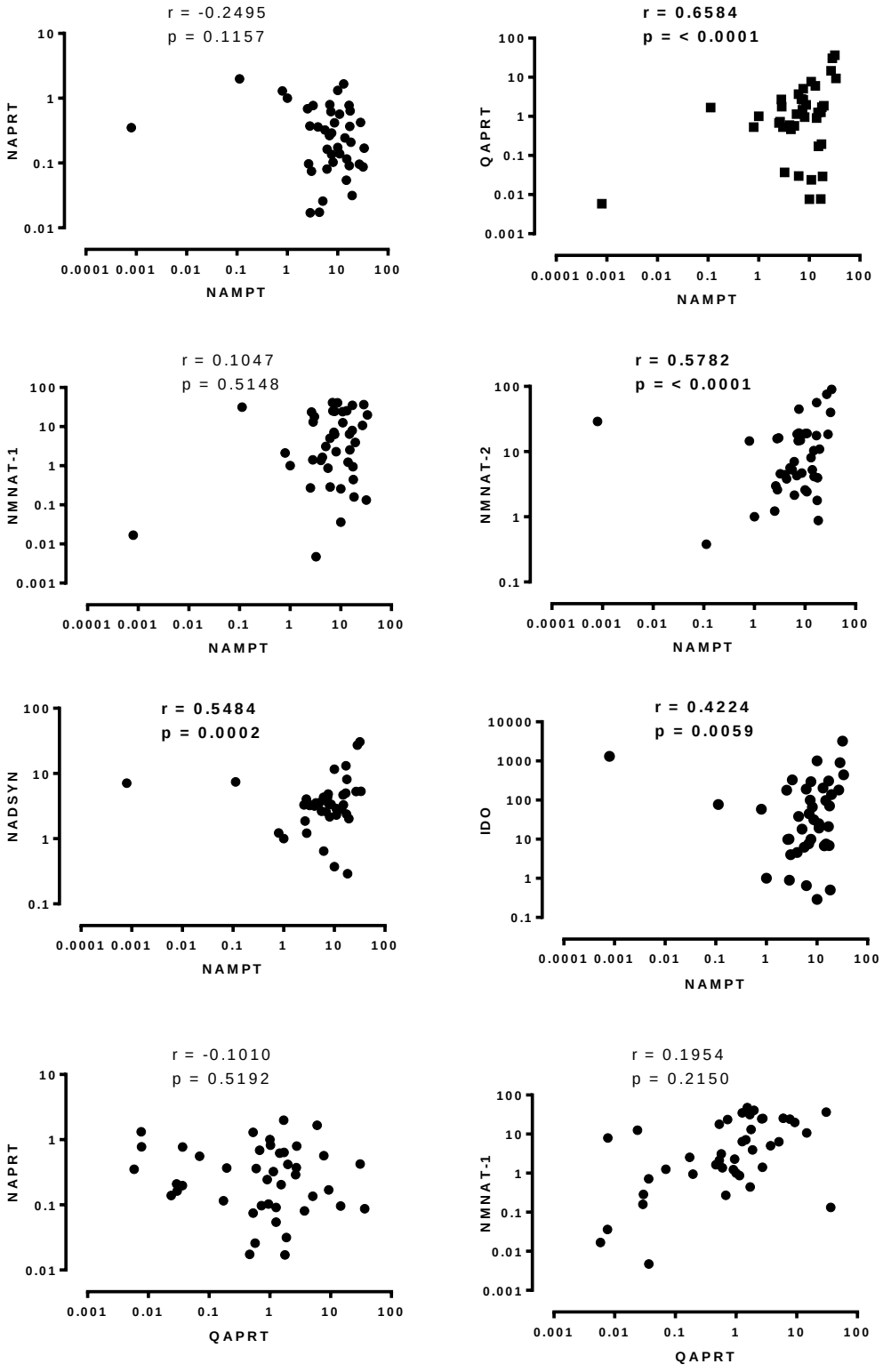


Fig 3.6 (a). Correlation between NAD^+ biosynthesis enzymes investigated in this study. RQ values of NMNAT 1-2, NADSYN, IDO, NAPRT and NAMPT were plotted against each other. Correlation of NAMPT/QAPRT, NAMPT/NMNAT2, NAMPT/NADSYN, NAMPT/IDO were found to be significant ($p<0.05$).

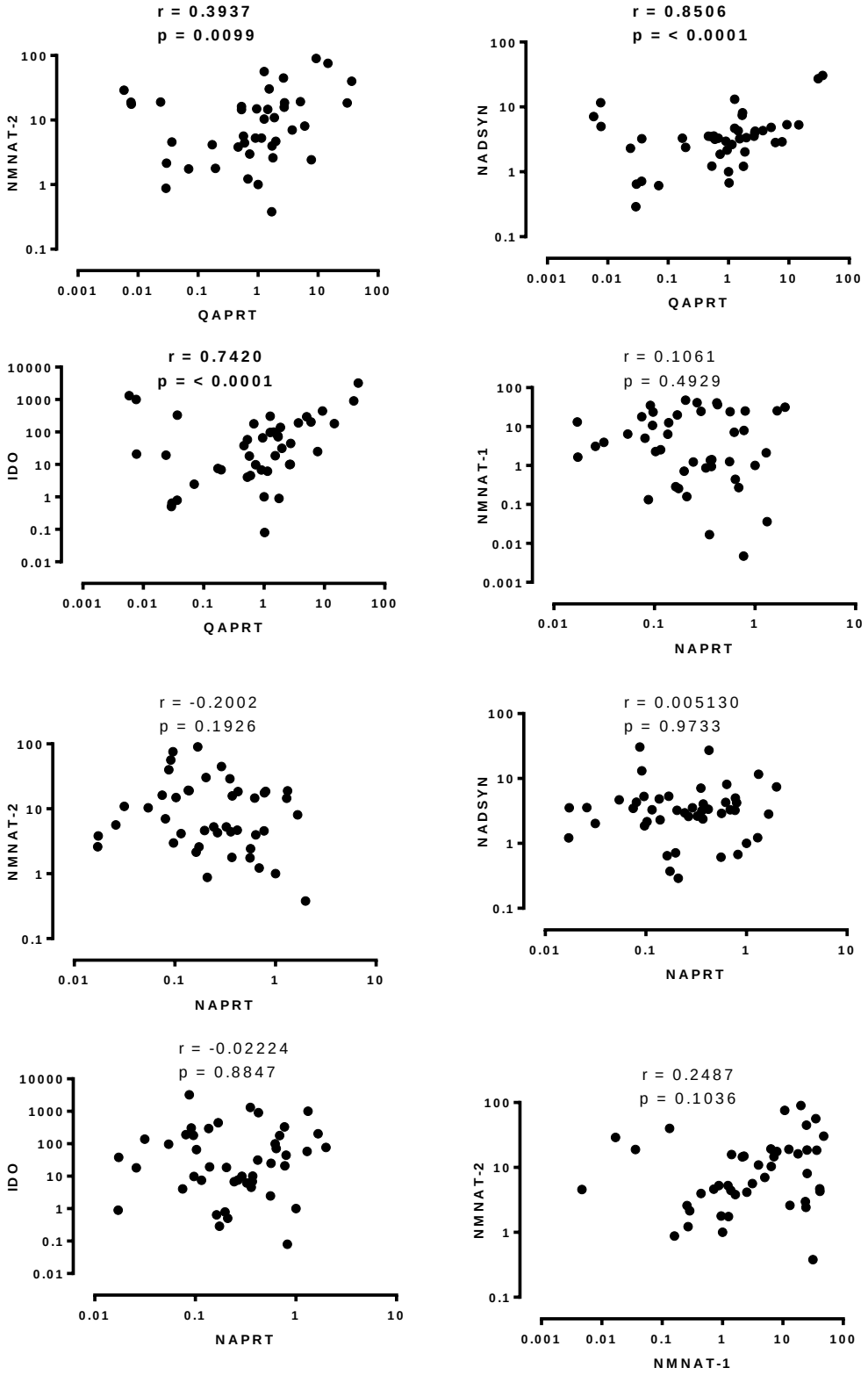


Fig 3.6 (b). Correlation between NAD^+ biosynthesis enzymes investigated in this study. RQ values of NMNAT 1-2, NADSYN, IDO, NAPRT and NAMPT were plotted against each other. Correlation of QAPRT/NMNAT2, QAPRT/NADSYN, QAPRT/IDO were found to be significant ($p<0.05$).

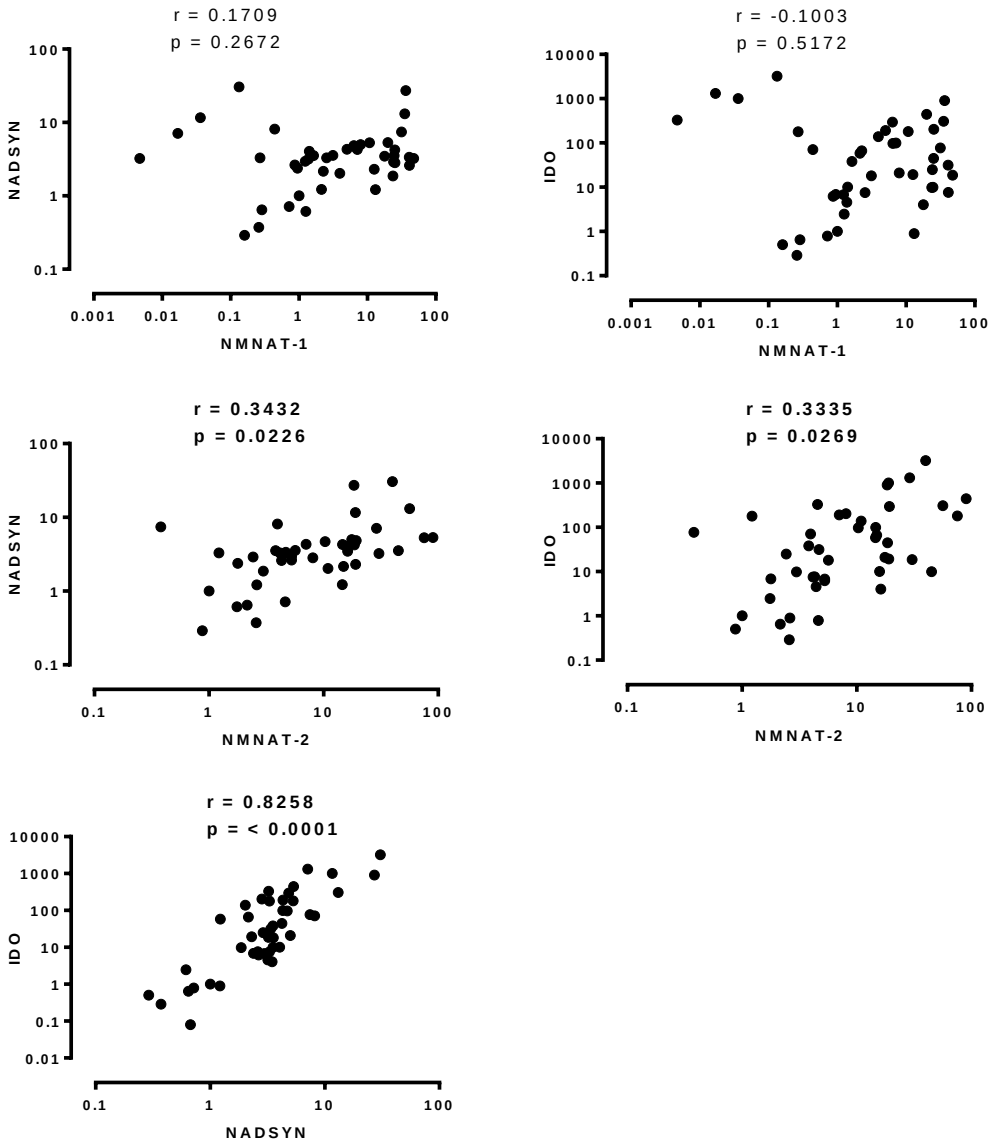


Fig 3.6 (c). Correlation between NAD^+ biosynthesis enzymes investigated in this study. RQ values of NMNAT 1-2, NADSYN, IDO, NAPRT and NAMPT were plotted against each other. Correlation of NMNAT2/NADYN, NMNAT2/IDO, NADSYN/IDO were found to be significant ($p<0.05$)

3.6 Discussion

3.6.1 NAD^+ biosynthesis enzyme expression in synovial tissue

This study has explored for the first time, the gene expression of NAD^+ biosynthesis enzymes in normal healthy and diseased synovial tissues.

A number of studies have shown NAMPT to be elevated in inflammation (Dahl et al. 2007; Moschen et al. 2007; Moschen et al. 2011) including RA (Nowell et al. 2006; Otero et al. 2006; Brentano et al. 2007; Matsui et al. 2008; Rho et al. 2009). In RA, NAMPT has been shown to be elevated in serum (Otero et al. 2006; Brentano et al. 2007; Matsui et al. 2008; Rho et al. 2009), synovial fluid (Nowell et al. 2006; Brentano et al. 2007; Meier et al. 2012) and synovial tissues (Nowell et al. 2006; Brentano et al. 2007; Matsui et al. 2008; Meier et al. 2012). Previous studies have used a number of different methods to quantify NAMPT expression, including detection by immunohistochemistry (Nowell et al. 2006; Brentano et al. 2007; Meier et al. 2012), western blot of tissue (Brentano et al. 2007), *in situ* hybridisation (Brentano et al. 2007), ELISA (Rho et al. 2009; Meier et al. 2012), enzyme immunoassay (EIA) (Nowell et al. 2006; Otero et al. 2006; Matsui et al. 2008) and RT-PCR (Brentano et al. 2007; Matsui et al. 2008).

Some studies have also investigated the expression of IDO in RA synovial tissue (Malone et al. 1994; Zhu et al. 2006; Park et al. 2011) but there have been no studies in literature (at the time of this writing) which have characterised the expression of QAPRT, NAPRT, NADSYN and NMNAT in synovium. In this study qPCR was used to study the gene expression of NAD^+ biosynthesis enzyme in synovium and for the first time has compared the gene expression of NAD^+ biosynthesis enzyme from synovial tissues of patients with RA with patients with OA and normal healthy subjects.

3.6.2 NAMPT in synovial tissue

3.6.2.1 NAMPT in developing healthy synovial tissue

In this study, the expression of NAMPT was found to be high in synovial tissue of young actively-developing healthy subjects, which gradually decrease over time with age. Studies in literature have shown NAMPT dependent NAD^+ biosynthesis regulates many cellular and physiological function of Sirt1 (Imai 2009a, b) and the NAMPT/Sirt1 pathway plays an important role in cellular differentiation and maturation. Over expression of NAMPT is observed in maturing human vascular

smooth muscle cells (SMC) (van der Veer et al. 2005) and increasing the expression of NAMPT in these cells, aids in their maturation (van der Veer et al. 2005) and lifespan (van der Veer et al. 2007). In contrast, decreasing the level of NAMPT impairs survival and maturation of SMC *in vitro* (van der Veer et al. 2005). It's possible therefore, that like SMCs, NAMPT is similarly overexpressed in young actively-developing and maturing synovial tissues and may well be related to the differentiation and maturation state of the synovial tissue under investigation.

3.6.2.2 NAMPT in normal and diseased synovial tissue

In this study the expression of NAMPT was found to be high in the synovium of patients with RA compared to OA & normal healthy patients. On closer inspection it was evident that some of the RA patients (n = 4) had received anti-TNF medications (e.g., etanercept). When these patients were excluded from analysis, NAMPT expression in synovial tissue from RA patients was statistically significantly enhanced when compared to tissue from OA and normal patients (Fig 3.5a). TNF is known to regulate the expression of NAMPT in neutrophils and macrophages *ex-vivo* (Jia et al. 2004; Iqbal and Zaidi 2006) and it is possible that the anti-TNF medication in these patients prevented the over expression of NAMPT in the synovial tissues. A study performed by Klaasen *et* al (2012) showed a significant reduction in serum NAMPT levels after patients with RA were treated for 16 weeks with adalimumab, a recombinant human monoclonal antibody specific to TNF. Furthermore, this reduction in serum NAMPT has been observed in patients treated with other biologics – for example, treatment with rituximab (a chimeric monoclonal antibody against the protein CD20 on B-cells) significantly reduces the serum levels of NAMPT in patients with RA with levels comparable to healthy controls, notably, a lack of change of serum NAMPT in these patients predicts worsening of disease activity (Šenolt et al. 2011).

In this study the expression of NAMPT was found to be high in synovial tissue from OA when compared to normal healthy patients, however it was not statistically significant. Laiguillon *et al* (2014) have also shown NAMPT to be expressed in human OA synovium, cartilage and subchondral bone with increased expression in synovial tissue. NAMPT was also found to be highly expressed in synovial fluid of patients with OA and NAMPT expression positively correlated with severity of OA, degradation marker of collagen II and aggrecan (Duan et al. 2011).

3.6.3 NMNAT in synovial tissue

All enzymes studied (NAMPT, QAPRT, NAPRT, NMNAT 1 & 2, NADSYN and IDO were detected with the exception of NMNAT3. The lack of detection of this particular enzyme may be due either to low expression of NMNAT3 in synovial tissue or an issue with the design of the primer used in the qPCR analysis.

NMNAT is present as three isoforms in humans. NMNAT-1 is a homohexamer consisting of 279 amino acids with a molecular mass of 31.9 kDa (Werner et al. 2002; Berger et al. 2005; Di Stefano et al. 2010). NMNAT-2 is a homodimer consisting of 307 aminoacids and has 34% sequence homology to NMNAT-1 with a molecular mass of 34.4 kDa (Raffaelli et al. 2002; Di Stefano et al. 2010). NMNAT-3 is a homotetramer and contains 252 amino acids. It has 50% and 34% sequence homology to NMNAT-1 & -2 respectively with molecular mass of 28.3 kDa (Zhang et al. 2003; Di Stefano et al. 2010). NMNAT-1 is localised to the nucleus and NMNAT-2 & -3 are localised to Golgi complex and mitochondria respectively (Zhang et al. 2003; Berger et al. 2005). Interestingly, the tissue distribution of all three isoforms are different (table 3.2). Also of interest is that NMNAT-2 expression is high in tissues where NMNAT-3 is not detected and vice versa. This mirrors the observations in the RA synovial tissue as this study showed increased expression of NMNAT-2 in RA synovial tissue whilst NMNAT-3 could not be detected.

This study showed elevated expression of NMNAT-2 in RA synovium compared to normal healthy tissue. It is natural to hypothesise that any increase in NAMPT expression would need to be associated with an increased expression of NMNAT to convert the NAMPT product, NMN, to NAD^+ (Fig 3.7). Indeed, this study showed a significant correlation between the expression of NMNAT-2 and NAMPT mRNA in RA synovial tissues [Fig 3.6 (a)]. This observation suggests that increases in the enzyme expression of NAD^+ salvage pathway (i.e. both NAMPT and NMNAT) would likely be accompanied by an associated increase in NAD^+ production.

NMNAT-1 and -3 has been found to be weakly expressed in tumour cell lines (Emanuelli et al. 2001; Zhang et al. 2003) whereas NMNAT-2 activity is increased in liver cancer and neuroblastoma (Sorci et al. 2007). Pan *et al* (2014) have shown that p53 induces NMNAT-2 and to some extent NMNAT-1 & -3. These observations suggest that NMNAT may be regulated differently in diseased tissues and the next chapter will investigate the effect of cytokine stimulation of fibroblasts on NMNAT expression.

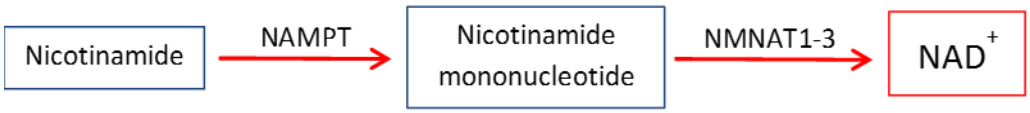


Fig 3.7. NAD^+ synthesis via the salvage pathway. NAMPT – nicotinamide phosphoribosyl transferase, NMNAT – nicotinamide mono nucleotide adenyl transferase, NAD – nicotinamide adenine dinucleotide.

Tissues	NMNAT1	NMNAT2	NMNAT3	Reference
Skeletal muscle	+++	++	+	(Emanuelli et al. 2001; Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Heart	+++	++	+	(Emanuelli et al. 2001; Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Kidney	+++	-	++	(Emanuelli et al. 2001; Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Lung	-	-	+++	(Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Spleen	+	-	+++	(Emanuelli et al. 2001; Zhang et al. 2003; Yalowitz et al. 2004)
Placenta	-	-	+	(Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Thymus	+	NT	-	(Emanuelli et al. 2001; Zhang et al. 2003)
Liver	++	-	-	(Emanuelli et al. 2001; Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Brain	+	+++	-	(Emanuelli et al. 2001; Fernando et al. 2002; Raffaelli et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)
Pancreas	++	++	-	(Fernando et al. 2002; Zhang et al. 2003; Yalowitz et al. 2004)

Table 3.2. Summary of tissue expression of different isoforms of NMNAT detected by Northern blot analyses with associated reference. +, weak expression to +++, strong expression; NT – not tested.

3.6.4 IDO in synovial tissue

In this study, it was found that IDO was also significantly expressed in synovial tissue from patients with RA compared to OA and normal synovial tissues (Fig 3.4f). This finding is similar to observations seen in other studies by Zhu *et al* (2006) and Park *et al* (2011).

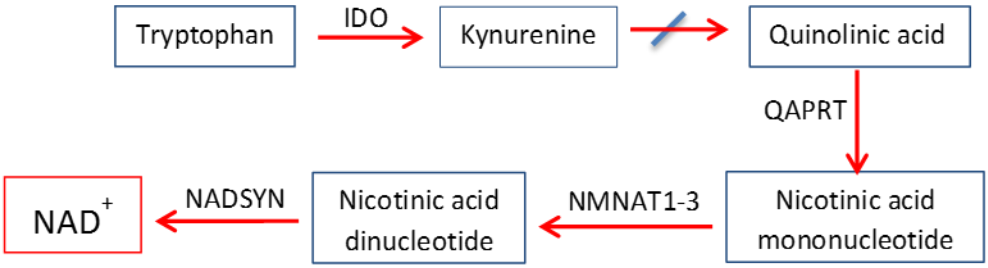


Fig 3.8. NAD^+ biosynthesis from Tryptophan via the *de nova* pathway. IDO – indole amine 2,3-dioxygenase, QaPRT – quinolinic acid phosphoribosyl transferase, NMNAT – nicotinamide mono nucleotide adenyl transferase, NADSYN – NAD synthetase.

IFN-γ is a well-known inducer of IDO (Mellor and Munn 2004). Indeed, in chronic immune activation, IFN-γ is released by TH-1 helper cells which leads to activation of IDO and increased degradation of tryptophan (Schroecksnadel et al. 2003). The extent of increased tryptophan degradation corresponds to the severity of RA (Schroecksnadel et al. 2006).

In addition, it is believed that tryptophan degradation via IDO leads to inhibition of cell proliferation resulting in modulation of cellular immune response; studies have shown inhibition of T cell proliferation by macrophages (Munn et al. 1999) and dendritic cells (Hwu et al. 2000) following tryptophan degradation by IDO. IFN-γ mediated activation of IDO and enhanced tryptophan depletion has shown to inhibit IL-1β induced collagenase and stromelysin gene expression in fibroblast which were reversed by the addition of exogenous tryptophan (Varga et al. 1995). This suggests that inhibition of IL-1β induced collagenase and stromelysin gene expression in fibroblast was directly due to reduction of local tryptophan concentration rather than the accumulation of kynurenine and other tryptophan metabolites.

3.6.4.1 IDO – the good?

In collagen induced arthritis (CIA) in mice, IDO was found to be significantly upregulated in the dendritic cells in draining lymph nodes and the severity of the disease was found to be high in IDO deficient ($Indo^{-/-}$) mice when compared to C57BL6/J mice (Criado et al. 2009). IDO gene transfer using adenoviral vectors encoding IDO ameliorated ankle arthritis in CIA by reduction of synovial IL-17 production and induction of CD+ T-cell apoptosis (Chen et al. 2011b). Following CIA in $Indo^{-/-}$ mice there was increased production of IFN-γ and IL-17 in the lymph nodes and increased infiltration of Th1 and Th17 cells in the arthritic joints suggesting that IDO mediated tryptophan catabolism regulates Th1/Th17 in collagen induced arthritic joints (Criado et al. 2009).

3.6.4.2 IDO – the bad?

Inhibition of IDO by 1-methyl-tryptophan (1-MT) *in-vivo* resulted in increased incidence and severity of collagen induced arthritis in mice when compared with vehicle treated mice (Szanto et al. 2007; Criado et al. 2009). From above studies it is clear that increased IDO activity leads to suppressive effects on the activation of T cells. However, the effect of IDO on B cell function is not clearly understood. When K/BxN murine RA model was treated with 1-MT there was amelioration of arthritis symptoms rather than exacerbation (Scott et al. 2009). There was no difference in the percentage of T regulatory cells nor in the levels of Th1/Th2/Th17 cytokines, but there was a decrease in autoantibody titres (Scott et al. 2009).

These findings suggest that the activity of IDO is very complex in not only being immunosuppressive but also supporting the development of B cell mediated inflammation and therefore warrants further investigation.

3.6.5 QAPRT in synovial tissue

In this study there was increased expression of QAPRT in RA synovial tissue when compared to synovial tissues in OA and normal healthy subjects, however there was no statistically significant difference (Fig 3.4). QAPRT is the rate limiting enzyme which catalyses the conversion of quinolinic acid to nicotinic acid mononucleotide (Fig 3.8 above).

There are no studies in the literature at the time of writing this thesis showing that expression of QAPRT differs in diseased synovial tissues. However, studies have shown marked differences in QAPRT expression in other diseases – both increases and decreases; QAPRT is highly expressed in malignant gliomas with higher levels seen with increasing malignancy (Sahm et al. 2013). QAPRT was induced when glioma cells were treated with alkylating agents, irradiation and hydrogen peroxide in a dose dependant manner (Sahm et al. 2013). QAPRT was also induced when quinolinic acid was injected into rat striata (Foster et al. 1985). *Reduced* QAPRT activity has also been found at the epileptic foci in human brain (Feldblum et al. 1988) whereas *increased* activity is found in post-mortem brain of patients with Huntington disease when compared to normal subjects (Foster et al. 1985). When human skin fibroblasts were stimulated with IFN-γ and TNF-α there was no change in the expression of QAPRT, however, when stimulated simultaneously with both IFN-γ and TNF-α there was downregulation of QAPRT when compared to untreated controls (Asp et al. 2011).

Studies have shown that microglial cells over express QAPRT to synthesise NAD^+ when depletion of NAD^+ occurs following NAMPT inhibition, irradiation or when treated with alkylating agents (Sahm et al. 2013). There is also an increase demand for NAD^+ in highly proliferative tissue such as the RA synovial tissue. It is possible that the increased expression of QAPRT in this study could be to increase the synthesis of NAD^+ through the *de novo* pathway as the other enzymes required for NAD^+ synthesis through this pathway such as IDO, NMNAT and NADSYN are also highly expressed in the synovial tissue in RA (Fig 3.8). Also, there was significant correlation in the expression of QAPRT and IDO ($p<0.0001$), NMNAT2 ($p=0.009$) and NADSYN ($p<0.0001$) (Fig 3.6a) in this study. It is possible that QAPRT may be regulated by cytokines and this will be investigated in the next chapter by stimulating human synovial tissues with inflammatory cytokines found in RA (TNF-α, IL-1β, IFN-γ and OSM).

3.6.6 NADSYN in synovial tissue

In this study NADSYN was found to be significantly elevated in RA synovial tissues when compared to normal. So far the regulation and involvement of NADSYN in human diseases has not been characterised in any studies. In the next chapter the upregulation of NADSYN seen in RA synovial tissue in this study will be investigated further by looking at any regulation due to cytokines normally seen in RA (TNF-α, IL-1β, IFN-γ and OSM).

3.6.7 NAPRT in synovial tissue

This study found no statistically significant differences in NAPRT expression between RA, OA and normal synovial tissues. NAPRT converts nicotinic acid (NA) to NA mononucleotide (NaMN) which is then converted to NA adenine dinucleotide and finally into NAD^+ via the Preiss Handler pathway (Fig 3.9).

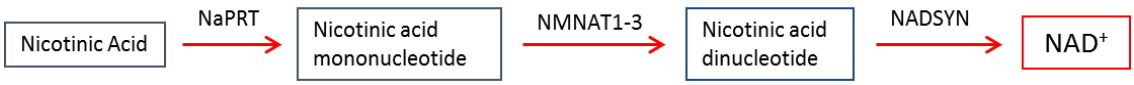


Fig 3.9. Synthesis of NAD^+ from nicotinic acid via Preiss-Handler pathway. NAPRT – nicotinic acid phosphoribosyl transferase, NMNAT – nicotinamide mono nucleotide adenyl transferase, NADSYN – NAD synthetase.

NA is known to be a better precursor in elevating NAD^+ levels than Nam (Hara et al. 2007). In cells expressing endogenous NAPRT, addition of NA at concentration as low as 5-10μM almost doubles (200%) the intracellular NAD^+ content whereas in cells

expressing NAMPT, addition of Nam at millimolar concentration only increases intracellular NAD^+ content by 130% (Hara et al. 2007). This is due to a lack of feedback inhibition of NAPRT by NAD^+ in contrast to NAMPT (Hara et al. 2007; Galassi et al. 2011). This property has been demonstrated to protect normal tissues from cytotoxicity of NAMPT inhibitors (such as APO866) by the co-administration of NA. For example, studies have shown that cancers and brain tumours demonstrating low NAPRT expression respond well to NAMPT inhibitors whilst normal tissues were rescued from death by replenishing the NAD^+ levels through the addition of NA (Watson et al. 2009; Olesen et al. 2010).

In this study the expression of NAPRT was very low in synovial tissue whilst the expression of NAMPT was high in RA synovial tissue, thus the protective action of NA on normal tissues by replenishing NAD^+ levels using NAPRT could potentially be a viable option if NAMPT inhibition is used for the treatment of RA.,

3.6.8 Correlation of NAD^+ biosynthesis enzymes in the salvage pathway

There was significant correlation between NAMPT and NMNAT-2, QAPRT, NADSYN & IDO in synovial tissue (Fig 3.6a). The relationship between NAMPT and NMNAT-2 is interesting, as any increases in intracellular NAMPT would need an associated increase in NMNAT to have an impact on intracellular NAD^+ (Fig 3.7).

As described above, there is a large body of evidence in literature demonstrating that NAMPT is an inducible enzyme, but there is little on NMNAT 1-3. The next chapter will investigate the regulation of expression of NMNATs by cytokine stimulation of the synovial fibroblast (i.e. TNF-α, IL-1β, IFN-γ and OSM).

NMNAT-3 was not detected in any of the synovial tissues hence no correlation could therefore be performed. Unlike NMNAT-2, there was no correlation between the expression of NAMPT and NMNAT-1 in this study. This suggests therefore that NMNAT-2 is the most important enzymes of the three NMNAT isoforms in synovial tissues for the NAD^+ salvage pathway.

3.6.9 Correlation of NAD^+ biosynthesis enzymes in the *de novo* pathway

QAPRT, NADSYN and IDO are all enzymes involved in the *de novo* pathway (Fig 3.8). It is well known that IDO is regulated by IFN-γ (section 3.6.4), and QAPRT has been shown to be regulated by cytokine activity (section 3.6.5). To date NADSYN have not been shown to be regulated by cytokine activity

All enzymes that were investigated in this study that are involved in the *de novo* pathway were correlated with each other (Fig 3.6b & c). Their related expressions may reflect their required *in tandem* expression for controlled NAD^+ availability via this pathway. The correlation of expression with NAMPT suggests that factors that are regulating NAMPT expression may also regulate the expression of QAPRT, NADSYN and IDO in a similar manner. The next chapter will specifically investigate the effect of a number of inflammatory cytokines (TNF-α, IL-1β, IFN-γ and OSM) although other factors such as oxidative stress, heat, NAD^+ and other metabolites within the pathway could also have a role to play in their regulation.

3.6.10 Correlation of NAD^+ biosynthesis enzymes in the Preiss-Handler pathway

In this study there was very low expression of NAPRT in RA, OA and normal synovial tissue and there was no correlation between the expressions of NAPRT with any of the NAD^+ biosynthesis enzymes tested. To date there are no studies in the literature which have investigated the regulation of expression of NAPRT. However, as with the other enzymes investigated in this chapter, stimulation of NAPRT by inflammatory cytokines usually seen in RA such as TNF-α, IL-1β, IFN-γ and OSM will be investigated further in the next chapter.

3.7 Summary

In this chapter the gene expression of various NAD^+ biosynthesis enzymes were characterised in RA, OA and normal synovial tissues.

The main findings were:

- NMNAT-3 could not be detected in any of the tissues. All other enzymes were detected *ex vivo*.
- NAMPT expression is highly expressed in pre-pubertal actively growing normal healthy synovial tissue, a characteristic that appears to be unique to NAMPT.
- When patients with RA receiving anti-TNF treatment for their arthritis are excluded from analyses, NAMPT expression is statistically significantly elevated in RA synovial tissue compared to tissue from patients with OA and normal joints.
- In synovial tissue, the expression of the enzymes involved in the NAD salvage pathway investigated in this study, namely NAMPT and NMNAT-2 significantly correlate with each other, but does not correlate with NMNAT-1
- NAMPT expression in synovial tissue is also significantly correlated with QAPRT, NADSYN and IDO
- In synovial tissue, the expression of enzymes involved with the *de novo* NAD pathway investigated in this study, namely, QAPRT, NMNAT-2, NADSYN and IDO, all significantly correlate with each other.
- Notably, NAPRT and NMNAT-1 do not correlate with any of the other enzymes investigated in this study.

Due to the known effect of cytokines such as TNF-α, IL-1β, IL-6, LPS and OSM on NAMPT expression in synovial fibroblasts (Nowell et al. 2006; Brentano et al. 2007), the effect of inflammatory cytokines common to RA upon the regulation of these enzymes will be explored in the next chapter.

Chapter 4 - Effect of cytokines on NAD^+ biosynthesis enzymes on RA synovial fibroblasts

4.1 Introduction

4.1.1 Normal Synovium

Synovial tissue (ST) consists of superficial intimal lining layer and deep synovial sublining layer. The intimal lining layers of normal synovium is of 1-2 cells thickness, predominantly consisting of $CD55^+$ fibroblast-like cells (mesenchymal origin) and $CD68^+$ macrophages (haemopoeitic origin) (Smith et al. 2003). Sublining cells consists of few blood vessels, fat cells, few fibroblasts and macrophages, $CD3^+$ T lymphocytes, HLA-DR^+ cells and no B lymphocytes (Lindblad and Hedfors 1987b; Smith et al. 2003; Singh et al. 2004). T lymphocytes are predominantly seen in the peri-vascular area and occasionally found to be diffuse (Lindblad and Hedfors 1987b; Singh et al. 2004). Pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β & IFN-γ are detected in normal synovium however its far less than in RA synovium (Smith et al. 2003). However, there is high expression of IL1ra (natural antagonist to IL-1 thus resulting in suppression of inflammation in normal synovial tissue (Smith et al. 2003).

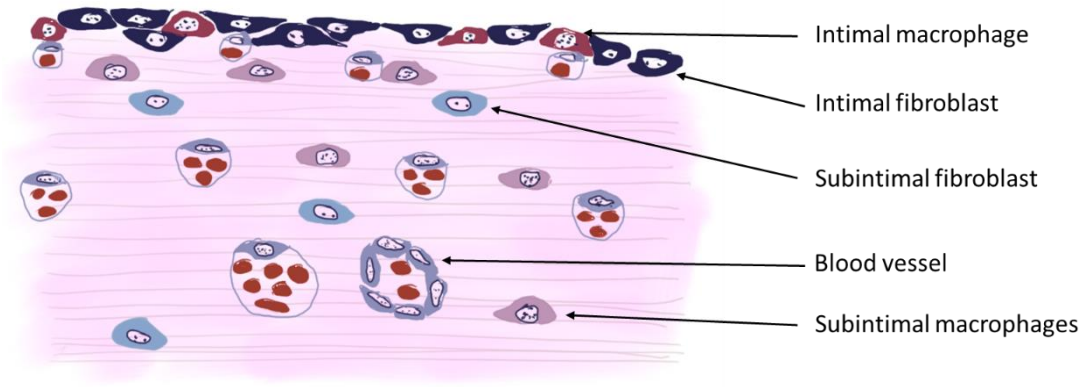


Fig 4.1. Schematic representation of normal synovium

4.1.2 Synovium in OA

Synovium in OA patients exhibit 3-4 layer thick synovial lining cells covered with fibrotic tissue (Lindblad and Hedfors 1987a; Haraoui et al. 1991). Macrophages are the predominant cells in the lining layer with T cells found in the sublining layer (de Lange-Brokaar et al. 2012). There are abundant $CD68^+$ cells followed by HLA-DR^+ cells in the lining and sublining layers (Saito et al. 2002). $CD4^+/CD8^+$ ratio is increased in OA synovial tissue (5:1) when compared to normal synovial tissue (2:1) (Saito et

al. 2002). B cells and plasma cells are also detected in OA ST but are low compared to RA (Lindblad and Hedfors 1987a; Revell et al. 1988). Various cytokines have been detected in OA ST such as IL-1β (Smith et al. 1997; Benito et al. 2005), TNF-α (Smith et al. 1997; Benito et al. 2005), IL-6 (Farahat et al. 1993; Doss et al. 2007), IFN-γ (Dolhain et al. 1996; Ishii et al. 2002), IL-8 (Furuzawa-Carballeda and Alcocer-Varela 1999), IL-10 (Furuzawa-Carballeda and Alcocer-Varela 1999), IL-18 (Tanaka et al. 2001) and TGF-β (Ning et al. 2011). In fact, the cytokine profiles between OA and RA synovium are similar with the difference being quantitative and not qualitative (Farahat et al. 1993; Schlaak et al. 1995). Indeed, the NAD^+ biosynthesis enzyme expression profile observed in the previous chapter was similar between OA and RA with increased expression in RA (section 3.5.2).

4.1.3 Synovium in RA

In RA, there is hyperplasia of the synovial lining cells that are 4-6 cells thick containing macrophages and fibroblasts and covered by fibrous tissue (Haraoui et al. 1991; Schumacher et al. 1994). $CD68^+$ and HLA-DR^+ cells are found infiltrating the lining and sublining layers. There is infiltration of T and B lymphocytes, plasma cells and macrophages in the sublining layers (Smeets et al. 1998) and $CD4^+$ T lymphocytes are found scattered throughout the synovium with clusters formed around blood vessels and sometimes forming lymphoid follicle like structures (Haraoui et al. 1991). $CD8^+$ T lymphocytes are frequently found in the deeper zones of lining cell layers. There is increased expression of VEGF in the synovial lining cells and increased neovascularisation of the subintimal layer (Fava et al. 1994). Synovial fibroblasts in RA also develop into an aggressive phenotype (pannocytes) where they hypertrophy and invade bone and cartilage leading to its destruction or erosion (pannus). There is an increased expression of phosphorylated signalling effectors such as phospho-p38, phospho-STAT-1, -3 and -5 in synovial fibroblasts (Galligan et al. 2009). Expression of cytokines are abundant in RA synovium compared to OA and normal tissue. IL-1β (CHU et al. 1992; Farahat et al. 1993), TNF-α (CHU et al. 1992; Farahat et al. 1993), IL-6 (CHU et al. 1992; Farahat et al. 1993), IL-10 (Kirkham et al. 2006), IL-16 (Kirkham et al. 2006), IL-17 (Kirkham et al. 2006), GM-CSF (CHU et al. 1992; Farahat et al. 1993), TGF-β (CHU et al. 1992), OSM (Okamoto et al. 1997) and IFN-γ (Kirkham et al. 2006) are all expressed highly in RA synovial tissue.

4.1.4 Synovial fibroblasts in *in vitro* studies

Cells obtained from synovial tissue following enzymatic dispersion contain both macrophages and fibroblasts. Following several passages, macrophages are eliminated leaving only the fibroblasts in the culture. Synovial fibroblasts proliferate and survive over a long period of time in culture. They can be easily stored in liquid nitrogen for prolonged periods and can be cultured again after thawing. However, after 10 – 12 passages the rate of growth diminishes and the cells become senescent (reviewed by Firestein 1996). Synovial fibroblasts obtained from arthritic or normal joints show anchorage independent growth *in vitro* (Lafyatis et al. 1989). They grow as monolayer cells in anchorage independent conditions during early passage and growth is not influenced by IL-1, TNF-α & IFN-γ (Lafyatis et al. 1989). Synovial fibroblasts from RA tissue can also sustain cytokine producing activity *in vitro* over several passages (Bucala et al. 1991). Interestingly, when RA synovial fibroblasts are engrafted into SCID mice, they have been shown to attach and invade cartilage suggesting that synovial fibroblasts maintain their invasive and destructive behaviour over long periods of time, even in the absence of T cells or other human cells (Müller-Ladner et al. 1996).

Synovial fibroblasts are thought to mediate many of factors involved in RA pathology and have been used as a model system for RA for many years. Their ease of culture makes them an ideal model to study the effects of cytokines on NAD enzyme synthesis.

4.1.5 Promoter regions of NAD^+ enzymes and their induction

4.1.5.1 NAMPT Promoter and induction

NAMPT has two distinct promoter regions which consists of binding sites for AP-2 (activator protein) and LF-1 (lymphoid enhancer binding factor) proximally; NF1 (nuclear factor) and NF-κB distally whereas SP1 (Specificity protein 1), NF-IL-6, AP-1, GR (glucocorticoid receptor), HRE (hypoxia inducible factor responsible elements) and cAMP response element (CRE) binding protein were found throughout (Ognjanovic et al. 2001; Luk et al. 2008) (Fig 4.2). There are also two STAT binding sites in the proximal and distal promoter regions (Nowell et al. 2006).

NAMPT can be induced by a number of cytokines including TNF-α, IL-1β, IL-6 (with sIL-6R) and OSM *in vitro* (Ognjanovic et al. 2001; Nowell et al. 2006; Brentano et al. 2007).

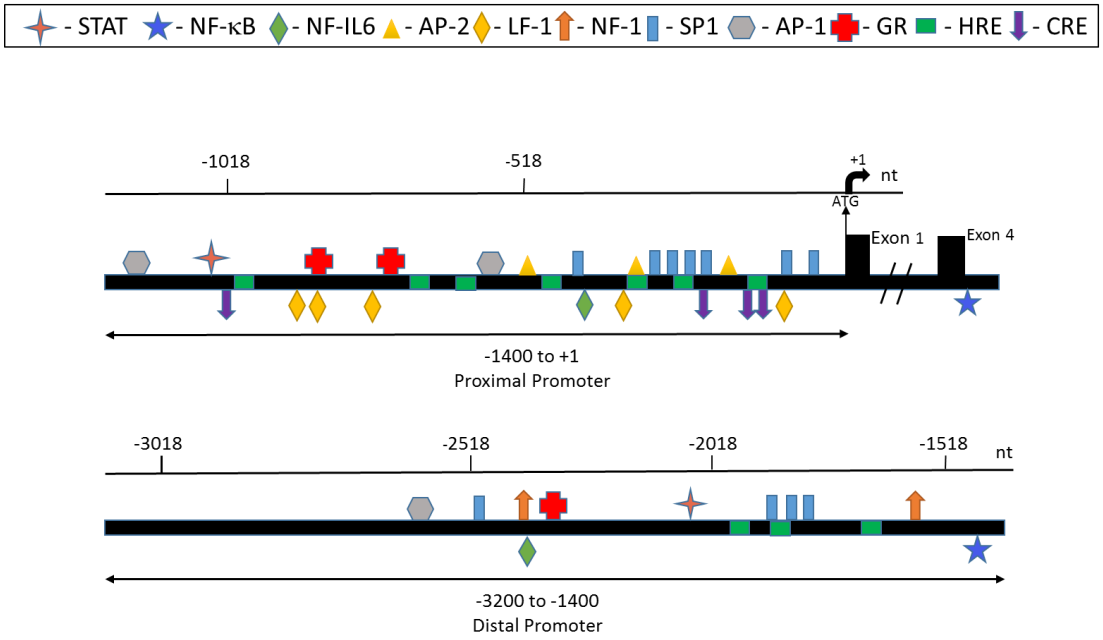


Fig 4.2. Schematic representation of promoter region in NAMPT gene. The line marked nt (nucleotides) represents the distance from the transcription initiation site. NF – nuclear factor. (Adapted from Luk *et al* (2008)).

4.1.5.2 IDO promoter and induction

IDO has a distinct promoter region with two interferon-stimulated response elements (ISREs) and three gamma-interferon activating sequence (GAS) element with binding sites for interferon responsive factor (IRF)-1, STAT-1 and NF-κB (CHON et al. 1995; Konan and Taylor 1996; Fujigaki et al. 2006; Robinson et al. 2006) (Fig 4.3)

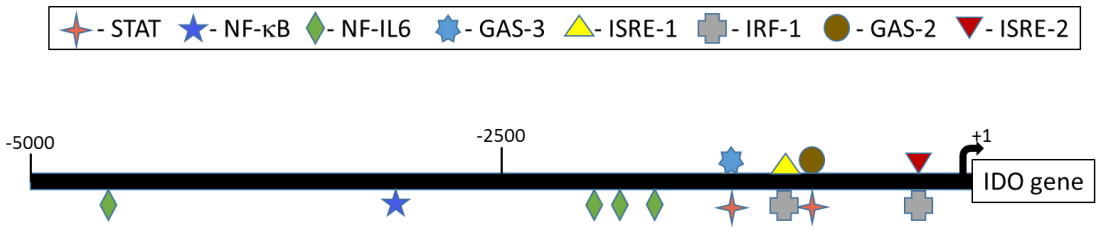


Fig 4.3. Schematic representation of promoter region in IDO gene. GAS - gamma-interferon activating sequence, ISRE - interferon-stimulated response element, IRF - interferon responsive factor. Adapted from Chon *et al* (1995), Konan and Taylor (1996), Fujigaki *et al* (2006) and Robinson *et al* (2006).

IDO is highly induced by IFN-γ and its expression is synergistically enhanced in the presence of TNF-α, IL-1β and LPS *in vitro* (Hissong and Carlin 1997; Babcock and Carlin 2000; Currier et al. 2000; Robinson et al. 2003; Fujigaki et al. 2006).

4.1.5.3 NMNAT promoter and induction

In *Drosophila* there is a single NMNAT gene which contains a heat shock element in its promoter region and has been shown to be inducible by heat shock factor and hypoxia inducible factor 1α (Ali et al. 2011). In humans, NMNAT2 has two p53 response elements in its promoter region (Pan et al. 2014). P53 significantly induces NMNAT-2 and to lesser extend NMNAT-1 & -3 in human non-small lung cancer cell line compared to normal tissue (Pan et al. 2014). However, there are no studies in the literature that has examined the induction of NMNAT by cytokines.

4.1.5.4 QAPRT, NADSYN & NAPRT promoters and induction

To date there are no studies in the literature which have characterised response elements in the promoter regions of QAPRT, NADSYN nor NAPRT. Unlike NADSYN and NAPRT however, QAPRT has been shown to be sensitive to IFN-γ and TNF-α, co-stimulation (Sahm et al. 2013).

4.1.6 Choice of cytokines for synovial fibroblast stimulation

Both NAMPT and IDO have STAT and NF-κB binding sites in their promoter regions and IDO has known binding sites for IRF-1 (see section 4.1.5). TNF-α and IL-1β both result in binding of NF-κB to DNA with IFN-γ activating the binding of STAT-1 and OSM STAT-3 & -5 (see table 1.2). TNF-α, IL-1β, IFN-γ and OSM are commonly seen in synovial fluid and synovial tissues in RA (CHU et al. 1992; Farahat et al. 1993; Kirkham et al. 2006) and are all known to induce NAMPT (Nowell et al. 2006; Brentano et al. 2007) and synovial fibroblasts have been shown to express their respective cell receptors on their cell surface (Nowell et al. 2006).

The known effect of these cytokines on NAMPT and IDO in synovial fibroblasts and their known dysregulation in RA made them the choice cytokines for use in this study.

4.1.7 Aims of chapter 4

The effect of cytokines such as TNF-α, IL-1β, IFN-γ and OSM on NAMPT and IDO is well known, however, the regulation of the other NAD^+ enzymes such as NMNAT, NADSYN and NAPRT by these cytokines is unknown.

This chapter aims to characterise the effect of OSM, IFN-γ, TNF-α and IL1-β on the NAD^+ biosynthesis enzymes to gain insight into their regulation in diseased synovial tissue.

4.2 Methods

4.2.1 Stimulation with Cytokines and qPCR analysis

Synovial fibroblasts (SF) from patients with rheumatoid arthritis (n = 3) were cultured in SFCM in 6 well plates and serum starved for 48 hours (refer to section 2.2.9). SF were stimulated with either 10ng/ml Oncostatin M (OSM), Interferon γ (IFN), Interleukin - 1β (IL-1β) or Tumour Necrosis factor α (TNF-α) for 0, 2, 4, 6, 8 and 10 hours.

At the end of time course, supernatant was removed and mRNA extracted from the cells using the hybrid method (refer to section 2.2.3).

mRNA thus obtained was quantified using Nanodrop method (refer to section 2.2.4) and reverse transcripted into cDNA (refer to section 2.2.5).

qPCR analysis performed as described in section 2.2.6.4 and NAD enzyme expression was normalised against h18s, ACTB and UBC (refer to section 2.2.6.1).

4.3 Results

4.3.1 NAD^+ biosynthesis enzyme expression in RA synovial fibroblasts

All enzymes in the NAD^+ biosynthesis pathway were constitutively expressed *in vitro* (with the exception of NMNAT-3).

4.3.2 Induction of NAMPT and IDO by cytokines.

Following stimulation with OSM the expression of NAMPT rapidly increased in the first two hours after which the levels remained steady for up to eight hours when levels were observed to decrease. Only mild increases in expression of NAMPT were observed following stimulation with IFN-γ, IL1-β & TNF-α (Fig 4.4). In contrast, following stimulation with IFN-γ, IDO expression was very rapid and substantial and continued to be upregulated even after 10hrs. (Fig 4.10). IDO also showed significant expression following stimulation with OSM, however, there was no change in IDO expression following stimulation with either TNF-α or IL1-β.

4.3.3 Induction of other NAD enzymes by cytokines

NMNAT-2 was weakly upregulated following stimulation with TNF-α and IL1-β, however, no upregulation was seen with either OSM & IFN-γ (Fig 4.9). In synovial fibroblasts, neither NAPRT, QAPRT, NADSYN nor NMNAT-1 were upregulated following stimulation by OSM, IFN-γ, TNF-α and IL1-β (Fig 4.5 – 4.8)

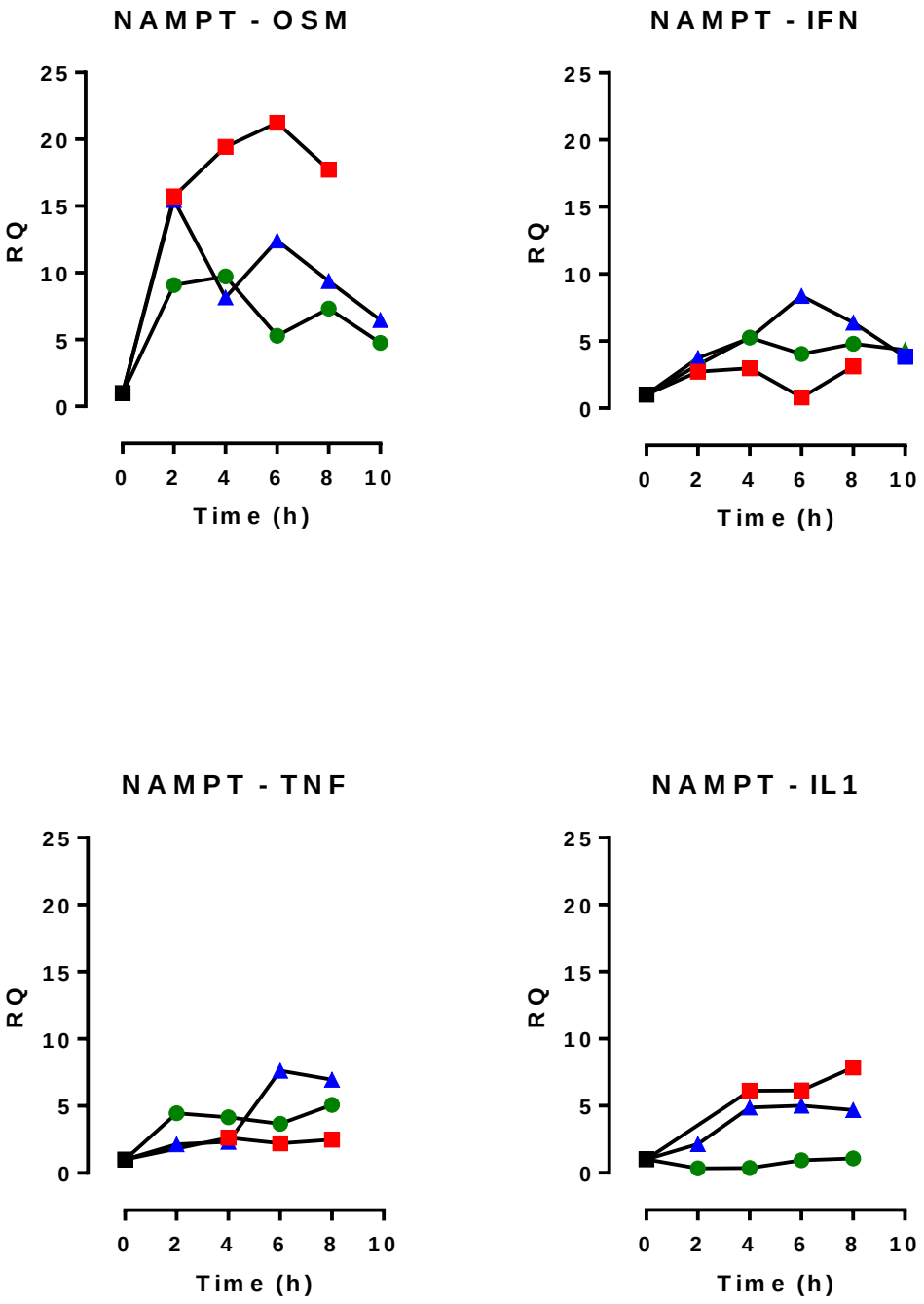


Fig 4.4. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, NAMPT, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

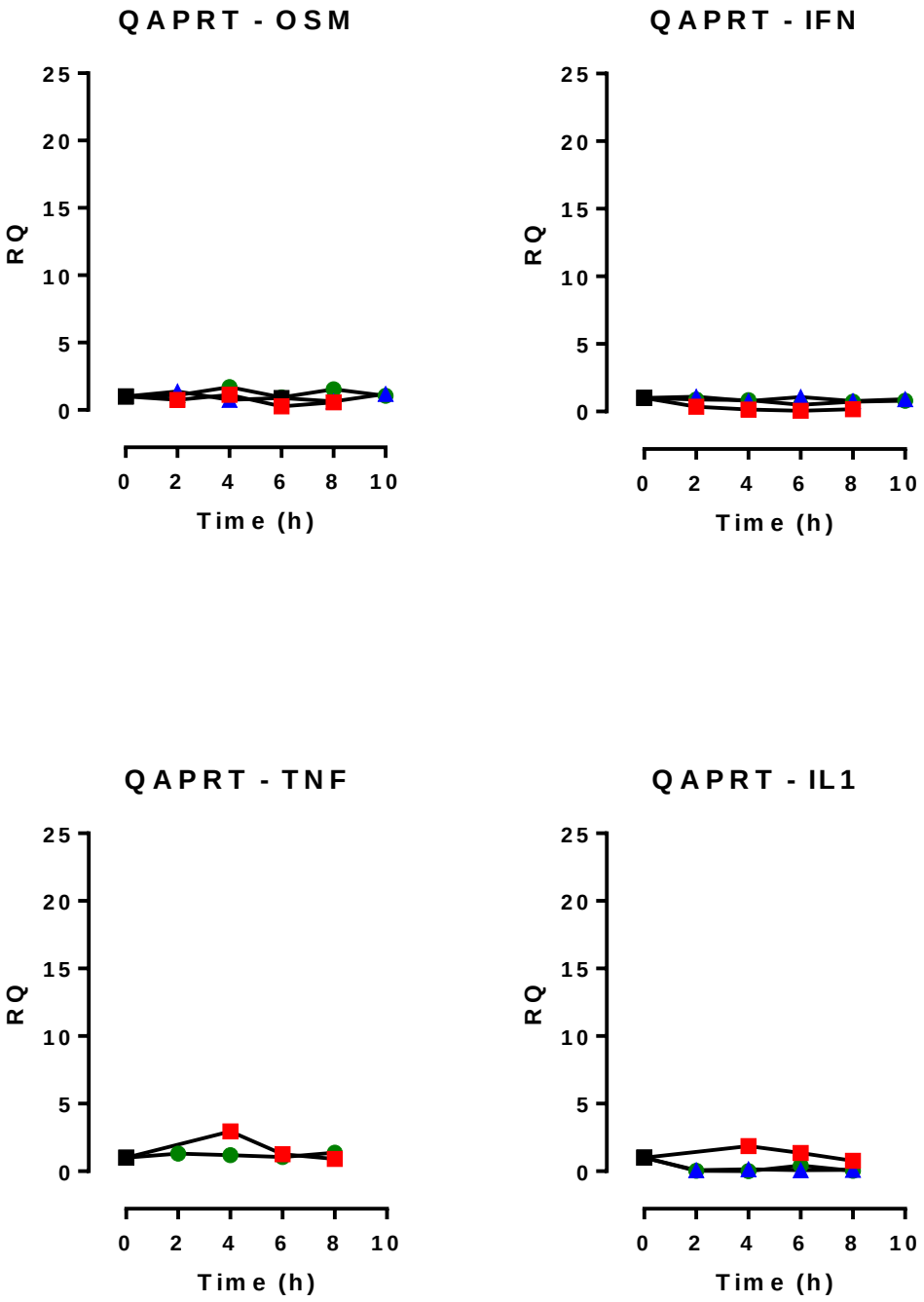


Fig 4.5. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, QAPRT, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

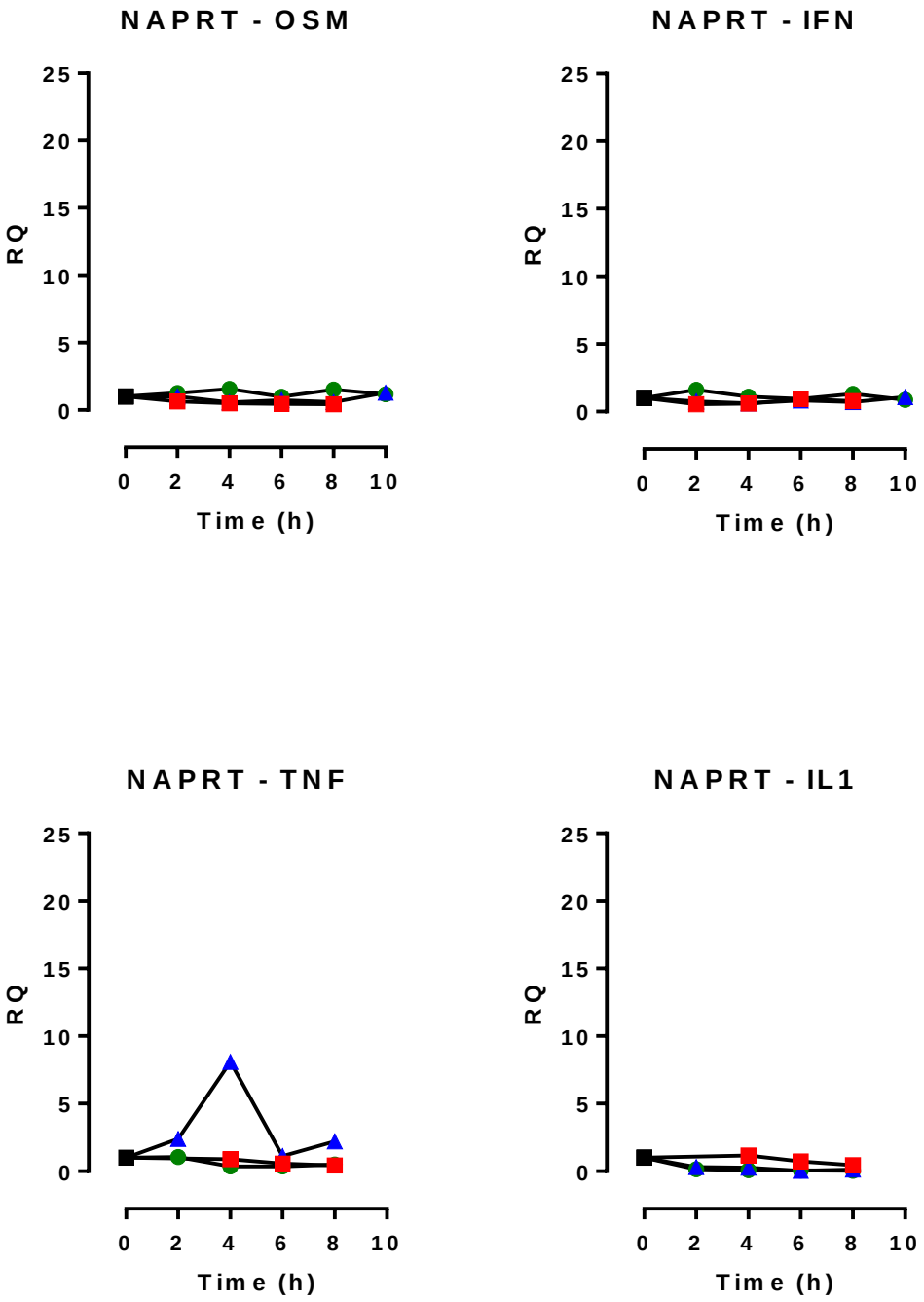


Fig 4.6. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, NAPRT, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

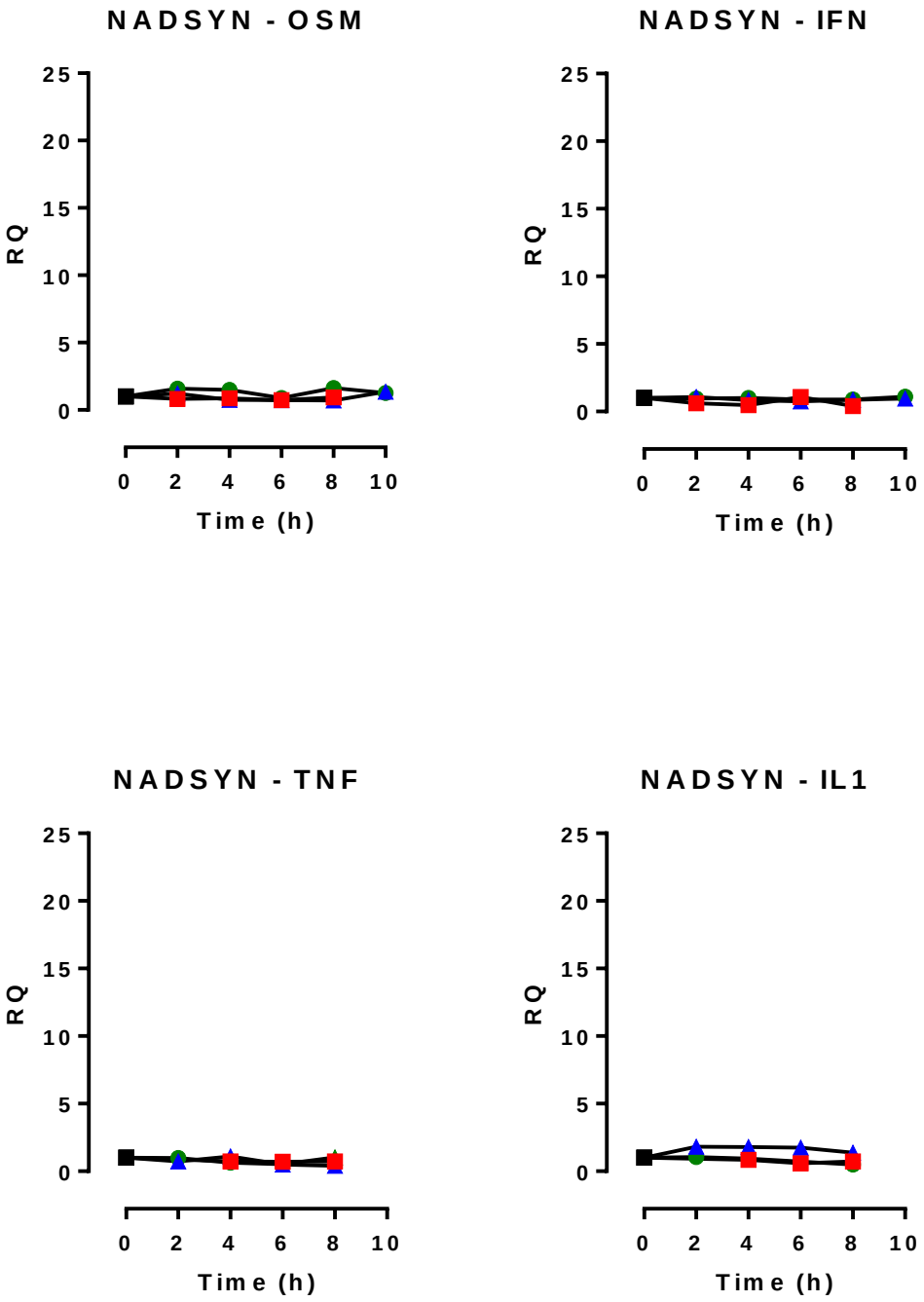


Fig 4.7. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, NADSYN, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

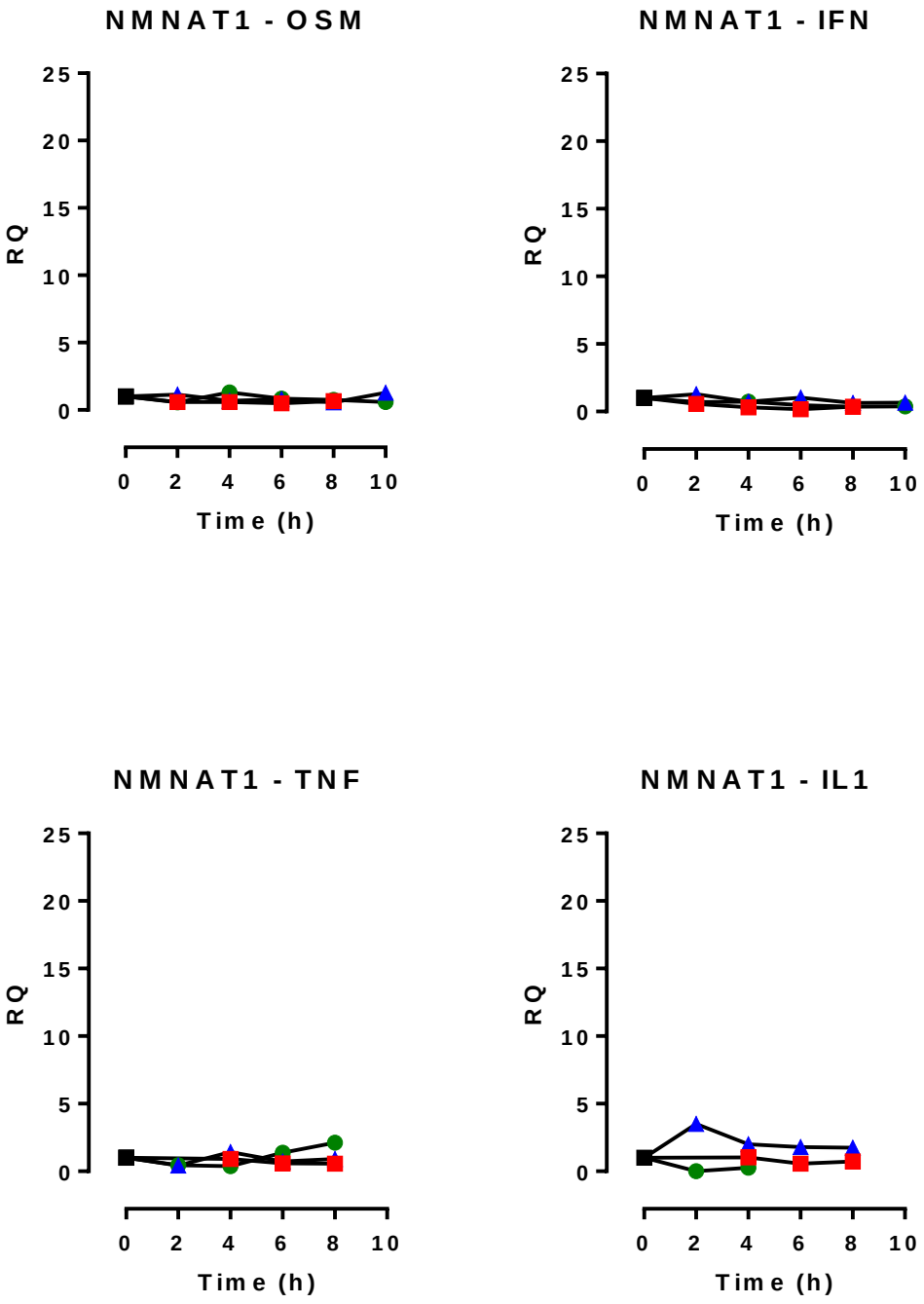


Fig 4.8. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, NMNAT1, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

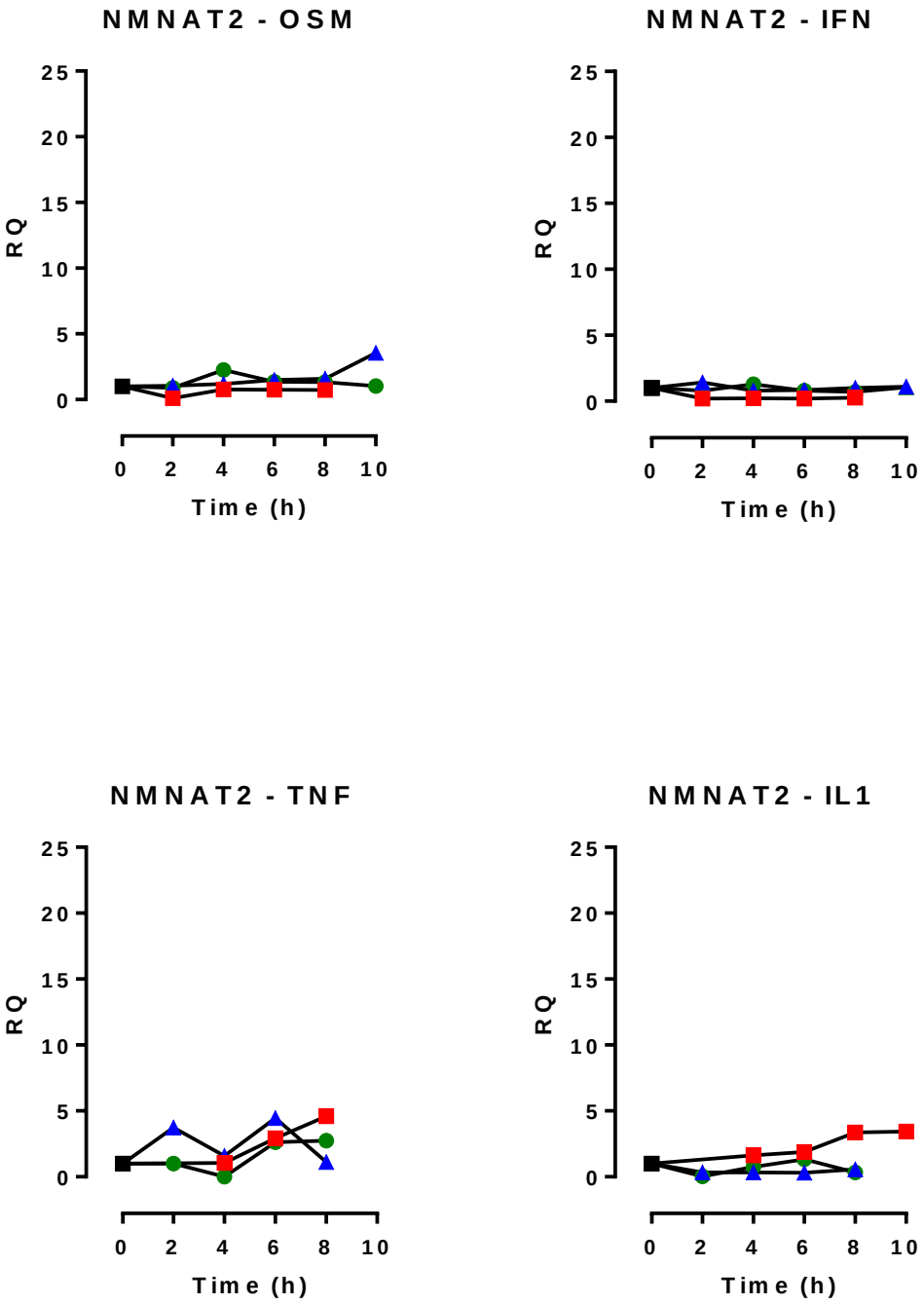


Fig 4.9. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, NMNAT2, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

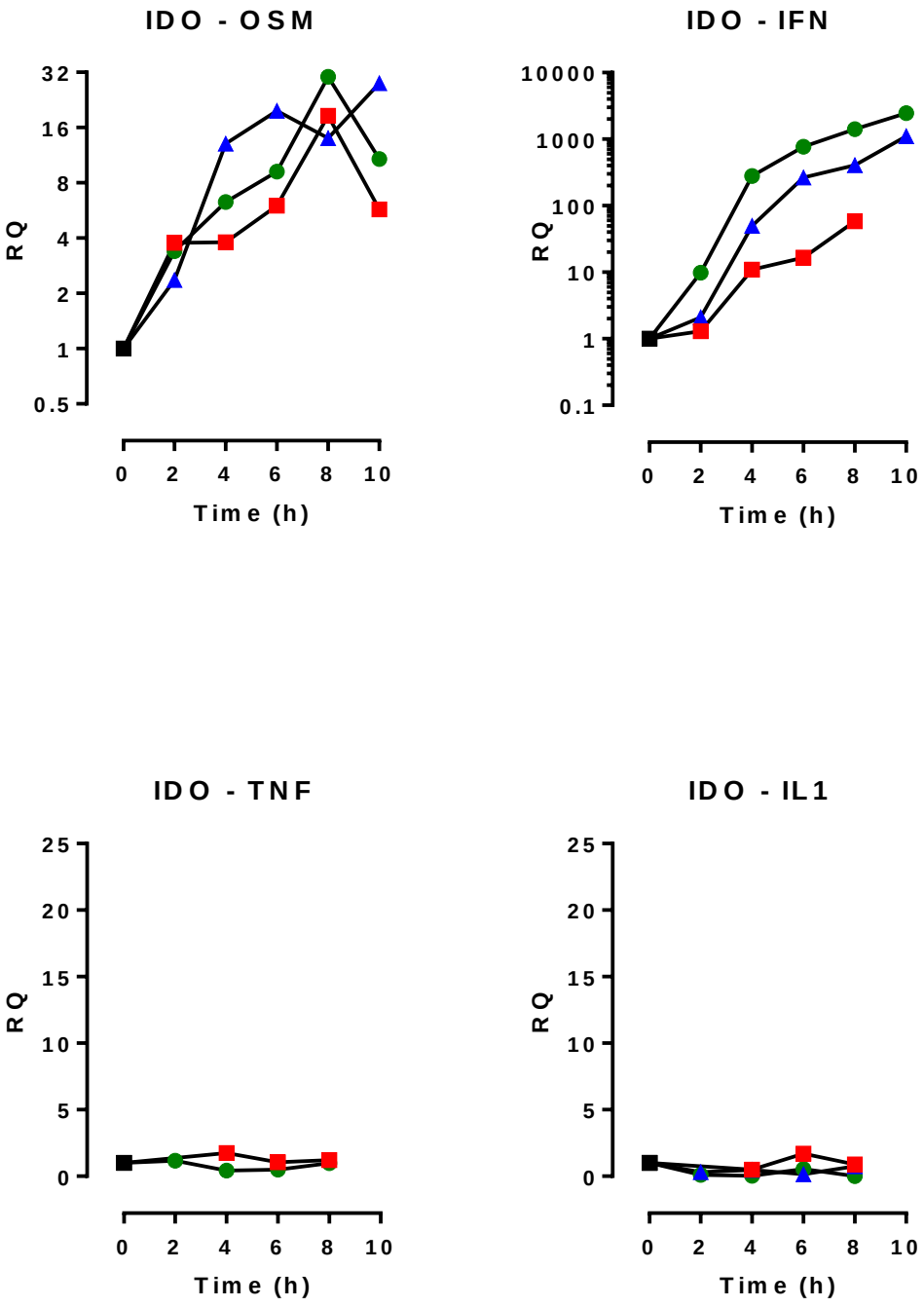


Fig 4.10. In vitro analysis of the relative quantity (RQ) of the NAD^+ biosynthesis enzyme, IDO, in synovial fibroblasts derived from RA patients synovial tissue (n=3 independent patients). Serum starved cells were stimulated with 10ng/ml of OSM and IFNγ (STAT activating cytokines) and 10ng/ml of TNFα and IL1β. RQ was calculated using 18s, UBC and ACTB as the reference genes.

4.4 Discussion

In this study it was found that in synovial fibroblast monolayer culture, the NAD^+ enzymes were not all regulated by the cytokines tested in the same manner; NAMPT was upregulated by all four (OSM, IFN-γ, TNF-α and IL-1β) cytokines; IDO was upregulated by both IFN-γ and OSM; NMNAT-2 was upregulated (albeit very weakly) by TNF-α & IL-1β; QAPRT, NAPRT, NMNAT-1, -3 and NADSYN expression was unaffected by any of the cytokines tested *in vitro*.

4.4.1 NAMPT regulation by cytokines in synovial fibroblasts

The NAMPT gene has large promoter region that contains binding sites for NF-κB, STAT-1 & STAT-3 (Ognjanovic et al. 2001; Nowell et al. 2006; Luk et al. 2008). In synovial fibroblasts, NAMPT has been shown to be upregulated *in vitro* by IL-6/sIL-6R and OSM - two STAT-activating cytokines (Nowell et al. 2006). The observation of OSM-induced NAMPT expression in synovial fibroblasts is not new (Nowell et al. 2006) and Brentano *et al* (2007) have also demonstrated increased expression of NAMPT following stimulation of synovial fibroblasts with IL-1β and TNF-α. Studies have also shown NAMPT to be highly expressed following stimulation by TNF-α, IL-1β and IL-6 in various cell lines (table 4.1).

Cytokines	Cell Types
TNF-α	Neutrophils, peripheral blood monocytes, adipose tissue, amniotic epithelial cell line, activated immune cells such as monocytes, macrophages, dendritic cells, T cells and B cells
IL-1β	Neutrophils, peripheral blood monocytes, human gingival fibroblasts, amniotic epithelial cell line, activated immune cells such as monocytes, macrophages, dendritic cells, T cells and B cells.
IL-6	Amniotic epithelial cell line, activated immune cells such as monocytes, macrophages, dendritic cells, T cells and B cells

Table 4.1. Upregulation of NAMPT in different cell lines by TNF-α, IL-1β a and IL-6 (Ognjanovic et al. 2001; Rongvaux et al. 2002; Jia et al. 2004; Iqbal and Zaidi 2006; Hector et al. 2007; Busso et al. 2008; Damanaki et al. 2014).

In the previous chapter NAMPT was found to be significantly elevated in RA synovial tissue when compared to normal healthy subjects and when patients who were on anti-TNF medications were excluded from the study the expression of NAMPT was found to be statistically significant in RA synovial tissue when compared to OA synovial tissues (refer to section 3.5.2.2). In this study NAMPT was observed to be

upregulated following stimulation with OSM, IFN-γ, TNF-α and IL-1β in RA synovial tissue. As there is an increased expression of TNF-α, IL-1β, and OSM in synovial fluid and tissue of RA patients (refer to section 1.6), these may well contribute to maintaining high levels of NAMPT observed in the RA tissue in the previous chapter.

4.4.2 IDO regulation by cytokines in synovial fibroblasts

IDO has two distinct promoter regions with binding sites for interferon responsive factor (IRF)-1, STAT-1 and NF-κB (see section 4.1.5.2). IDO was found to be upregulated by IFN-γ & OSM in this study, suggesting strong regulation by STATs. Although IDO has a binding site for NF-κB, the results from this chapter showed no increased expression of IDO following stimulation with either TNF-α or IL-1β in synovial fibroblasts. A study by Park *et al* (2011) showed similar findings where the IDO expression remained unchanged in RA synovial fibroblasts following stimulation with TNF-α. This is also similar to other studies in macrophage and monocytes which indicate no upregulation of IDO following stimulation with TNF and IL-1 alone. Interestingly in these cells, IDO expression is increased when TNF-α & IL1-β are co-stimulated with IFN-γ (see section 4.1.5.2), however co-stimulatory experiments of this kind were not carried out during the course of this study.

4.4.3 NMNAT-2 regulation by cytokines in synovial fibroblasts

In this study, it appeared that NMNAT-2 was weakly upregulated by TNF-α & IL-1β but not by IFN-γ or OSM. However, the results are not conclusive and warrant further investigation.

Interestingly, there are presently no studies in the literature that indicate that NMNAT-2 is upregulated by *any* cytokines.

Human NMNAT-2 has two P53 response elements in its promoter region and it is inducible by P53 (Pan et al. 2014). Furthermore, NMNAT-2 and P53 are positively correlated in colorectal cancer cells (Cui et al. 2016). P53 is overexpressed in RA synovial tissue and in RA synovial fibroblasts (Firestein et al. 1996; Tak et al. 1999). Hence, it is plausible that overexpression of NMNAT-2 observed in synovial tissue described in the previous chapter (refer to section 3.5.2) could be P53 dependant.

NMNAT-2 is also inducible by heat shock factor and hypoxia inducible factor (HIP) 1α (Ali et al. 2011). As there is also enhanced expression of heat shock protein and HIP-1α in RA synovial tissue (Schett et al. 1998; Roelofs et al. 2006; Huang et al. 2009) expression of NMNAT-2 may therefore be HIP1a and/or Heat shock dependent.

4.4.4 QAPRT regulation by cytokines in synovial fibroblasts

In the previous chapter QAPRT was found to be elevated in RA synovial tissue compared to synovial tissue in OA and normal healthy subjects. However, this study showed no evidence of induction of QAPRT in RA synovial tissue by stimulation with neither OSM, IFN-γ, TNF-α nor IL-1β.

Other studies have indicated that QAPRT can be induced by quinolinic acid (Foster et al. 1985). Therefore, it is possible that the increased IDO seen in this study (refer to section 3.5.2) could lead to increased quinolinic acid in the RA synovial tissue which may impact the expression of QAPRT *in vivo*. This hypothesis could easily be addressed *in vitro* in follow-on studies.

Studies involving human dermal fibroblasts have also shown that QAPRT expression is unchanged when stimulated by IFN-γ or TNF-α alone but, in combination, QAPRT has been found to be downregulated in these cells (Asp et al. 2011). Again, the effect on co-stimulation could be performed in follow-on studies.

4.4.5 NADSYN regulation by cytokines in synovial fibroblasts

NADSYN was found to be upregulated in RA synovial tissue compared to OA and healthy synovial tissue (refer to section 3.5.2) however, *in vitro* analysis was unable to demonstrate any increase in expression of NADSYN following stimulation with OSM, IFN-γ, TNF-α and IL-1β. There are currently no studies in the literature which have characterised the regulation of NADSYN. Oxidative stress, metabolites, heat stress factors, hypoxia inducible factors are all good candidates and further studies would be required to evaluate NADSYN induction.

4.4.6 Other NAD enzyme regulation by cytokines in synovial fibroblasts

Similar to the expression observed in synovial tissue described in the previous chapter, NMNAT-3 was not detected in synovial fibroblasts.

In this study there were no observed changes in the expression of NMNAT-1 and NAPRT following stimulation with neither TNF-α, IL-1β, IFN-γ nor OSM.

The previous chapter showed that neither NMNAT-1 nor NAPRT were differentially regulated in RA synovial tissue compared to normal synovium (refer to section 3.5.2) and these studies showed no induction by OSM, IFN-γ, TNF-α and IL-1β.

4.5 Summary

In this chapter the induction of NAD^+ biosynthesis enzymes following stimulation with OSM, IFN-γ, TNF-α and IL-1β was characterised in human RA synovial fibroblasts.

The main findings were

- NMNAT-3 was not detected *in vitro* in synovial fibroblasts.
- NAMPT was upregulated *in vitro* following stimulation with OSM, IFN-γ, TNF-α and IL-1β with the highest expression of NAMPT seen following stimulation with OSM.
- IDO was upregulated *in vitro* following stimulation with IFN-γ and OSM, but not TNF-α and IL1-β, with exponential increases seen in synovial fibroblasts upon IFN-γ stimulation
- NMNAT-2 was weakly upregulated *in vitro* following stimulation with TNF-α and IL-1β but not by OSM and IFN-γ.
- QAPRT, NAPRT and NADSYN were detected in synovial fibroblasts *in vitro*, but showed no change in expression following stimulation with OSM, IFN-γ, TNF-α and IL-1β.

Chapter 5 – General Discussion and conclusions

Rheumatoid arthritis is a chronic inflammatory condition of the synovial joint affecting around 1% of the adult population (Gabriel 2001) with substantial economic burden for patients and health services (Barrett et al. 2000; Young et al. 2002). It is well established that one drug doesn't fit all patients, and identification of new and more cost-effective therapies, as well as characterizing patients to target for personalized treatment is actively being pursued.

NAMPT is a rate limiting enzyme in the synthesis of NAD^+ via salvage pathway. It is known that NAMPT is elevated in synovial tissue in RA and its involvement in the pathogenesis of RA could possibly be due to either by directly acting as an inflammatory cytokine (eNAMPT) or by maintaining a high NAD^+ levels in the inflamed cells (iNAMPT). It is also known that NAMPT is regulated by inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α and the synthesis of TNF-α and IL-6 in monocytes (when stimulated by lipopolysaccharide *in vitro*) relies on intracellular NAD^+ levels produced via the NAD salvage pathway (Van Gool et al. 2009).

IDO is known to be upregulated in RA (Zhu et al. 2006; Park et al. 2011), however, the consequence of its upregulation is debated in the literature. Some authors believe IDO to be anti-inflammatory where as others believe IDO to be involved in the pathogenesis of inflammatory arthritis (refer to section 3.6.4).

However, it was not known, prior to this thesis, whether the expression of some of the other NAD^+ biosynthesis enzymes (NMNATs, NAPRT, QAPRT and NADSYN) differed in their expression in rheumatoid arthritis tissue and if they were sensitive to cytokine induction *in vitro*.

In this study, in order to gain insight into the regulation of NAD^+ biosynthesis enzymes in synovial tissue, the expression of the principle enzymes involved in NAD^+ biosynthesis were characterised by quantifying the mRNA expression using qPCR *ex vivo* in RA, OA and normal 'healthy' synovial tissues; and *in vitro* following cytokine (OSM, IFN-γ, TNF-α and IL1-β) induction of RA synovial fibroblasts.

5.1. The principles finding in this study were:

- All the NAD^+ biosynthesis enzymes investigated in this study were expressed *ex vivo* in rheumatoid synovial tissue and *in vitro* in synovial fibroblasts except NMNAT-3.
- NAMPT is highly expressed in pre-pubertal actively growing normal healthy synovial tissue, a characteristic that appears to be unique to NAMPT.
- The expression of the enzymes involved in the NAD^+ salvage pathway investigated in this study, namely NAMPT and NMNAT-2 are significantly elevated in RA synovial tissue when compared to normal, healthy synovial tissue and their expression significantly correlate with each other.
- There was no statistical significant difference in the expression of enzymes in the salvage pathway between RA and OA. However, when patients with RA receiving anti-TNF treatment for their arthritis were excluded from analyses, NAMPT expression was statistically significantly elevated in RA synovial tissue compared to tissue from patients with OA. However, there was no change in the expression of rest of the NAD^+ biosynthesis enzymes that were investigated following exclusion of these patients.
- NAMPT is upregulated following stimulation with OSM, IFN-γ, TNF-α and IL-1β and NMNAT-2 was weakly upregulated following stimulation by TNF-α and IL-1β but not by OSM or IFN-γ.
- The expression of enzymes involved with the *de novo* NAD^+ pathway investigated in this study, namely, QAPRT, NMNAT-2, NADSYN and IDO, were elevated in RA synovial tissue when compared to normal healthy synovial tissue and significantly correlate with each other in synovial tissue.
- IDO was upregulated following stimulation with IFN-γ and OSM with exponential increase seen with IFN-γ and NMNAT-2 was weakly upregulated by TNF-α and IL-1β, however, QAPRT and NADSYN showed no change in expression following stimulation with OSM, IFN-γ, TNF-α and IL-1β.
- Of the expression of enzymes involved with the Preiss-Handler NAD pathway, namely, NAPRT, NADSYN and NMNAT1-3, only NADSYN and NMNAT-2 were significantly elevated in RA synovial tissue compared to normal. Neither NAPRT nor NMNAT-1 were significantly elevated in RA synovial tissue and did not correlate with any of the other enzymes investigated in this study.

5.2. NAD^+ Biosynthesis enzymes via salvage pathway

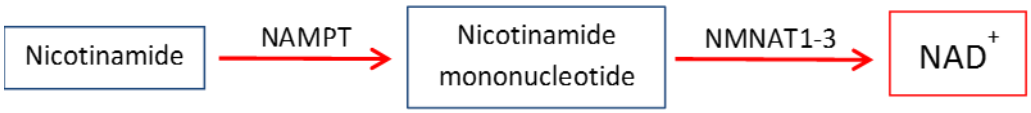


Fig 5.1. NAD^+ synthesis via the salvage pathway. NAMPT – nicotinamide phosphoribosyl transferase, NMNAT – nicotinamide mono nucleotide adenyl transferase, NAD – nicotinamide adenine dinucleotide.

In this study, the expression of NAMPT was found to be high in synovial tissue of young actively-developing healthy subjects, which gradually decrease over time with age. This finding is quite unique to this study as no other studies have described this type of finding before. It's possible that the overexpression seen could be related to the differentiation and maturation state of the synovial tissue as similar upregulation of NAMPT has been observed during maturation of smooth muscle cells (SMC) (van der Veer et al. 2005). This warrants further study to understand the relevance of NAD^+ in developing tissues as this may have profound implication in future therapies targeting NAD/NAMPT inhibition in Juvenile idiopathic arthritis as this may impair their growth and maturation.

In this study both salvage pathway NAD^+ enzymes, NAMPT and NMNAT-2, were elevated in RA synovial tissue when compared to normal and the expression of two enzymes significantly correlated with each other. NMNAT-3 could not be detected in synovial tissue and there was no increased expression of NMNAT-1 in the RA synovial tissue when compared to OA and normal healthy synovium.

The relationship between NAMPT and NMNAT-2 is interesting, as any increases in intracellular NAMPT would be associated with an increase in NMNAT to have an impact on intracellular NAD^+ (Fig 5.1). It would be of interest to further investigate this association by investigating the impact of increased NAMPT / NMNAT-2 levels and NAD^+ concentration in both *in vitro* and *ex vivo studies*. It would also be of interest to assess the correlation between cytokines and NAD^+ biosynthesis enzymes in RA synovial tissues.

In vitro studies have shown that TNF-α upregulates the expression of NAMPT. Interestingly when some of the patients in the study receiving anti-TNF medications were eliminated from analyses, NAMPT expression in RA synovial tissue was significantly elevated when compared to OA and normal synovial tissue. However, NMNAT-2 is weakly upregulated by TNF-α and eliminating patients who were receiving anti-TNF made no difference to the expression of NMNAT-2 in RA synovial

tissue when compared to OA. In the literature so far there have been no studies which investigated the correlation of cytokine expression in serum or synovial tissue with NAD^+ biosynthesis enzyme expression and this warrants further investigation.

The evidence presented in Chapter 4 clearly show NAMPT and NMNAT-2 are regulated differently in synovial fibroblasts. The NAMPT gene has a large promoter region that contains binding sites for NF-κB, STAT-1 & STAT-3 (Ognjanovic et al. 2001; Nowell et al. 2006; Luk et al. 2008), whereas NMNAT-2 is inducible by P53, heat shock factor and hypoxia inducible factor 1α (Ali et al. 2011; Pan et al. 2014). In RA there is increased expression of TNF-α, IL-1β, and OSM in the synovial fluid and synovial tissue and these may well contribute to maintaining high levels of NAMPT observed in the RA tissue. There is also evidence of increased expression of P53, heat shock protein and HIP-1α in RA synovial tissue (Firestein et al. 1996; Schett et al. 1998; Tak et al. 1999; Roelofs et al. 2006; Huang et al. 2009) hence, it is plausible that overexpression of NMNAT-2 observed in RA synovial tissue in this study could be dependent on these factors and could easily be investigated in future studies.

Finally, NAMPT expression in RA synovial tissue and serum correlates with c-reactive protein (CRP) and disease activity (Brentano et al. 2007) and its expression in serum is associated with increased levels of radiographic joint damage in RA (Rho et al. 2009). Therefore, it would be interesting to investigate if increasing NAD^+ concentration in serum or synovial tissue is associated with increased disease activity as measured by CRP and disease activity score in 28 joints.

5.3. NAD^+ Biosynthesis enzymes via *de novo* pathway

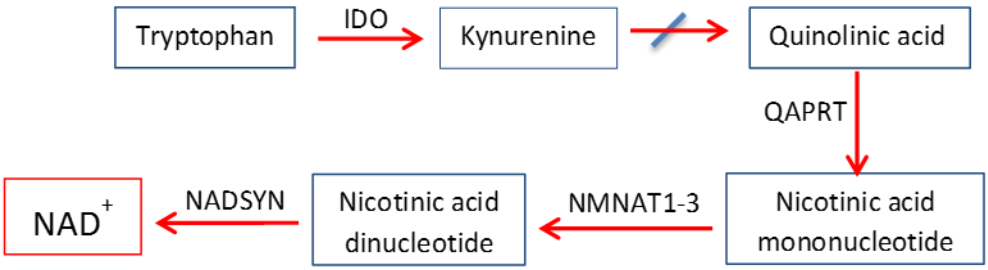


Fig 5.2. NAD^+ biosynthesis from Tryptophan via the *de nova* pathway. IDO – indole amine 2,3-dioxygenase, QaPRT – quinolinic acid phosphoribosyl transferase, NMNAT – nicotinamide mono nucleotide adenyl transferase, NADSYN – NAD synthetase.

In this study it was found that there was increased expression of NAD^+ biosynthesis enzymes of the *de novo* pathway such as IDO, NMNAT-2 and NADSYN in RA synovial tissue when compared to normal. QAPRT was also found to be

overexpressed in RA synovial tissue when compared to normal, however, it was not found to be statistically significant and it's possible that the sample size was not large enough to find a meaningful difference. Expressions of all these enzymes in *de novo* pathway investigated in this study correlated with each other. Their related expressions may reflect their required *in tandem* expression for controlled NAD^+ availability via this pathway. It is plausible that the increased expression of enzymes involved in the *de novo* pathway in the RA synovial tissue found in this study may replenish the NAD^+ used up in the actively proliferating tissue such as that seen in RA. Further investigation would be required to prove this hypothesis.

In this study, IDO was found to be upregulated following stimulation with IFN-γ and OSM. Although IDO has a binding site for NF-κB, the results from chapter 4 showed no increased expression of IDO following stimulation with either TNF-α or IL-1β in RA synovial fibroblasts. Interestingly, IDO is upregulated when cells are co-stimulated with TNF-α or IL-1β in the presence of IFN-γ (Babcock and Carlin 2000; Robinson et al. 2003; Fujigaki et al. 2006). Co-stimulatory experiments of this kind were not carried out during the course of this study and would be of interest for future studies.

Although QAPRT and NADSYN were overexpressed in RA synovial tissue they could not induced in RA synovial fibroblasts *in vitro* following stimulation with inflammatory cytokines. QAPRT is inducible by quinolinic acid (Foster et al. 1985), therefore, it is possible that the increased IDO seen in this study could lead to increased quinolinic acid in the RA synovial tissue which may impact the expression of QAPRT *in vivo*. This hypothesis could easily be addressed in *in vitro* follow-on studies. Sahm *et al* (2013) have demonstrated that QAPRT expression is increased when there is depletion of NAD^+ in microglial cells by NAMPT inhibition, irradiation and alkylating agents . This finding further endorses the hypothesis that increased expression of *de novo* enzymes seen in RA synovial tissue is required for increased requirement for NAD^+ in this tissue.

The correlation of expression of enzymes of the *de novo* pathway with NAMPT outlined in chapter 3, suggests that factors that are regulate NAMPT expression may also regulate the expression of IDO, QAPRT, NMNAT-2 and NADSYN in a similar manner. Therefore, there are a number of factors other than the inflammatory cytokines used in this study which may be involved in the regulation of NAD^+ biosynthesis enzymes such as oxidative stress, heat, and hypoxia inducible factors and this warrants further investigation.

Unfortunately, there are currently no studies in the literature that characterise the regulation of NADSYN, which could be used to explain the observed increase in NADSYN expression seen in RA synovial tissue. Again it is possible that factors such as oxidative stress, heat, and hypoxia inducible factors, may have a role to play in its regulation and further studies could reveal the mechanism of induction.

5.4. NAD^+ biosynthesis enzyme via Preiss-Handler pathway

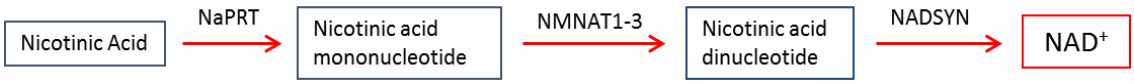


Fig 3.9. Synthesis of NAD^+ from nicotinic acid via Preiss-Handler pathway. NAPRT – nicotinic acid phosphoribosyl transferase, NMNAT – nicotinamide mono nucleotide adenyl transferase, NADSYN – NAD synthetase.

In this study it was found that there was very low expression of NAPRT in RA, OA and normal synovial tissue. It was also observed that there was no change in the expression of NAPRT following stimulation by TNF-α, IL-1β, IFN-γ or OSM and the expression of NAPRT do not correlate with the expression of other NAD^+ biosynthesis enzymes. To date there are no studies in the literature which have characterised the regulation of expression of NAPRT. NAPRT is normally highly expressed in liver, kidney, heart and small intestine with moderate expression in lungs (Hara et al. 2007) and NAPRT is unlikely to have any impact on RA disease pathology because of lack of change seen in RA synovial tissues and synovial fibroblasts.

Nicotinic acid (NA) is known to be a better precursor for NMN in elevating NAD^+ levels than Nam (Hara et al. 2007) (refer to section 3.6.7). Inhibition of NAMPT would affect both normal and highly proliferative tissues such as cancer and RA tissues. However, if the proliferative tissue expresses low NAPRT levels, addition of nicotinic acid along with the NAMPT inhibitor may be able to replenish NAD^+ concentrations via the Preiss Handler pathway in normal healthy tissue but not in proliferative diseased tissue, thus protecting the normal tissue from cytotoxicity of NAMPT inhibitors. Indeed, *in vitro and in vivo* studies have shown that cancers and brain tumours demonstrating low NAPRT expression respond well to NAMPT inhibition whilst normal tissue is rescued from cell death by replenishing intracellular NAD^+ levels through the addition of NA (Watson et al. 2009; Olesen et al. 2010). In this study the expression of NAPRT was low in synovial tissue and the expression of NAMPT was high in RA synovium, thus the protective action of co-administration of NA (i.e. healthy cells are able to replenish

NAD^+ levels via NAPRT) could potentially be a viable option if NAMPT inhibition is used for the treatment of RA.

5.5. Future directions

This study has demonstrated that the NAD^+ biosynthesis enzyme mRNA expression is elevated in the synovial tissue samples in RA patients and some are also elevated following stimulation with cytokines. However, further work is required to investigate if elevated mRNA levels translate to functional enzymes being over expressed in these tissues and ultimately NAD^+ synthesis and its related compounds. Thus it would be interesting to measure the levels of cellular NAD^+ and its related compounds in normal and RA synovial fibroblasts and the impact on the concentration of NAD^+ and NMN in RA synovial fibroblasts upon increasing concentrations of NAMPT to the synovial culture medium. It would also be interesting to investigate the concentration of NAD^+ and its related compounds in synovial fibroblasts following stimulation with cytokines. Previous studies have demonstrated that liquid chromatography/tandem mass spectrometer (LC/MS/MS) employing an electrospray ionisation (ESI) is very sensitive and specific to detect and quantify NAD^+ and related compounds such as NMN, NAMN, NAM or NA (Yamada et al. 2006) and this would be the method of choice for such experiments.

In RA there is increased expression of NAMPT in synovial tissue and its expression is also increased following stimulation with TNF-α, IL-1β, and OSM. Previous studies have shown that expression of NAMPT is increased with increasing dose of TNF-α, IL-1β, and OSM in the synovial tissue (Evans and Nowell; unpublished). Therefore, it would be interesting to investigate the effect of titrating the doses of inflammatory cytokines on the RA synovial fibroblasts and assessing the expression of NAD^+ biosynthesis enzymes with qPCR analysis. Studies have shown that IDO expression was synergistically increased when co-stimulated with a TNF-α or IL-1β in the presence of IFN-γ in monocytes and macrophages (Babcock and Carlin 2000; Robinson et al. 2003; Fujigaki et al. 2006). As there are a number of cytokines which are expressed inside a rheumatoid joint, future studies involving co-stimulating RA synovial fibroblasts with various different cytokines and investigating the NAD biosynthesis enzyme expression would be important.

In this study QAPRT and NADSYN were found to be highly expressed in RA synovial tissue but we were unable to demonstrate their expression following stimulation of RA synovial tissue with inflammatory cytokines. Therefore, it would be interesting to

determine the principle pathways involved in their induction and this could be achieved by exposing the synovial fibroblasts to a combination of cytokines, oxidation, heat and hypoxia inducible factors.

The enzymes involved in the *de novo* pathway of NAD^+ biosynthesis which were tested such as IDO, QAPRT, NMNAT-2 and NADSYN were all elevated and their expression correlated with each other. The increased expression of the enzymes involved in the *de novo* pathway in the RA synovial tissue found in this study is likely to replenish the NAD^+ but could easily be confirmed by using an IDO inhibitor such as 1-methyl-tryptophan, then measuring the concentration of NAD^+ in the cells.

The expression of NAPRT was low in the RA synovial tissue in this study and therefore addition of nicotinic acid is unlikely to replenish NAD^+ via Preiss Handler pathway in the RA synovial tissue. This could be confirmed by adding a NAMPT inhibitor and nicotinic acid to RA synovial fibroblast culture and measuring the concentration of NAD^+ using LC/MS/MS.

Finally, animal experiments could also be performed involving mice with CIA and identifying the maximum dose of NAMPT inhibitors which can be used either alone or in combination with nicotinic acid and assessing whether the combination therapy reduces the cytotoxic effect of NAMPT inhibitors whilst reducing the symptoms associated with CIA. Upon sacrificing the animal, it would be interesting to look at various tissue distribution of NAPRT and also the concentration of NAD^+ in them.

5.6. Conclusions

Taken together, the results presented in this thesis and observations published in the literature suggest that NAD^+ biosynthesis enzymes are upregulated in rheumatoid arthritis and may maintain an activated phenotype by increased NAD^+ availability. Observation in animal models have shown that use of NAMPT inhibitors such as APO866 caused a significant reduction of arthritis in CIA murine model (Evans et al. 2011) and clinical scores can be reduced in a dose dependant manner (Busso et al. 2008).

Although NAMPT inhibitors effectively reduced NAD^+ levels and caused tumour cell death in *in vitro* and *in vivo* studies, clinical trials involving at least three NAMPT inhibitors such as APO866, CHS-828 and GMX1777 have been limited by toxicities such as thrombocytopaenia, gastrointestinal symptoms and skin rash (reviewed by Sampath et al. 2015). Additional toxicities involving retina and heart have been

observed in rodent safety studies involving newer NAMPT inhibitors such as GNE-617. Therefore, alternative strategies for improving the therapeutic index of NAMPT inhibitors need to be explored.

Olesen *et al* (2010) have shown that in tissues which express high NAMPT and low NAPRT, addition of nicotinic acid to a NAMPT inhibitor will protect normal tissues from cytotoxicity of NAMPT inhibitors. Observations within this thesis have shown that RA synovial fibroblasts express low NAPRT with high NAMPT and therefore nicotinic acid could possibly be used to protect normal tissues in the event of a NAMPT inhibitor being used as a potential treatment strategy.

In this thesis it was observed that expression of NAMPT in normal synovial tissues was found to be high in young and actively developing individuals. Any future therapies targeting NAD/NAMPT would therefore need to be carefully considered in younger patients (e.g. in patients with JIA) and these preliminary observations warrants further investigation in a larger cohort of younger patients to understand the relevance of NAD^+ in developing tissues.

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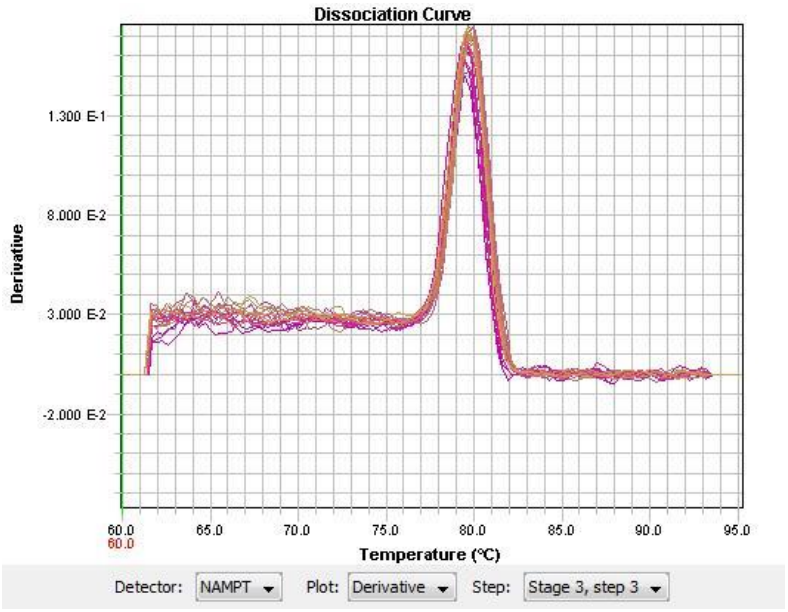
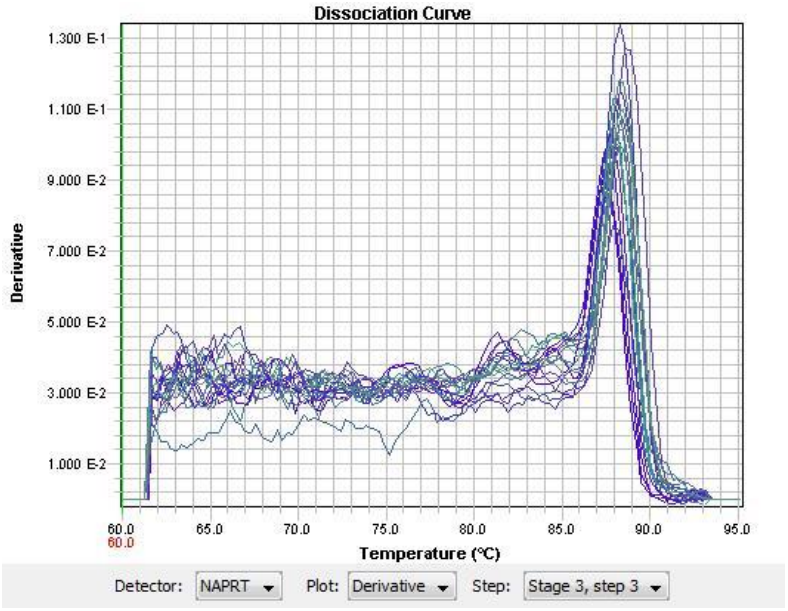
Appendix

Appendix 1a



Appendix 1b



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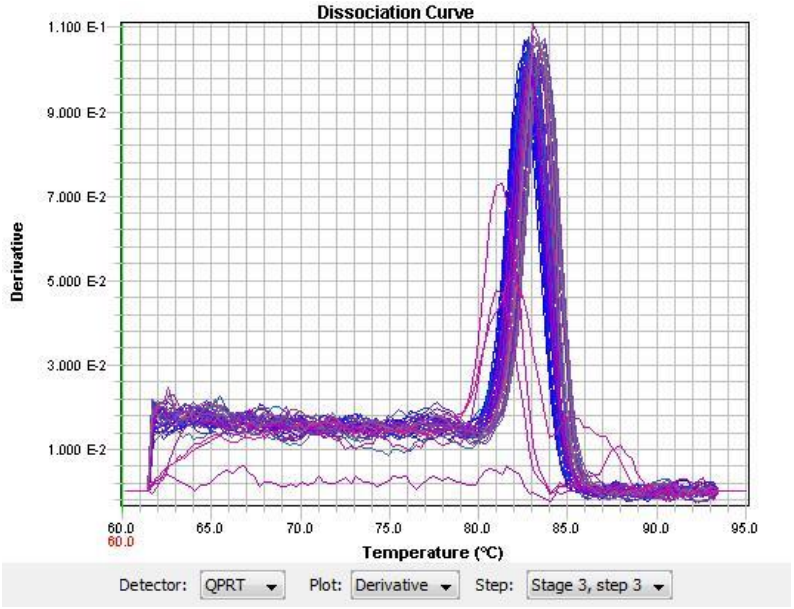

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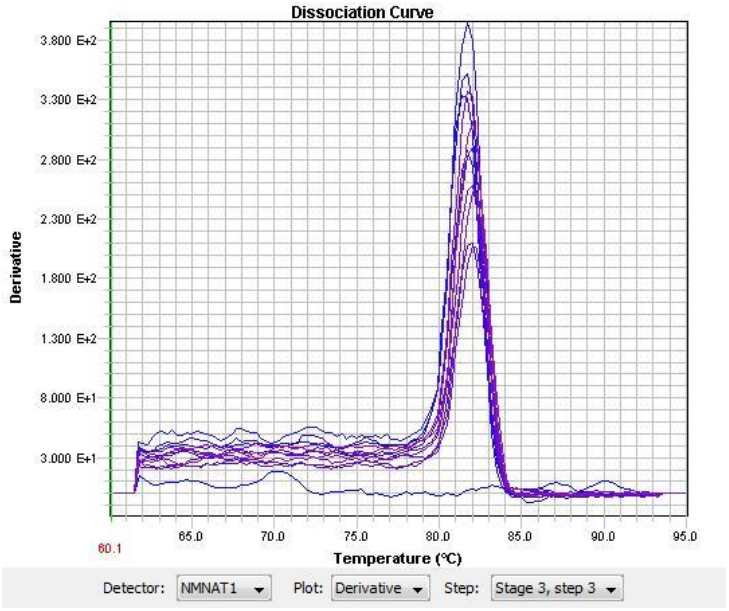
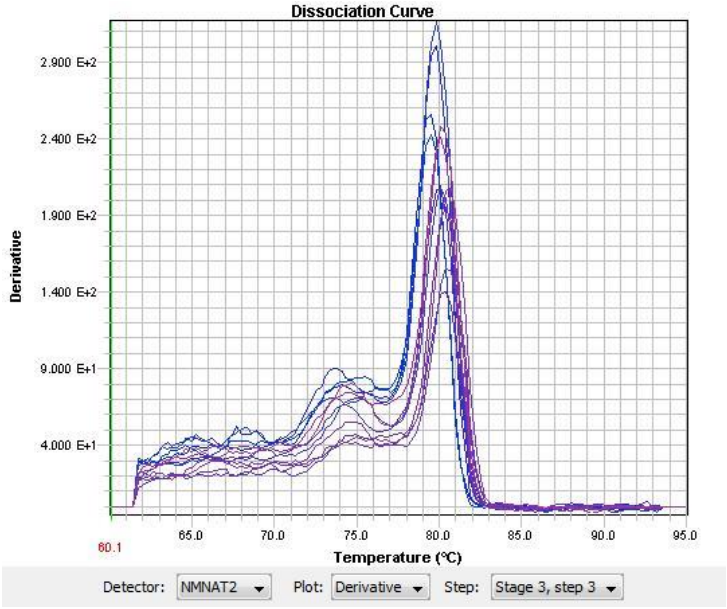


Appendix 1e



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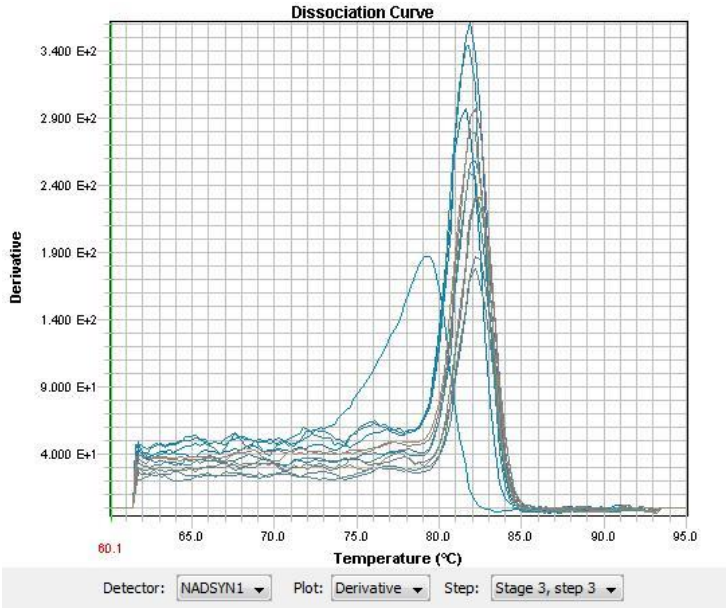
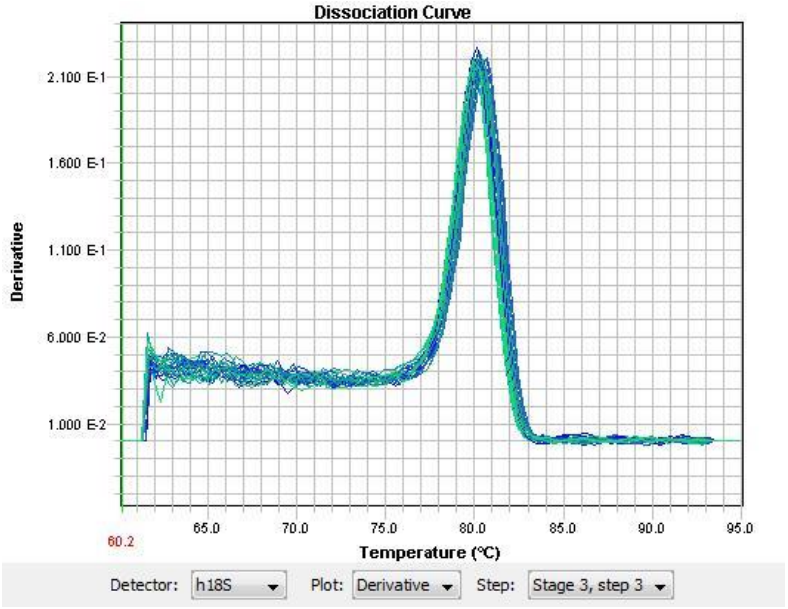
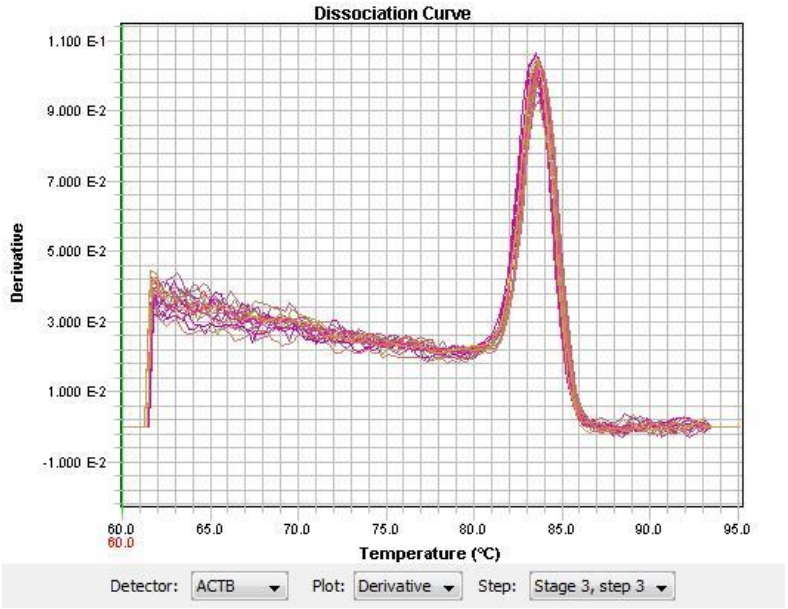


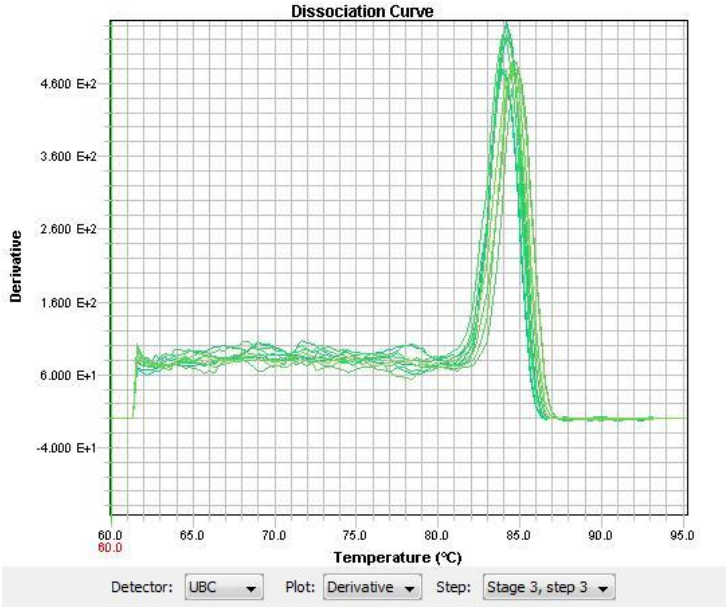
Appendix 1g



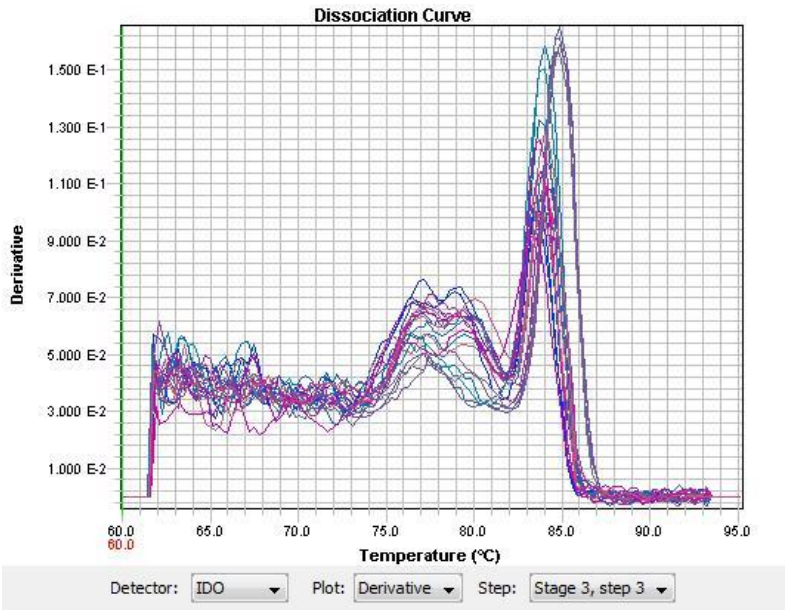
Appendix 1h



Appendix 1i



Appendix 1j



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PATIENT INFORMATION SHEET

The collection, storage and analysis of clinical waste

We would like to invite you to take part in our research studies by donating your clinical waste samples to the Arthritis Research UK Biomechanics and Bioengineering Centre (Arthritis Research UK BBC) at Cardiff University. Before you decide, we would like you to understand the purpose of you donating samples, and what being part of our research would mean for you. Someone from our team will go through the information sheet with you and answer any questions you may have. If English is not your preferred language and you would like this information in another language, please ask and it will be provided, or an interpreter called. You are encouraged to take this document home and discuss your decision to donate tissue for research with friends and family. If you wish to take part, you will be asked to sign a Consent Form. By signing this form, you indicate that you understand this information and you give consent to donate samples as a gift to the Arthritis Research UK BBC for research purposes.

What is the purpose of obtaining my clinical waste?
The aim of analysing samples obtained following Orthopaedic or Rheumatology clinical procedures will help us research into the causes, diagnosis and treatment of joint diseases, such as arthritis. Research with such samples can help us to find out more about what causes joint diseases, how to prevent and treat them. The samples you donate might be used for a number of related research studies associated with Arthritis Research UK BBC.

What is involved in the storage of my samples?
Cardiff University has a Human Tissue Authority (HTA) licence for the storage of human material for research purposes. This allows us under the Human Tissue Act to store human samples under strict and robust guidelines. Arthritis Research UK BBC will be collecting and storing samples of bone, other tissue from joints and synovial fluid in the secure laboratories at Cardiff University. Some of these samples are stored in a special freezer or fridge; others are stored preserved in paraffin wax blocks. The human sample storage facilities at Cardiff University are used to collect tissues for research only.

Why have I been invited?
You have been invited to take part because you are having a clinical procedure which involves the removal of bone, joint tissue or fluid around your joint. These samples would normally be incinerated after the clinical procedure. We are asking your permission to use your samples to study degenerative bone and joint diseases. We

hope that by doing this we can improve the care we provide for patients with degenerative bone and joint conditions in the future.

Do I have to take part?
It is up to you to decide to join the study. We will describe the study and go through the information sheet with you. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?
We will ask your consultant to save the samples removed during your Orthopaedic or Rheumatology clinical procedure for us. Please ensure you have initialled this box if you are happy to do this.

What other types of information do we want?
For our studies we would like to collect information from your medical record, such as your weight, and record details of your condition, such as diagnosis and the results of various tests. We would also like to follow your progress after your procedure by looking at information that your doctor has collected from you during your routine follow-up visits. You will not have to make a special trip or appointment for this. Your doctor may record information such as the medications you are taking and whether your level of pain has changed. We would also like to ask your permission to send you ethically approved surveys or questionnaires to complete. This enables us to collect standardised information about large numbers of tissue donors. They ask questions about lifestyle choices such as "Do you smoke?" or "Do you exercise?" No personal information will be collected without your permission.

How long will my tissue be stored?
Samples collected are usually kept at very cold temperatures, and can be stored this way for a very long time. The tissue that is stored in paraffin wax is stabilised before it is stored in the wax and can be stored at room temperature. Your samples will be stored indefinitely or until it is used up. If you want to withdraw from the study, you can request that your sample is removed and destroyed. However, any research data already obtained from your sample would remain in the study.

What types of research may be done with my tissue?
Many different types of research rely on the use of human samples. They can be used to develop new tests or help diagnose diseases, or can be used to help develop new ways to treat or even cure diseases. Some of the research may lead to new medical products, such as diagnostic tests and drugs, or new procedures.

Who gets to use my tissue?
Access to your tissue and any personal data that may be associated with your tissue is strictly controlled and all members of staff undertaking future studies will bide by the data protection act 1998 with any medical information related to you kept confidential. The sample you donate may be given to other groups within Cardiff University as well as external research collaborators for approved medical research but the samples will not be sold for profit to you or the researchers. Such researchers will only receive your donated samples and when appropriate, information about you (such as your sex, age and the reason for your clinical procedure) from your hospital

record. The researcher will not receive your name, address, phone number or any other personal identifying information. This is done to protect your confidential information.

Why do you need information from my health records?
In order to do research with your samples, researchers may need to know some things about you. For example: Are you male or female? What is your race or ethnic group? How old are you? Have you ever smoked? What medications are you taking? What kind of operation did you have? What joint did you have your operation on? What condition did you have before your operation? Do you have any other bone or joint problems? How much pain were you in before you had your operation? This helps the researcher answer questions about diseases.

The information that may be given to the researcher includes, but is not restricted to: your age, sex, race, medical history, diagnosis, treatments and possibly some medical history. This information will be collected from your health record by the study staff. They may also look at your medical record in the future in order to update your personal health information. Neither your name nor any other identifying information will be shared with researchers.

Will anything go into my health record relating to this research?
Sometimes a note may be placed in your medical records that says you took part in this research study. This is so we can be informed if you are visiting clinic or having any other Orthopaedic / Rheumatology related procedures. No research results will be placed in your medical record. Research results will not be shared with your doctor, you or your family members or used for your medical care.

What are the possible disadvantages and risks of taking part?
Samples will be collected during your routine clinical procedure and therefore there are no obvious disadvantages to taking part in the study.

Will I benefit from the research using my tissue?
There will be no direct benefit to you because your sample will not be used for your immediate medical care. However, it is hoped that the results of the research on your tissue and tissues from other patients will provide information that will help patients with arthritis in the future.

What if something goes wrong?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have grounds for concerns about any aspect of the way your have been approached or treated during the course of this study the normal National Health complaints procedure is available to you. The Complaints Officer can be contacted on (029) 20746296.

Will my taking part in the tissue bank be kept confidential?
All information that is collected about you during the course of the research will be kept strictly confidential. No information about you will leave the hospital with your name and address on it so you cannot be recognised.

Will my GP be informed of my involvement in this study?
With your permission, we may send a letter to your GP informing him or her of your participation in this study. If we would like to invite you to take part in any future studies, we may contact your GP first to ensure it is appropriate for us to do so. We will ask your permission to do this on your consent form.

What will happen if I don't want to carry on being a part of the study?
You can decide to withdraw from the study at any point. If you want to withdraw you can contact the research team and make that request and the samples and information we have obtained can be withdrawn and destroyed. However, any research data already obtained from your sample would remain in the study.

What will happen to the results of the research using samples I have donated?
The results from these studies will be submitted for presentation at scientific conferences and publication in scientific journals. You will not be identified in any presentation or publication.

Who is organising and funding the research?
The tissue storage is organised and administered by the Arthritis Research UK Biomechanics and Bioengineering Centre (Arthritis Research UK BBC) at Cardiff University under the HTA licence. The Centre is funded by Arthritis Research UK and Cardiff University.

Who has reviewed the study?
This study has been reviewed by the Research Ethics Committee (REC) for Wales.

What if I wish to lodge a complaint?
If you wish to make a minor complaint regarding the way you were approached or treated during the trial, please contact the Arthritis Research UK Biomechanics and Bioengineering Centre Research Coordinator at the contact details below or you can contact the Cardiff University Research Governance Team on 029 208 79277.

Contact for further information
Research Coordinator
Arthritis Research UK Biomechanics and Bioengineering Centre
Cardiff School of Biosciences
Cardiff University
Cardiff
CF10 3AX
Tel: 029 2087 5419
Email: Robertshc@cf.ac.uk or Longmanaj@cf.ac.uk

Thank you for reading this information sheet.
If you agree to take part you will be given a copy of the information sheet and a signed consent form to keep.

Helen Roberts / Dr Andrea Longman
search Coordinators
hritis Research UK Biomechanics and Bioengineering
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medical Sciences Building
diff University
seum Avenue
diff
10 3AX
: 029 20875419

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

PATIENT CONSENT FORM

The collection, storage and analysis of clinical waste

Study Number:
Patient Identification Number for this trial:

You DO NOT have to sign this document. Please DO NOT sign this document unless you fully understand it. If there is ANYTHING which you do not understand please do not hesitate to ask for a full explanation.

Please initial EACH box

1. I confirm that I have read and understand the information sheet dated 11/03/2011 (Version 3) for the above study and have had the opportunity to ask questions ☐

2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐

3. I agree to donate my samples from my Orthopaedic or Rheumatology clinical procedure as a "gift" to the Arthritis Research UK BBC at Cardiff University according to the conditions in the information sheet. ☐

4. I understand that researchers from other institutions may access my samples, that research may take many years and the information gained will not benefit me or my family directly. ☐

5. You may contact me in the future to take part in other research projects and surveys ☐

6. I agree to my hospital number being used to track my tissue on your secure system. ☐

7. I agree to my GP being informed of my participation in the study and for my GP to be contacted prior to me taking part in any future studies ☐

8. I agree to take part in the above study. ☐

Name of Patient: _____

Signature: _____ Date: _____

I confirm that I have fully explained the experimental protocol and purpose of the study

Name of Researcher: _____

Signature: _____ Date: _____

Name of person taking consent: _____
(If different from researcher)

Signature: _____ Date: _____

1 copy for the patient; 1 copy for the researcher