From DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

IMMUNOGENICITY OF BIOPHARMACEUTICALS IN CHRONIC INFLAMMATORY DISEASES

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Stockholm 2018

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Published by Karolinska Institutet.
Printed by E-print AB 2018

ISBN 978-91-7831-003-6

Immunogenicity of Biopharmaceuticals in Chronic Inflammatory diseases

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i **CMM lecture hall**, L8:00, Karolinska Universitetssjukhuset, Solna

Friday 23rd March 2018, at 9.00

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ABSTRACT

Multiple sclerosis (MS) and rheumatoid arthritis (RA) are chronic inflammatory diseases where genetic and environmental factors influence the pathogenesis. MS is a disease of the central nervous system, while RA primarily affects the joints. Biopharmaceuticals such as interferon beta (IFNβ) and tumor necrosis factor-alpha (TNF-α) inhibitors are widely used treatments to achieve a reduction in disease activity in people with MS and RA respectively. Over time, however, some of the treated patients develop anti-drug antibodies (ADA) or neutralizing ADA (NAb) that can reduce or abrogate the drug efficacy and subsequently lead to loss of clinical response.

Five studies are included in this thesis, which assess endogenous immune processes affected and evaluates laboratory methods used for monitoring immunogenicity of IFNβ and TNF-α inhibitors. Collectively, the findings presented in this thesis aim to optimize methods for drug level and ADA screening to allow for easier treatment decisions. Additionally, the thesis highlights the skin site as a potential contributor to ADA development.

In **study I**, we studied the immunomodulatory role of IFNβ and how it was affected by NAb. We found a 3-fold increase of serum IL-7 (genetically associated with MS) in IFNβ treated MS patients and this was related to the lowered IL-7Rα expression on cell surfaces. The presence of high NAb titers to IFNβ resulted in significantly lower serum IL-7 levels compared to NAb negative patients as measured with the myxovirus resistance protein A (MxA) gene expression assay (MGA). Since the MGA method is cumbersome we decided to evaluate a new method, iLite (in **study II**). We found that the NAb titers had a high degree of correlation between the two assays and that NAb titers of 150 TRU/mL were suggestive of significant neutralization of the drug. However, in the iLite assay (and MGA) NAb titers are calculated using the Kawade principle and this method has statistical limitations. In **study III**, we therefore continued to validate the iLite assay using a cut-point approach designed to be more sensitive and statistically accurate. By using the cut-point approach, we identified 12% more NAb positive samples compared to using the Kawade method, showing the increased sensitivity achieved with the cut-point design. In **study IV**, we evaluated different methods for ADA screening of the TNF-α inhibitor infliximab. We showed that ADA could be detected in the majority of samples with low drug levels using ELISA, but that samples with detectable drug levels also tested ADA positive using an acid and dissociation assay (PandA). Thus, the PandA proved useful as a complement to the routinely available ELISA to monitor immunogenicity. Lastly (in **study V**), to understand the mechanism of ADA induction, we investigated the primary immune response against repetitive injections with biologicals in skin cells. Using a human skin model, we found that the IFNβ injection enhanced dendritic cell maturation and elevated the expression of several inflammatory cytokines, which suggest that the administration triggers an immune response at the injection site.

LIST OF PUBLICATIONS

I. Wangko Lundström, **Christina Hermanrud**, Maria Sjöstrand, Susanna Brauner, Marie Wahren Herlenius, Tomas Olsson, Virginija Karrenbauer, Jan Hillert and Anna Fogdell-Hahn.
Interferon beta treatment of multiple sclerosis increases serum interleukin-7.
Multiple Sclerosis Journal, November 2014, 20(13): 1727–1736

II. **Christina Hermanrud**, Malin Lundkvist Ryner, Elin Engdahl, and Anna Fogdell-Hahn.
Anti-Interferon Beta Antibody Titers Strongly Correlate Between Two Bioassays and In Vivo Biomarker Expression, and Indicates That a Titer of 150 TRU/mL Is a Biologically Functional Cut-Point.
Journal of Interferon & Cytokine Research, July 2014, 34(7): 498-504

III. **Christina Hermanrud**, Malin Ryner, Thomas Luft, Poul Erik Jensen, Kathleen Ingenhoven, Dorothea Rat, Florian Deisenhammer, Per Soelberg Sørensen, Marc Pallardy, Dan Sikkema, Elisa Bertotti, Daniel Kramer, Paul Creeke, Anna Fogdell-Hahn.
Development and validation of cell-based luciferase reporter gene assays for measuring neutralizing anti-drug antibodies against interferon beta.
Journal of Immunological Methods, March 2016, 430: 1–9

IV. **Christina Hermanrud**, Malin Ryner, Rille Pullerits, Karen Hambardzumyan, Nancy Vivar Pomiano, Per Marits, Inger Gjertsson, Saedis Saevarsdottir, Anna Fogdell-Hahn.
Measurement of serum infliximab levels and detection of free and bound anti-infliximab antibodies in patients with rheumatoid arthritis.
Manuscript

V. **Christina Hermanrud**, Toni M.M van Capel, Michael Auer, Virginija Karrenbauer, Florian Deisenhammer, Esther C. de Jong and Anna Fogdell-Hahn.
Different interferon beta preparations induce the same qualitative immune response in human skin.
Manuscript

ADDITIONAL PUBLICATIONS

I. Clemens Warnke, **Christina Hermanrud**, Malin Lundkvist, Anna Fogdell-Hahn
Anti-drug antibodies
Drugs and Therapy Studies 2012; volume 2:e11

II. Jenny Link, Malin Lundkvist Ryner, Katharina Fink, **Christina Hermanrud**, Izabella Lima, Boel Brynedal, Ingrid Kockum, Jan Hillert, and Anna Fogdell-Hahn.
Human leukocyte antigen genes and interferon beta preparations influence risk of developing neutralizing anti-drug antibodies in multiple sclerosis.
PLoS One, March 2014, 9(3):e90479

III. Kathleen Ingenhoven, Daniel Kramer, Poul Erik Jensen, **Christina Hermanrud**, Malin Ryner, Florian Deisenhammer, Marc Pallardy, Til Menge, Hans-Peter Hartung, Bernd C. Kieser, Elisa Bertotti, Paul Creeke, Anna Fogdell-Hahn.
Development and Validation of an Enzyme-Linked Immunosorbent Assay for the Detection of Binding Anti-Drug Antibodies against Interferon Beta.
Frontiers in Neurology, July 2017, 8:305

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LIST OF ABBREVIATIONS

α	alpha
ABIRISK	Anti-Biopharmaceutical Immunization prediction and clinical relevance to reduce the RISK
ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
ADA	anti-drug antibody
ALP	alkaline phosphatase
β	beta
BAb	binding antibodies
CCP	cyclic citrullinated peptide
CD	cluster of differentiation
CIS	clinically isolated syndrome
CNS	central nervous system
COX-2	Cyclooxygenase-2
CXCL	C-X-C motif chemokine
DC	dendritic cell
$^{\Delta}$Ct	delta cycle threshold
DIS	lesions in space
DIT	lesions in time
DMARD	disease-modifying anti-rheumatic drugs
DMT	disease-modifying therapies
EBV	Epstein-Barr virus
EC	effective concentration
ECL	electrochemiluminescent
EDSS	Expanded Disability Status Scale
ELISA	enzyme-linked immunosorbent assays
EULAR	European league against rheumatism
Fab	fragments of antigen binding
Fc	fragment crystallizable

FDA	Food and Drug Administration
γ	gamma
HHV	human herpesvirus
HLA	human leukocyte antigen
HMGB	high mobility group box
IC	immune complex
IFN	interferon
IFX	infliximab
Ig	immunoglobulin
IL	interleukin
IL-7R	IL-7 receptor
i.m.	intramuscular
IRF	interferon regulatory factor
ISGF	IFN-stimulated gene factor
ISRE	IFN-stimulated response element
JAK	janus activated kinase
JCV	John Cunningham virus
LC	langerhans cell
mAb	monoclonal antibody
µg	microgram
MGA	myxovirus resistance protein A (MxA) gene expression assay
mL	milliliter
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MS	multiple sclerosis
MTX	methotrexate
MxA	myxovirus resistance protein A
NAb	neutralizing anti-drug antibody
PandA	precipitation and acid dissociation assay

PBMC	peripheral blood mononuclear cell
PEG	polyethylene glycol
PML	progressive multifocal leukoencephalopathy
PP	primary progressive
PR	progressive relapsing
RA	rheumatoid arthritis
RF	rheumatoid factor
RR	relapsing remitting
s.c.	subcutaneous
SP	secondary progressive
SRQ	Swedish Rheumatology Quality register
STAT	signal transducer and activator of transcription
Th	T helper
TIMP	tissue inhibitor metalloproteinase
TLR	toll-like receptor
TNF	tumor necrosis factor
TRU/mL	ten-fold reduction units per milliliter
TYK	tyrosine kinase
VCAM	vascular cell adhesion protein

1 BACKGROUND

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) and can cause significant disability (1, 2) and premature death (1). Symptoms can vary greatly since any part of the CNS can be affected. Symptoms can include impairment of balance, mobility, vision and cognitive function (3) (Fig. 1). Among adults under 40 years of age, MS is one of the most common neurological disorders and affects ≈ 2.3 million people worldwide (4). The average age of disease onset is approximately 30 years and woman are more than twice as likely as men to develop MS (the female to male ratio 2.35:1) (5) (Fig. 2).

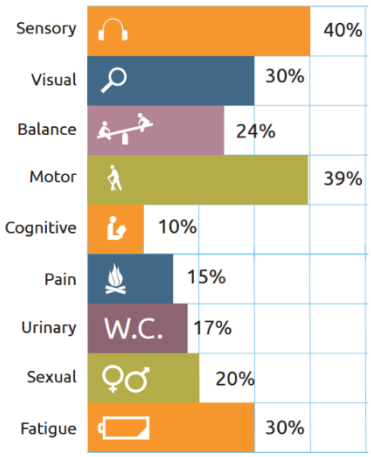


Fig. 1. **Frequency of MS symptoms**. Adapted with permission MS International Federation, MS Atlas 2013.

Fig. 2. **Gender ratio in MS**. Adapted with permission from MS International Federation, MS Atlas 2013.

1.1.1 Prevalence and incidence

The prevalence of MS varies greatly depending on the latitude (6). North America and Europe have the highest prevalence (140 and 108 per 100,000 respectively) while Sub-Saharan Africa and East Asia have the lowest prevalence (2.1 and 2.2 per 100,000). Sweden has the highest prevalence of MS in Europe (188.9 per 100,000) (5), and Albania has the lowest (22 per 100,000) (MS International Federation, MS Atlas 2013), showing that the north-south gradient is present even within Europe. In Sweden, the average MS incidence from 2001 to 2008 were 10.2 per 100.000 (7). The incidence is thus considerably higher than previous estimates of 4.3 (8) and 6.4 (9) making Sweden among the highest nationwide incidence estimates reported (7).

1.1.2 Diagnosis

Magnetic resonance imaging (MRI) of the brain together with the patient's disease symptoms, both specific for MS, are important for the diagnosis. Expanded Disability Status Scale (EDSS)

(10), together with MRI (11) are used as quantitative measures of disability and disease activity. According to the McDonald criteria (2001), diagnosis of MS is based on the finding of dissemination of lesions in space (DIS) and time (DIT) i.e. two separate MS specific CNS lesions on brain MRI that have occurred in two or more separate events (12, 13). The first event is referred to as a clinically isolated syndrome (CIS), and the second event confirms the diagnosis of MS provided that differential diagnoses are excluded. Immunoglobulin (Ig) production is a common feature in people with MS and is characterized by synthesis of IgG, IgM and IgA (14-16) and the presence of two or more oligoclonal bands in the cerebrospinal fluid. These bands are of diagnostic value since it can be found in up to 95% of people with MS (17). In 2017 the McDonald criteria was revised to allow earlier diagnosis of MS and treatment initiation. The new criteria suggests that an MS diagnosis of CIS patients can be done based on DIS and the presence of oligoclonal bands in the cerebrospinal fluid, which the latter can replace evidence of DIT (18).

1.1.2.1 Disease courses

Four MS disease courses were defined in 1996, including relapsing remitting (RR), primary progressive (PP), progressive relapsing (PR) and secondary progressive (19) (Fig. 3). However, the disease phenotypes of MS are constantly being re-examined and new recommendations were provided in 2013 (20). The main changes were the addition of CIS and removal of PRMS. Around 85% to 90% of people with MS present with an RR disease course (RRMS) (21, 22). RRMS is characterized by disease episodes known as relapses that are followed by periods of remission where complete or partial clinical recovery of symptoms is gained (22). Relapses are caused by inflammation and demyelination in the CNS (23, 24). One to two decades following an MS diagnosis most of the people with RRMS (80%) proceed to develop a secondary progressive phase (SPMS). The disease course worsens without periods of recovery due to increased axonal loss and decrease in brain volume. Approximately 10% to 15% of the people with MS present with a primary progressive disease course from the onset of the disease (PPMS) (1, 23, 25), and a decreased diagnosis of PPMS have been observed in Sweden after introduction of disease-modifying therapies (DMT) (26). Progressive relapsing MS (PRMS) is the least prevalent form and only affects around 5% of the people diagnosed with MS.

Fig. 3. **Classification of MS.** Adapted with permission from MS International Federation, MS Atlas 2013.

1.1.3 The immunopathology

The cause of MS is, to date, unknown but evidence suggests that the natural tolerance of the immune system breaks when antigen presenting cells present self-antigen, such as parts of myelin together with co-stimulatory signals, and thereby can begin to mount an immune response to these self-antigens (27).

Loss of self-tolerance has been suggested to occur through molecular mimicry (28), for example, if foreign pathogens share structural similarities with self-antigens. Once the peripheral immune cells are activated they can infiltrate the CNS through the compromised blood brain barrier. Infiltration allows acute inflammation, myelin destruction, axonal injury (29) and neurodegeneration (30). The infiltrating cells, mostly macrophages and T cells, form perivascular inflammatory lesions (plaques) and promote disruption of neurological axonal signaling (24). When infiltrating cells interact with activated CNS-resident cells including local tissue resident antigen presenting cells such as microglia it leads to destruction of oligodendrocyte-produced myelin. Cross-reactivity between an endogenous protein and a pathogenic protein might also initiate activation of T cells (31).

MS was previously considered to be a T cell-mediated disease since autoreactive myelin-specific cluster of differentiation (CD)4^+ T cells (32, 33) and $CD8^+$ T cells (34, 35) were found in MS lesions. More recently interleukin (IL)-17 producing T cells have also been suggested as key players in the pathogenesis of MS (36-38). Furthermore, MS is characterized by the elevated production of T helper (Th) 1 proinflammatory cytokines TNF-alpha (TNF-α), IL-2, and IFN-gamma (IFNγ) (39, 40), whereas anti-inflammatory Th2 cytokines IL-4 and IL-10 are downregulated (41). However, B cells have also now been recognized as important in the pathogenesis of MS (42, 43). Two randomized placebo-controlled phase 2 trials have shown that the disease activity in RRMS and PPMS were reduced after B cell depletion using anti-CD20 monoclonal antibody (mAb) (rituximab) (44, 45). Another anti-CD20 mAb (ocrelizumab) was associated with lower rates of disease activity and disability worsening than IFNβ-1a treatment of RRMS and PPMS in three phase 3 trials (46). These drugs are thought to exert their effect on B cells by reducing their capacity to present antigens and ability to secrete cytokines (42).

1.1.4 Risk factors

The etiology of MS is considered multifactorial where both genetics and environment influence pathogenesis (47).

1.1.4.1 Genetic factors

In terms of genetic association MS is not considered to be inherited. However, there is evidence that genetic variations affect the risk of MS (48) and whole genome association studies have traced more than 200 genetic regions outside the human leukocyte antigen

(HLA) to be associated to MS (49). A haplotype within the HLA region (*DRB1*15:01*) is associated with a three-fold increased risk of the disease (50), whereas *HLA-A*02* has shown to have a protective effect (51, 52).

The IL-2 receptor (*IL2R*) and IL-7 receptor (*IL7R*) genes are also well established genetic factors contributing to MS risk (53). The IL-7R is one of the most studied gene polymorphisms and may play a role in MS (54, 55). The IL-7R is a heterodimer that consists of the IL-7Rα chain (CD127), which is shared with thymic stromal lymphopoietin and the common cytokine γ chain (CD132) (Fig. 4) (56).

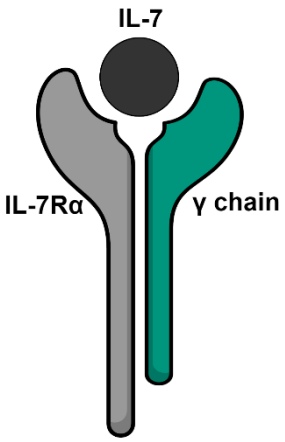


Fig. 4. **The IL-7 receptor.**

1.1.4.2 Environmental factors

Epstein-Barr virus (EBV) infection causing infectious mononucleosis, and cigarette smoking, are the best confirmed environmental contributors associated with MS (57). Moreover, vitamin D deficiency (6, 58) has also been suggested to contribute to disease pathogenesis.

1.1.4.2.1 Virus

A higher frequency of EBV seropositivity has been reported in MS patients compared to controls (59). A majority of people who become infected with EBV are asymptomatic but the virus can in some individuals cause a lymphoid infection, clinically known as infectious mononucleosis (60), which has been associated with a 2-fold risk of developing MS (61).

Infection with human herpesvirus 6 (HHV-6) has also been indicated to play a role in MS pathology. One of the first studies that showed an association between HHV-6 and MS was presented by Challoner and colleagues that showed that HHV-6 was expressed in plaques of individuals with MS (62).

1.1.4.2.2 Cigarette smoking

Cigarette smoking contributes to the risk of developing MS, with an odds ratio of 1.5 for smokers versus non-smokers (63) and contributes to accelerated disease progression (64-66).

Smokers that are carriers of the HLA-DRB1*15 allele have a further increased risk of developing MS (67). In contrast, oral tobacco users are thought to have a decreased risk to develop MS (68). Therefore it is suggested that risk of MS may be associated with lung irritation (69), which is supported by that smoking causes inflammation in the lung (70).

1.1.4.2.3 Vitamin D

The link between autoimmune diseases and poor vitamin D levels is still unclear but low vitamin D levels and limited sun exposure have been linked to an increased risk of developing MS (6, 58). This notion was further supported in a recent paper which evaluated the association between vitamin D and the risk of MS using a nested case-control design (71). Using the Finnish maternity cohort, they analyzed serum samples from 1,092 women diagnosed with MS and 2,123 women without MS. They found that 1 in 2 women (with and without MS) had vitamin D deficiency, and 1 in 3 had vitamin D insufficiency. On average, vitamin D levels were lower in MS patients than controls. Interestingly the authors found that with each 50 nanomol/liter increase in vitamin D the risk of MS was lowered by 39% (71). Despite several studies showing an association between low vitamin D levels and a higher risk of MS, the impact of vitamin D supplementation on MS activity still warrant further investigation (72).

1.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a heterogonous autoimmune disease characterized by synovial inflammation that can lead to joint destruction, but also systemic inflammation that can result in impaired movement and disability. Inflammatory symptoms include joint stiffness, swelling, pain and primarily affects the joints in the hands and feet (73). Before menopause women are three times more likely to develop the disease than men indicating that hormones play an important role in the pathogenesis (Fig. 5). RA is the most common inflammatory joint disease affecting 0.5% to 1% of the adult population globally (74). As RA progresses, continued inflammation results in permanent damage to cartilage, bone, tendons, ligaments and consequently results in joint destruction and disability.

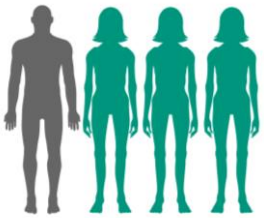


Fig. 5. **Gender ratio in RA**.

1.2.1 Diagnosis

Diagnosis of RA is based on different classification criteria, the older developed by the American College of Rheumatology (ACR) in 1987 (75), and the more recent by the ACR and European League Against Rheumatism (EULAR) that was designed to capture patients earlier at onset of disease (76). To be classified as RA using the older criteria set (ARC), patients were required to fulfill at least four out of seven criteria including morning stiffness, arthritis of ≥3 joints, arthritis of the hand joints, symmetric arthritis, rheumatoid nodules, rheumatoid factor (RF), and radiographic changes (75). However, some of the criteria are rarely fulfilled in the first year after RA onset (77, 78). Since an early diagnosis of RA, and subsequent early therapeutic intervention, is crucial to halt the disease progression and joint destruction there was a need to find a diagnostic method for early stage diagnosis (79). Thus, in 2010, the EULAR/ACR criteria was launched which captured about 50% of patients earlier, and included not only RF (as in the older criteria), but also the more specific anti-citrullinated protein antibodies (ACPA) (80, 81). The presence of RF or ACPA (usually measured with the anti-cyclic citrullinated peptide (CCP) test), is associated with greater joint damage (82). ACPA can be present up to 10 years prior to RA diagnosis and is thus a valuable biomarker. Today individuals with joint pain and positive anti-CCP are often monitored and sometimes treated early, despite not meeting the ARC diagnostic criteria (83).

1.2.2 The immunopathology

Production of autoantibodies, including RF and ACPA, that target self-proteins constitute a significant part of RA disease pathogenesis. RF of the IgM isotype are most common, but the

IgA and IgG class of RF can also be detected (84), where IgA has been associated to a more destructive disease course (85). The prevalence of RF in healthy individuals is less than 5%, whereas RA patients have a frequency of 70% to 90% (86). RF are generally of low affinity and directed to the fragment crystallizable (Fc) region of IgG (82). Disease symptoms can progress by the formation of immune complexes between the RF and IgG (Fig. 6) (87), which may in turn, activate the complement system resulting in increased inflammation.

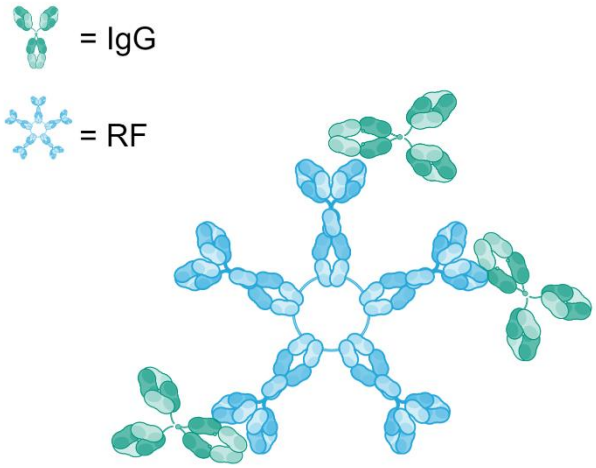


Fig. 6. **Immune complex formation between IgG and RF.**

ACPA are strongly associated with RA. ACPA-producing B cells and plasma cells can be found in both the synovium and circulation and are of IgG, IgA, or IgM isotype (88). These antibodies are directed against peptides and proteins that are citrullinated (conversion of arginine on the B cell epitope into citrulline) and are found not only in the joints of patients with RA but also in the gum and lungs. Smoking, a strong risk factor for RA as described below, is known to induce citrullination. ACPA are pathogenic as they can trigger immune cell activation and several studies have showed that immune complexes (containing ACPA) triggered TNF-α secretion by macrophages (83).

In addition to the characteristic autoantibodies (RF and ACPA) observed in two-thirds of the patients, the pathophysiology of RA is characterized by an overproduction of proinflammatory cytokines, where TNF-α is thought to be the most dominant (89). TNF-α induce an increased production of other proinflammatory cytokines including IL-1, IL-6, and IL-8. These cytokines promote T cell activation and induce expression of adhesion molecules on endothelial cells, and the process leads to increased T cell infiltration. Additionally, proinflammatory cytokines increase the expression of vascular growth factors that stimulate angiogenesis and proliferation of keratinocyte that support osteoclast differentiation and maturation - the primary cell responsible for bone destruction (90).

Since there is a higher prevalence of women that develop RA it has been suggested that hormones could play a role in the disease. The disease often manifests during childbearing

years, with a peak post-partum, and the disease symptoms often improve during pregnancy (91). Breast-feeding, oral contraceptives or hormone replacement therapy post-menopausal are associated with decreased risk of developing RA (92, 93). Moreover, men with RA have been found to have decreased levels of sex hormones especially testosterone (94).

1.2.3 Risk factors

1.2.3.1 Genetics

The heritability of RA is estimated to be around 40% and is higher for sero-positive (RF+) than seronegative (RF-) RA (95), and the concordance rate in monozygotic twins is up to 15% (96). More than 100 genetic loci have been associated with RA (97), where HLA and protein tyrosine phosphatase non-receptor 22 have the strongest associations (98, 99).

The main genetic risk factor for RA has been known for over 30 years and is the so called 'shared epitope'. The term refers to that the majority of RA patients share a five amino acid sequence coded by several HLA-DRB1 alleles (100). The 'shared epitope' is associated with the ACPA positive RA subset (101).

1.2.3.2 Environmental factors

1.2.3.2.1 Cigarette smoke

Several epidemiological studies have identified smoking to be one of the strongest environmental risk factors for developing RA (102-104). A meta-analysis conducted by Sugiyama and colleagues showed that the risk of developing RA was about twice as high for smokers than for non-smokers, and that female smokers had a 1.3-times higher risk than for non-smokers (102). Furthermore, they showed that smoking is an even greater risk factor for RA in RF-positive men and for heavy smokers (102).

1.2.3.2.2 Microbiota

Some studies have indicated that changes in the function of gut microbiota are associated with RA (105). Chen and colleagues found that RA patients had a decreased diversity of gut microbiota compared with healthy individuals (106), and Vaahtovuo et al. found a reduced composition of microbiota in patients with early onset of RA (107). However, the largest epidemiological study to date found that common infections in the gastrointestinal or urogenital tract were associated with a strongly reduced risk of RA (108), thus it is yet unclear whether, and how, infections may eventually trigger or have a protective role in RA.

1.3 BIOPHARMACEUTICALS

Biopharmaceuticals, although expensive, have proved economically viable for treatment of incurable chronic diseases such as MS and RA by reducing the rate of disease progression and therefore the long-term health burden of these diseases. For MS and RA there are now several therapies targeting the immune system, the major agents being IFNβ and TNF-α-inhibitors, respectively.

1.3.1 Therapies for multiple sclerosis

DMT have been shown to significantly reduce the relapse rate and demyelination in MS, as measured by MRI scans. DMT are capable of modulating the immune system and have immunosuppressive properties. Currently there are eight injectable, three oral and three infused DMT available to treat MS. Those that are approved by the U.S. Food and Drug Administration (FDA) for treatment of relapsing MS include IFNβ which is often used as a first-line treatment.

1.3.1.1 Interferon beta

IFNβ is a naturally occurring pleiotropic cytokine that is secreted by fibroblasts, epithelial cells, NK cells, and leukocytes as a response mechanism to viral infections (109). The biological activity of IFNβ is initiated when IFN binds to the Type I IFN receptors (subunits IFNAR1 and IFNAR2) (110) that are located on the surface of most cell types (111). The binding activates the Janus Activated Kinase 1 (JAK1) and Tyrosine Kinase 2 (TYK2), that in turn phosphorylate transcription factors Signal Transducer and Activator of Transcription (STAT) 1 and STAT2 (112, 113). Phosphorylation leads to dimerization and association with Interferon Regulatory Factor 9 (IRF9) to form the IFN-Stimulated Gene Factor 3 (ISGF3). ISGF3 binds to IFN-Stimulated Response Elements (ISRE) in the cell nucleus (Fig. 7) (114). Even though the binding induces expression and transcription of genes, which function is to target viral infections (115), it is difficult to pinpoint the precise mechanism of IFNβ treatment.

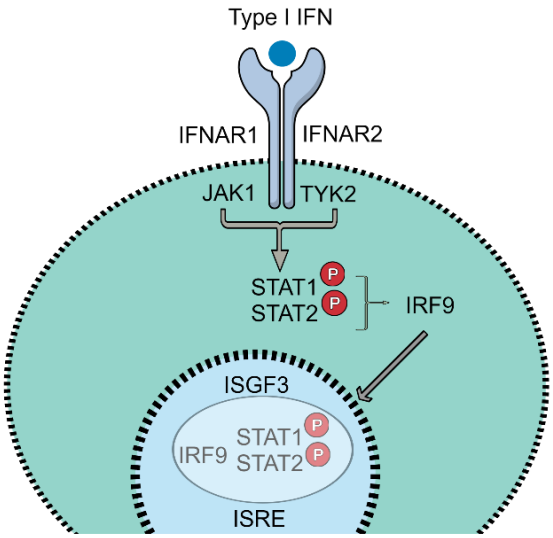


Fig. 7. **The JAK/STAT signaling pathway.**

1.3.1.1.1 IFNβ product properties

Two types of recombinant IFNβ preparations are available for treatment of MS; IFNβ-1a (Avonex®/Rebif®/Plegridy®), and IFNβ-1b (Betaferon®/Extavia®). Differences in terminology between the two recombinant proteins are based on the sequence difference (116). The recombinant versions of IFNβ show a high structural homology with the endogenous IFNβ. IFNβ-1a is produced in Chinese hamster ovary cells (117) and has the same amino acid sequence and glycosylation structure as the endogenous protein (118). The glycosylation reduces protein aggregation and increases protein stability and solubility (119). IFNβ-1a is administrated either intramuscularly (i.m.) of 6 million units (30 µg) once a week (Avonex®), or subcutaneously (s.c.) with 12 million units (44 µg) three times weekly (Rebif®). The most recently approved IFNβ-1a formulation is PEGylated (Plegridy®) and is administrated s.c. of 12 million units (125 µg) every fortnight. IFNβ-1b is produced in Escherichia coli (120) and differs from endogenous IFNβ protein as it is not glycosylated, lacks one amino acid, and has a replacement of the cysteine to a serine at position 17 (121-123). The amino acid changes are important for the stability of the molecule since it prevents the formation of incorrect disulfide bonds. IFNβ-1b (Betaferon®/Extavia®) is administrated of 8 million units (250 µg) s.c. every other day. Despite the fact that there is a 70% resemblance between the recombinant IFNβ preparations it has been suggested that IFNβ-1a has a 10-fold increased capacity over IFNβ-1b to induce antiviral, anti-proliferative and immunomodulatory activity. The difference between the molecules is thought to be caused by glycosylation-induced stabilization in IFNβ-1a (124). The s.c. injection is administered as a bolus into the subcutis and the i.m. injection delivers the medication deep into the muscle, allowing the medication to be absorbed into the bloodstream quickly (Fig. 8).

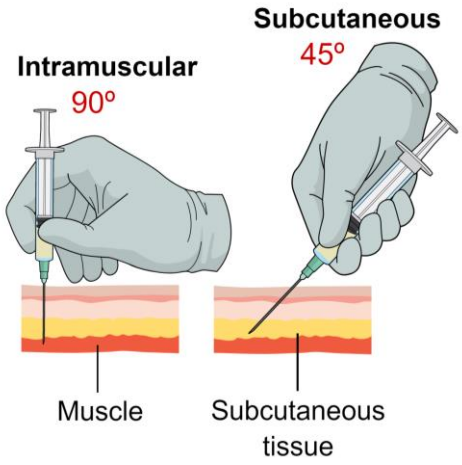


Fig. 8. **Routes of drug administration.**

1.3.1.1.2 Mechanism of action

In clinical trials, the use of IFNβ has been shown to decrease the relapse rate by about one-third (125, 126), reduce the development of new brain lesions (127-130), and slow

progression (131-134) as measured with the EDSS score (135-137). However, the clinical response rate to IFNβ is highly heterogeneous and some patients experience continued disease activity (138). It is suggested that MRI can be used to monitor the treatment effect of IFNβ where the development of MRI lesions within 6 to 24 months after treatment initiation defines a poor responder patient (138). IFNγ and IFNα have also been investigated as potential therapeutics in MS. However, IFNγ was found to worsen the disease symptoms, and IFNα did not exhibit as improved effect on the relapse rate as compared to IFNβ (139). The molecular mechanisms of IFNβ are complex and alter the expression of several hundreds of genes (140), including the IFNβ biomarker Myxovirus resistance protein A (MxA) used to identify treatment responders (141). Therapeutic effects of IFNβ includes **i)** increased anti-inflammatory cytokine production and reduced proinflammatory cytokine production, **ii)** reduced cell migration across the blood-brain barrier, **iii)** promoted CNS repair, and **iv)** anti-proliferative properties.

i. Treatment with IFNβ reduces Th1 proinflammatory cytokines IL-17 (142), TNF-α and IFNγ (143), and shifts the cytokine profile towards a Th2 profile and increased levels of anti-inflammatory cytokines such as IL-4 and IL-10 (144). For example, Liu and colleagues studied peripheral blood mononuclear cells (PBMCs) from MS patients before and after IFNβ-1a treatment and found significant changes in IL-10 expression two days after treatment initiation (145).

ii. Several *in vitro* studies have shown that T cells have a decreased capacity to interact with endothelial cells since IFNβ alters the expression of several adhesion molecules (146). For example, treatment with IFNβ lowered the serum levels of the matrix metalloproteinase (MMP)-9 and increased expression of tissue inhibitor metalloproteinase (TIMP)-1 (147, 148), which resulted in reduced numbers of MRI lesions (148). Furthermore, T cells subjected to IFNβ downregulate cell surface expression of the alpha 4 integrin (149) and increase expression of soluble vascular cell adhesion protein (VCAM) 1 in MS patients serum. Increases in soluble VCAM1 correlated with decreased MRI lesions suggesting that IFNβ interfere with the adhesion cascade and thus might prevent T cells from entering the CNS (150).

iii. IFNβ has been suggested to promote CNS repair by increased nerve growth factor mRNA after IFNβ treatment of astrocyte *in vitro* (151) and a study by Biernacki et al. saw that IFNβ could repair damage in the CNS by promoting the production of nerve growth factor in MS patients (152). The authors suggest that increased nerve growth might explain why early treatment with IFNβ can reduce the rate of brain atrophy (152).

iv. Studies have shown that leukocyte and lymphocyte counts were reduced as a result of IFNβ treatment (153) and a reduction of white blood cells has also been observed (154). Moreover, McKay et al. showed that treatment with IFNβ reduced the antigen presenting cells ability to present antigen, which led to reduced T cell responses (155).

1.3.1.1.3 Prediction of treatment efficacy

Identification of biomarkers that could be used to find patients who are likely to respond to IFNβ treatment is highly warranted (138). In 2010, Axtell and colleagues found that the efficacy of IFNβ could be predicted in RRMS where non-responders to IFNβ had higher serum IL-17F concentration before treatment initiation compared to responders (156). Moreover, a paper by Lee et al. that suggested that high serum IL-7 levels, when paired with low IL-17F serum levels, predicted a good response to IFNβ (157). However, when the same group replicated this finding in a validation cohort it could not be confirmed (158).

Several studies have investigated gene expression for use as biomarkers to evaluate the response to IFNβ (138). Two studies have shown that IL-8 gene expression was downregulated in MS patients who had a good response to IFNβ treatment (159, 160), and a genome-wide association study identified 47 genes that could distinguish IFNβ responders from non-responders (161).

1.3.1.1.4 Side effects related to IFNβ treatment

A recent case-control study showed that RRMS patients on IFNβ treatment had a 1.8- and 1.6-fold increased risk of migraine or stroke, and a 1.3-fold increased risk of developing depression and hematologic abnormalities, compared to MS patients on non-IFNβ disease-modifying drugs (162). Furthermore, a meta-analysis including nine clinical trials showed that IFNβ treated patients had a 2.8-fold increased risk of discontinuing treatment due to adverse events like flu-like symptoms, leukopenia, lymphopenia and elevated liver enzymes and injection site reactions (163).

Local cutaneous injection site reactions can cause symptoms such as eczema, swelling, pain and redness/erythema of the skin at the injection site (164). These symptoms can appear either immediately following IFNβ administration or several years after treatment initiation (165). As many as 96% of MS patients that are given s.c. administration with IFNβ get a skin reaction compared to i.m. IFNβ administration that only has a 33% reported incidence of skin reactions (166). Why such local skin reactions occur is not clear but may be due both to the administration and the biopharmaceutical composition. If the patient uses the wrong injection technique and the IFNβ is injected into the epidermis instead of the s.c. tissue it could potentially cause cutaneous adverse events. Also, the IFNβ formulation itself is observed to be inflammatory trough chemokine induction followed by immune cell extravasation. A study by Buttmann and colleagues showed that skin biopsies sampled 24

hours after IFNβ injection had strong Contaminant Candidate List 2 and CXC chemokine ligand (CXCL) 10 expression that initiated T cell trafficking from the circulation and infiltration into the skin tissue (167). It has also been reported that cutaneous psoriasis can be the caused by administration of IFNβ (164, 168). A study recently published showed that almost all regulatory T cells in normal human skin have a memory phenotype. Under steady-state conditions, the skin resident memory regulatory T cells are relatively unresponsive. However, in inflamed skin from psoriasis patients, memory regulatory T cells are highly proliferative and produce low levels of IL-17 (169). Immunohistochemistry of a skin biopsy from one MS patient that developed psoriasis after IFNβ injection revealed IL-17 expressing cells, suggesting that these cells might be defective (164).

1.3.1.2 Natalizumab

Natalizumab (Tysabri®) is a humanized monoclonal IgG4 antibody produced in murine myeloma cells. Treatment with natalizumab is a highly effective for RRMS and has shown in two randomized controlled trials to significantly reduce relapse rate by 68% and disease progression (170, 171). The drug is administrated intravenously with a dose of 300 mg every 4 weeks as recommended by the FDA and the European Medicine Agency. Natalizumab targets the very late antigen 4, composed of alpha 4 and beta 1 that is expressed on the surface of leukocytes and thereby prevents interaction with its ligand VCAM1 present on blood vessels (Fig. 9). Therefore, activated immune cells will be blocked to migrate into the inflamed brain (172, 173) as illustrated below.

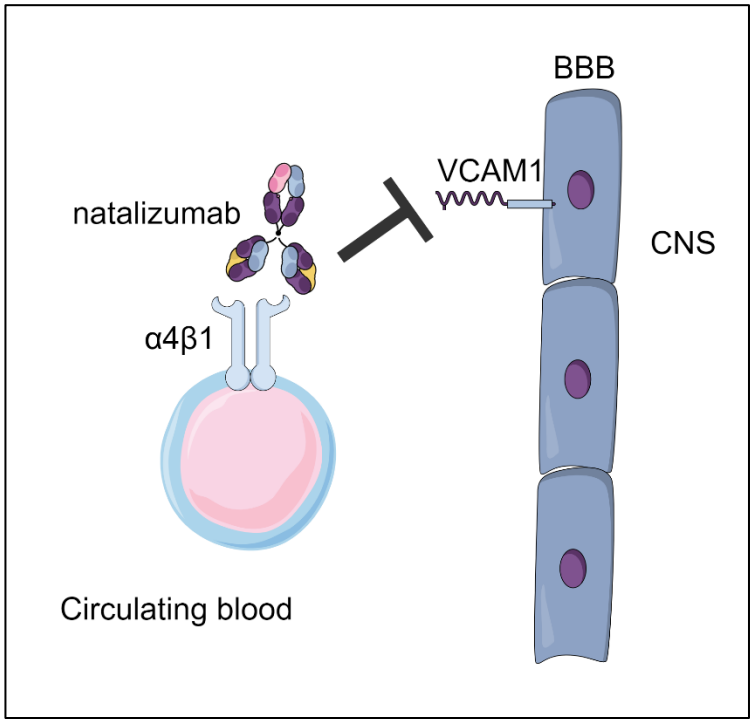


Fig. 9. **Natalizumab inhibits leukocytes migration across the blood brain barrier (BBB) into the CNS**.

1.3.2 Mechanism of action

A study by Kivisakk and colleagues showed that treatment with natalizumab increased the proportion proinflammatory cytokines including TNF-α, IFNγ, IL-17, and IL-2 in peripheral blood (174). Altered cytokine expression could be a consequence of preventing activated T cells to migrate from the peripheral circulation (174). MS patients treated with natalizumab have significant increased B cell counts in peripheral blood (175) suggesting interference with B cell homing. It´s been proposed that the interference could lead to impaired differentiation of plasma cells. This theory is support by Selter et al. who showed that IgM and IgG levels in serum were significantly decreased in natalizumab treated patients compared to treatment naïve patients (176).

1.3.2.1.1 Side effects of natalizumab therapy

Since natalizumab is associated with the risk of reactivation of latent John Cunningham virus (JCV) infection and thereby the development of progressive multifocal leukoencephalopathy (PML) it is often used only as a second-line treatment (177). PML is a disease of the CNS characterized by demyelination, oligodendrocytes with enlarged nuclei, and enlarged astrocytes (178). Infection with JCV is considered a prerequisite necessary to develop PML but not the only factor. Around 50% to 60% of people with MS and the healthy population are asymptomatic carriers of the JCV (179). There are three risk factors associated with PML during natalizumab treatment: 1) presence of serum anti-JCV antibodies, 2) previous use of immunosuppressive drugs (including IFNβ), and 3) use of natalizumab exceeding 24 months. The estimated risk to get PML has reached 4 per 1000 patients treated (180). A recent meta-analysis showed that JCV sero-positive patients with a low index value of anti-JCV antibodies sometimes revert to a sero-negative status in contrast to JCV sero-positive patients with a high index value of anti-JCV antibodies that almost never revert. Moreover, also a conversion from sero-negative to sero-positive occurred at a rate of 10.8% per year (181).

Other adverse events related to natalizumab treatment includes infusion- and hypersensitivity reactions. For infusion related reactions such as headaches, it has been shown to occur in 24% of the patients receiving the drug compared to 18% of the placebo group (170). Hypersensitivity reactions occurred in 4% of the patients receiving natalizumab, compared to 0% of the placebo patients (170).

1.3.3 Therapies for rheumatoid arthritis

Disease-modifying anti-rheumatic drugs (DMARD) are the first-line therapy in RA, in combination with methotrexate (MTX) as the main drug if the patients have no contraindications (182). About one-third of patients respond well to MTX (183) and the vast majority of those who respond well to MTX tend to have a better disease course (184). However, although this form of treatment is quite effective in reducing the disease symptoms, two-thirds of patients do not respond sufficiently and continue to develop joint destruction. Biological therapy has revolutionized the outcome of RA during the last 15 years, where the TNF-α inhibitors were the first on the market and currently the second-line therapy of choice. TNF-α is an inflammatory cytokine known to have a role in the joint damage and TNF-α inhibitors effectively inhibits joint destruction (185). Today there are five different TNF-α inhibitors approved for the treatment of RA: infliximab (Remicade®), adalimumab (Humira®), etanercept (Enbrel®), certolizumab pegol (Cimzia®), and golimumab (Simponi®).

Infliximab (IFX) was the first TNF-α inhibitor on the market and is currently favored on cost-effectiveness grounds and for practical reasons including intravenous administration, which improves patient compliance. This, and the fact that it can induce immunogenicity, is the reason for it being the agent studied in the current thesis.

1.3.4 Product properties of infliximab

IFX was the first biologic drug that was shown to be effective for treatment of RA (186). IFX is a chimeric mouse-human monoclonal IgG1 antibody, where the variable region is from a murine origin and the Fc part (constant domain) is humanized (Fig. 10). There are now several IFX biosimilars on or about to reach the market, the first sold under the trademarks Remsima® and Inflectra® (with the same substance). Biosimilars have the same formulation as the generic drug and similar chemical properties, but since it is a biological substance there can, theoretically, be subtle differences. Therefore separate trials were needed to compare those to the original IFX and they were found to have equivalent treatment efficacy in RA patients (187, 188). IFX is administered intravenously every 4 to 8 weeks with a half-life of 8 to 10 days and is detectable in plasma up to 12 weeks after the latest dose.

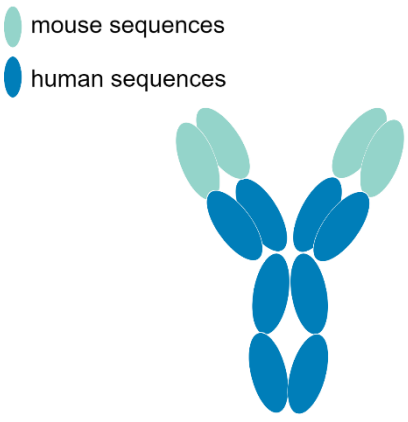


Fig. 10. **IFX is a chimeric mouse-human monoclonal antibody.**

1.3.4.1.1 Mechanism of action

RA patients have elevated concentrations of TNF-α in the joints and this correlates with increased disease activity. TNF-α is a pleiotropic pro-inflammatory cytokine that affects various cell types. There are two forms of TNF-α; transmembrane bound TNF-α and soluble TNF-α. Transmembrane TNF-α is mainly produced by activated macrophages and lymphocytes and is a precursor of the soluble TNF-α as depicted in the simplified illustration below (Fig. 11). The TNF-α converting enzyme (TACE) cleaves biologically active soluble TNF-α from transmembrane pro-TNF-α and exerts its biological function through binding to TNF-α receptor-1 and receptor-2, which are present on almost all nucleated cells (189). Transmembrane TNF-α exercises its biological activity through cell-to-cell contact, whereas soluble TNF-α can act at remote sites from the TNF-α-producing cells (190). Binding of transmembrane and soluble TNF-α to the TNF-α receptors mediates pleiotropic effects including apoptosis, cell proliferation and cytokine production (190), which all promote inflammation (191).

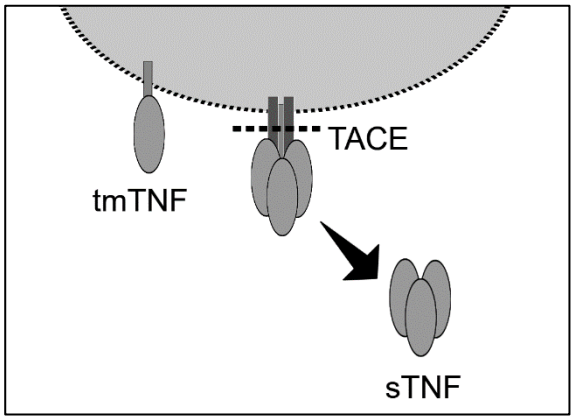


Fig. 11. **Transmembrane bound (tm) TNF-α and soluble (s) TNF-α.**

Treatment with IFX controls inflammation by high-affinity neutralization of both soluble and transmembrane bound TNF-α (Fig. 12; adapted by permission from Macmillan Publishers Ltd: Nature (192), copyright 2010). Neutralization prevents TNF-α from binding to the cellular receptors and thereby inhibits the bioactivity of TNF-α and the induction of activated T cells.

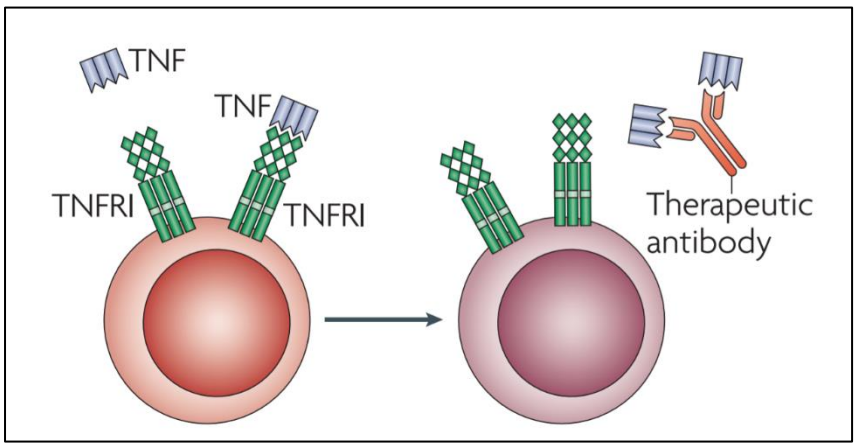


Fig. 12. **Soluble TNF-α blockade with IFX**, Macmillan Publishers**.**

By controlling TNF-α, IFX hinders inflammatory cells from infiltrating inflamed areas of the joints (190). Concomitant administration of MTX sustain serum IFX concentrations for longer and combination therapy is thus recommended instead of monotherapy (193), both to diminish immunogenicity and reduce joint damage (194).

1.3.4.1.2 Dose optimization

Treatment with TNF-α inhibitors are not effective in all RA patients. Around 30% of RA patients treated with TNF-α inhibitors have a primary response failure (194) and only one-third have a good response according to the recommended EULAR response criteria. An even greater proportion of patients experience secondary failure, as seen with a loss of efficacy (secondary response failure) (195). To achieve optimal treatment efficacy with TNF-α inhibitors dose optimization strategies can be applied, although currently there are no general guidelines with recommendations (196). Both the US and European labeling of IFX recommends a dose of 3 mg/kg every 8 weeks. However, the suggested dose for non-responders differs between US and Europe, where US labeling suggests an increased dose up to 10 mg/kg as often as every 4 weeks, but the European guidelines recommend a maximum IFX dose of 7.5 mg/kg every 8 weeks or 3 mg/kg every 4 weeks (197).

1.4 IMMUNOGENICITY OF BIOPHARMACEUTICALS

1.4.1 Anti-drug antibody formation

Prolonged administration of biopharmaceuticals can have an immunogenic effect in the form of anti-drug antibody (ADA) formation in treated patients. ADA can be either non-neutralizing or neutralizing (198, 199). In 2015 a paper was published as part of the Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK (ABIRISK) consortium with the aim to standardize definitions related to immunogenicity (200). Binding anti-drug antibodies (BAb) and ADA includes all antibodies, regardless of their function, that bind to the biopharmaceutical drug and ADA was decided to be the preferred acronym. Neutralizing anti-drug antibodies (NAb) are antibodies that reduce or abrogate the biological functions of the therapeutic and cause loss of product efficacy with treatment failure as a consequence (201, 202). Factors by which ADA/NAb contributes to low or undetectable drug levels include drug neutralization or by formation of immune complexes that promote the clearance of the drug (Fig. 13).

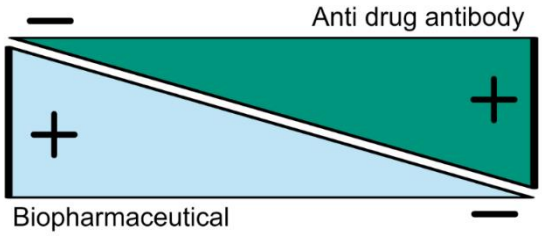


Fig. 13. **The serum drug concentration inversely correlates with the ADA titer.**

Immune reactions to biopharmaceuticals occur when an antigen from the drug is presented together with a danger signal to T cells, which in turn activate B cells that will differentiate into antibody secreting B cells (203, 204). Even biopharmaceuticals that are almost identical to the native human proteins can induce ADA.

1.4.2 Antibody structure and function

Antibody molecules are Y-shaped proteins that consist of two fragments of antigen binding (Fab) regions and an Fc region (Fig. 14). The Fab region is composed of two pairs of light chains that pair up with the two heavy chains, and the Fc part contains the constant region of the heavy chains. The Fab region is responsible for antigen binding and has variable domains to recognize a diverse repertoire of pathogens. The idiotype is part of the variable region and has a unique binding specificity for each antibody clone, i.e. mAb produced by the same B cell clone. The Fc part mediates the effector functions such as antibody dependent cellular cytotoxicity, phagocytosis, and degranulation. When an immune response is initiated, IgM and IgD antibodies are the first antibody isotypes to be produced. Immunoglobulin class switching occurs when activated B cells encounter new specific stimulation. Alteration of the IgM constant heavy chain, or Fc part, allows class switching into another immunoglobulin such

as IgG, IgE and IgA. IgA consists of two subclasses (IgA1 and IgA2) and IgG can be divided into four subclasses (IgG1, IgG2, IgG3, and IgG4). Each antibody class and subclass shapes a different antibody response to efficiently clear off different pathogens.

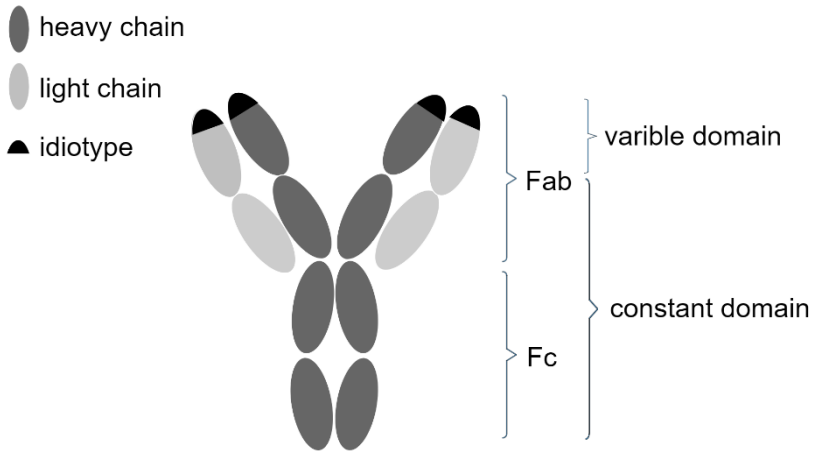


Fig. 14. **Antibody structure.**

1.4.3 Anti-drug antibody development

There are different factors associated with the immunogenicity of biopharmaceuticals that can be classified into the following three categories; **i)** product **ii)** patient and **iii)** treatment properties (116, 205, 206). In short, **i)** product associated factors that can influence immunogenicity includes the molecular properties of the drug such as glycosylation and half-life. Other product-related factors such as the presence of impurities and aggregates and that can occur during the manufacturing process can also contribute to immunogenicity. **ii)** Examples of patient-related risk factors include disease state and polymorphisms in HLA (207), which can affect the T cell responses (208). Epidemiologic studies have identified that smoking increases the risk of developing ADA in MS (209, 210). However, in a study by Auer and colleagues where they measured the nicotine metabolite cotinine in the serum from MS patients on IFNβ treatment, they found smoking not to be associated with increased risk of developing NAb (211). **iii)** Treatment-associated factors include the treatment duration, frequency of administration, and the route of administration (212). The risk of developing NAb increases with prolonged duration of treatment and thereby with increased exposure. Increased incidence of NAb also occurs if the drug is administered s.c. or i.m. in comparison to intravenous administration of the drug.

1.4.4 Immunological mechanisms involved in immunogenicity

Theoretically, most protein therapeutics will be able to trigger an immune reaction, which leads to antibody formation. Based on the trigger, low-titer, low-affinity, transient IgM antibody responses or high-titer, high-affinity IgG responses can be induced. Thus, the causes

of immunogenicity of protein therapeutics are complex and the generation of antibodies to therapeutics are multifactorial (213).

A recent paper by Kalluri and colleagues studied the T cell responses to recombinant IFNβ and showed that NAb development was associated to IFNβ specific T cells in treated MS patients (208). They found that, despite all patients displaying T cell responses to IFNβ, NAb positive patients had a higher frequency of T cell responses compared to the NAb negative patients and untreated controls (208). They further identified two IFNβ immunodominant regions. The T cell responses to the region located at $IFN\beta_{1-40}$ were observed in all IFNβ treated patients regardless of NAb status, whereas region $IFN\beta_{125-159}$ was stronger in the NAb positive patients compared to those with a NAb negative state (208).

1.4.4.1 T cell dependent pathway

ADA are mainly of the IgG isotype, which suggests a T cell dependent pathway as class switching requires T helper cell interaction. Two steps are necessary for the naïve B cell to become activated and differentiate into an antibody secreting plasma cell. Firstly, the naïve B cell receives primary activating signals when it binds the antigen to it's B-cell receptor (membrane bound Abs), mediates antigen endocytosis and processes the antigen into peptides. These peptides are presented on the HLA class II molecules on the cell surface. Secondly, T cells with a receptor specific for the presented peptides from the antigen will interact with the activated B cell and give the signals required for class switching. The interaction occurs via B7 on B cells that binds to CD28 on T cells. The contact between the B- and T cell leads to upregulation of the costimulatory molecule CD40 ligand on the surface of T helper cells and secretion of B cell stimulatory cytokines by the T helper cells. When the costimulatory molecule CD40 ligand on the T cell surface binds to CD40 on the B cell surface this instructs the B cells to switch from IgM and IgD production to a downstream antibody class on the Ig gene. The cytokines further activate the B cell and stimulate the differentiation of B cells into antibody secreting plasma cells. Some of the activated B cells differentiate into memory cells with the capacity to react rapidly to re-challenge of the specific antigen by production of short-lived plasma cells. T cell dependent antibody responses are generally long lasting and of high titers.

1.4.4.1.1 Affinity maturation

The process of affinity maturation allows the antigen binding site of the antibody to develop a higher affinity for the antigen through somatic hypermutation and selection in the germinal center (214). In the rapidly proliferating B cell, affinity maturation occurs randomly within the DNA that codes for antigen binding region (heavy and light chain) of the antibodies that are produced. The mutations will generate antibodies with different affinities for the same antigen and only B cells producing antibodies with the highest affinity will survive the

selection in the affinity maturation process, resulting in high affinity antibodies being generated.

1.4.4.2 T cell-independent pathway

In the T cell-independent antibody responses naïve B cells can be activated by large carbohydrates that are constructed with a backbone that contain multiple antigenic determinants. If the carbohydrate backbone binds to the naïve B cells IgM and IgD receptors, expressed on the cell surface, it forces a strong clustering and crosslinking of the immunoglobulins and the signal promotes B cell activation with production and release of low titers of IgM and IgD. However, as no T cells are involved there will be no class switching and the antibodies are in some instances transient.

1.4.4.3 The human skin

As administration routes of biopharmaceuticals have been suggested to influence the risk for an immunological response in form of ADA (206, 215), the immunological matrix composition and immune cells in the skin are of interest to study. The skin is the human body´s largest organ and harbors a complex structure to protect against antigen entry (216). Epidermis and dermis are the two main segments of the skin (Fig. 15). The outer layer of the skin consists of the epidermis which is abundant of keratinocytes (217) and provides a physical barrier between the body and the outer environment by resisting penetration by microorganisms. The epidermis is separated from the dermis by a basement membrane which prevents interaction between the cells in the epidermis and dermis. An injury to the membrane can, however, allow the epidermal cells to come in direct contact with the dermis. The dermis is the inner layer formed of collagenous connective tissue and blood vessels. This layer provides nutrients and structural support to the epidermis (216). The adipose tissue, which is the s.c. fatty region below the dermis functions as the insulation for the body (218).

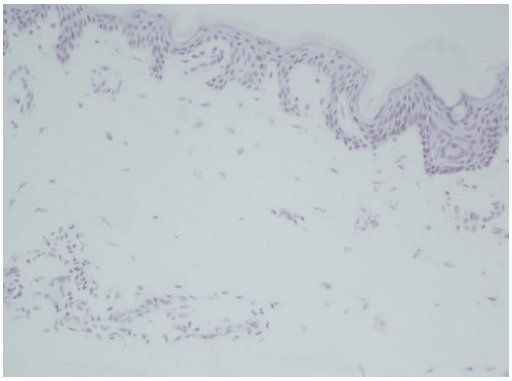


Fig. 15. **Epidermis and dermis are the main segments of the skin.**

1.4.4.3.1 Immunity in the skin

Other than acting as a physical barrier, the skin provides an immunological barrier to the external environment. There is a constant interplay between keratinocytes, immune cells, and microorganisms in response to wounding and infection. The immune system is initiated by recognition of pathogen-associated molecular patterns that activate keratinocyte pattern recognition receptors, resulting in the secretion of antimicrobial peptides, cytokines, and chemokines (216, 219, 220). The cytokines initiate effector mechanisms (221), whereas the chemokines recruit T cells and innate effector cells such as monocytes to the skin (222). In addition to keratinocytes the epidermis contains memory T cells, Langerhans cells (LC), Merkel cells, and melanocytes. The dermis is composed of elastin- and collagen fibers and a matrix produced mainly by fibroblasts (223). The cell types that contribute to immune function in dermis are mast cells, macrophages, dendritic cell (DC) subsets, innate lymphoid cells and T cells (223). Healthy human skin contains a large population of resident-memory T cells, which are believed to be non-recirculating T cells which mediate protective immunity in the skin. Nearly all of these T cells have the skin homing receptor cutaneous leucocyte-associated antigen that enable binding to E-selectin expressed on cutaneous blood vessels (7). Healthy skin contains as much as 1×10^6 T cells/cm^2, and thus almost twice the number compared to the T cells in circulation (8).

1.4.4.3.2 Skin resident DC

DC are critical regulators for the control of immunity and tolerance and they are present throughout the body. The DC system is composed of a variety of subsets exerting different types of immunity (224). DC subsets can circulate the blood, reside in the lymphoid organs or the peripheral tissues. In the peripheral tissue DC capture antigens and then migrate to the lymph nodes and present antigens both directly to T cells via HLA class I and II, and also through the non-classical CD1 antigen presenting molecule (225). Human skin contains at least two myeloid DC subtypes: epidermal LC and dermal DC. The dermal DC can be divided into at least two subsets: $CD1a^+$ DC and $CD14^+$ DC. Since antibody responses are suggested to be mediated by $CD14^+$ dermal DC (226), it has been proposed that $CD14^+$ DC would be a suitable target to induce a potent humoral immune response in vaccination (227, 228). This hypothesis is supported by mouse studies which show that activated dermal DC migrate towards B cell follicles, whereas activated LC migrate to the T cell paracortex in the cutaneous lymph node (229). The cellular mechanism behind regulation of immunity at the skin site has been shown by Klechevsky and colleagues (230). They found that $CD14^+$ DC induced differentiation of naïve T cells into T follicular helper cells, which in turn regulate humoral immunity (231). Thus, $CD14^+$ DC prime $CD4^+$ T cells to induce B cells which in turn produce larger amounts of IgM than those B cells induced by $CD4^+$ T cells primed with LC. Moreover, they showed that $CD4^+$ T cells could induce naïve B cells to an Ig class switch toward IgG and IgA (230), and these findings were supported by another study (232). Based on this notion

they suggested that targeting $CD14^+$ dermal DC could potentially induce an enhanced antibody response. Data have also showed that LC, expressing marker $CD207^+$, could stimulate T cells to become T follicular helper cells (233). LC can furthermore activate T cells to become efficient helpers for the activation of cytotoxic T lymphocyte responses (230, 234, 235) (Fig. 16, adapted with permission from Drug discovery today (235), copyright 2011).

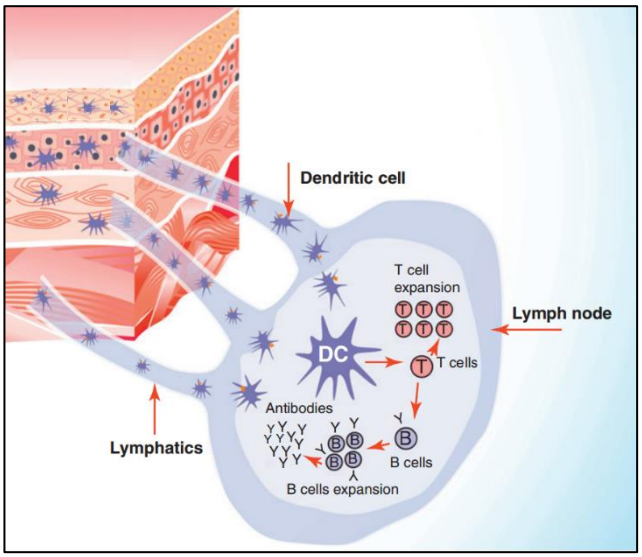


Fig. 16. **Skin immunity,** Drug discovery today**.**

1.4.5 Occurrence of anti-drug antibodies

1.4.5.1 Interferon beta immunogenicity

ADA occur in nearly all the IFNβ treated patients (236) and up to half of them will become NAb positive (215). NAb generally develop between 6 to 18 months after therapy initiation (237). For the IFNβ treated MS patients there are different rates of immunogenicity for each preparation, where IFNβ-1a (i.m.) and IFNβ-1a (s.c.) are less immunogenic than IFNβ-1b (s.c.) (Table I) (123, 238). NAb against IFNβ-1a occur at a lower incidence but often generate higher NAb titers which often persist, while NAb to IFNβ-1b occur more frequently but are of lower titers and can be transient (239). The most recently approved IFNβ-1a formulation (Plegridy®) was found to have a very low immunogenicity with less than 1% that became NAb positive during a follow-up period of two years (240).

1.4.5.2 Natalizumab immunogenicity

ADA against natalizumab have been found in up to 9% of the treated patients (241) and can be detected as early as three months after treatment initiation (Table I) (242). These antibodies can be either confirmed persistent or transiently positive (241, 243, 244), where half of the ADA positive group are transient (241). In persistently positive patients, significantly higher ADA levels can be detected compared to the transiently positive.

Moreover, the level of total ADA in the first sample can be used to predict patients at risk for being persistently ADA positive (242).

Table I. **Immunogenicity of Biopharmaceuticals.**

Product	IFNβ-1a	IFNβ-1a	IFNβ-1a	IFNβ-1b		natalizumab
Trade name	Avonex®	Rebif®	Plegridy®	Betaferon®/Extavia®		Tysabri®
Type	cytokine	cytokine	cytokine	cytokine		IgG4
Route of administartion	i.m	s.c	s.c	s.c.		i.v.
Dose	30 µg	22 or 44 µg	125 µg	250 µg		300 mg
Units (10^5)	6	12	12	8		n/a
Frequency	once weekely	three times weekely	every 14 days	every other day		every 4th week
NAb (%)	6	28	<1	47	ADA (%)	9

IFNβ = interferon beta; i.m. = intramuscular; s.c. = subcutaneous; i.v. = intravenous; ADA = anti-drug antibodies; NAb = neutralizing ADA

1.4.5.3 TNF-α inhibitor immunogenicity

Several studies have shown that ADA reduce the bioavailability of the targeted TNF-α inhibitor in the circulation of the treated RA patients (245-249), and this in turn is associated with failure to respond to the drug (250-252). ADA negative patients usually display normal to high serum drug trough levels in contrast to ADA positive patients that have very low or undetectable serum drug trough levels (249, 253, 254). The frequency of ADA positivity varies significantly between studies and can be a result of different factors such as the patients' concurrent medications, the timing of sampling, and which bioassay used for ADA detection (255, 256). Furthermore, ADA binding to the therapeutic can result in immune complex formation and become undetectable using standard laboratory techniques (257). Acid dissociation assays are used to overcome immune complex formation as these assays can dissociate any drug and ADA complexes (258).

ADA development is reported in up to 44 percent of patients treated with adalimumab (247, 259, 260) and in up to 17% of the patients treated with IFX (261), but concomitant immunosuppressant treatment with MTX is suggested to reduce ADA development (262). ADA to etanercept has been reported (260), whereas other studies have been unable to detect any ADA (263).

1.4.6 Clinical relevance of anti-drug antibodies

1.4.6.1 Multiple sclerosis

The clinical significance of NAb is sometimes difficult to assess in MS as many studies have a short follow-up time (less than two years), resulting in the late appearing NAb and the time it would take for the NAb to exert clinical relevance, not being accounted for (264). However, several studies confirm that NAb positive MS patients treated with IFNβ have increased

disease activity relative to NAb negative patients (137, 265, 266). For example, in a pivotal trial on IFNβ-1b, the NAb positive group had a relapse rate during years 2 and 3 that was higher than for the NAb negative group (267). Furthermore, another study found that IFNβ NAb positive patients had higher annual relapse rates than ADA negative patient during years 3 and 4 (268), and a large Danish study saw the same phenomenon after 42 and 48 months of treatment (269). In addition, IFNβ NAb positive patients have been shown to have more gadolinium (radiocontrast agent) enhancing lesions on MRI compared to NAb negative patients (270). To overcome immunogenicity of IFNβ protein modifications to eliminate aggregation-prone regions and epitopes by point mutation are implemented during the drug development process (116).

For anti-natalizumab antibodies, studies have shown that persistent antibody positivity resulted in low natalizumab levels and induced infusion-related adverse events, including hypersensitivity reactions (170, 241). Furthermore, Vennegoor and colleagues showed that antibodies that persist are associated with increased occurrence of relapses and brain lesions (271). An additional study by Vennegoor and colleagues showed that MS patient with high antibody titers to natalizumab had very low or undetectable concentrations of natalizumab and was associated with relapses and gadolinium enhanced lesions on MRI (271).

1.4.6.2 Rheumatoid arthritis

For ADA to TNF-α inhibitors, a study by Pascual-Salcedo et al. showed that anti-IFX antibodies were associated to loss of clinical response (245). A meta-analysis by Garces and colleagues showed that ADA to TNF-α inhibitors significantly decreased the drug response rates (272). Moreover, for IFX treated patients, immune complexes can form between the therapeutic and ADA. Depending on the form, size and interaction of the immune complexes, adverse events such as hypersensitivity reactions (255, 273) and IgE-mediated anaphylaxis can occur in a proportion of patients receiving IFX (274). In addition, the circulation of immune complexes in the bloodstream results in lysosomal degradation of the immune complexes and this phenomenon becomes an additional factor that contributes to that the protein might be cleared from the body before it can elicit its therapeutic effect. Risk factors for the formation of immune complexes have been identified and are based on the therapeutics molecular constitution, route of delivery, and binding to HLA alleles.

1.5 ASSESSMENT OF IMMUNOGENICITY

Depending on the biopharmaceutical drug used for treatment different strategies for ADA/NAb detection can be undertaken. Since IFNβ is a cytokine, and thus has a short half-life, the serum samples are screened for the presence of NAb instead of measuring the drug level that often is implemented for mAb therapy. The NAb positive samples are thereafter titrated as a confirmatory step to validate the positive result. For RA patients on mAb treatment such as TNF-α inhibitors, the drug concentration in trough level can indicate whether ADA testing is needed. If the drug level is low, the sample is screened for the presence of ADA followed by titration as a confirmatory step. It is recommended to retrieve the patient's serum sample just before the next dose (trough) to avoid drug interference. Measuring the immunogenicity of biopharmaceuticals can be done using various methods including:

- Enzyme-linked immunosorbent assays (ELISA) that determine the presence of all antibodies but cannot separate binding from neutralizing;
- Radioimmunoassay (RIA) uses radioactively labeled drug to detect ADA;
- Cell-based bioassays that identify the presence of NAbs; and
- Electrochemiluminescent (ECL) immunoassays using Meso Scale Discovery technology that have the advantage of not being drug sensitive (when using acid disociation) and identifies both free and bound ADA (immune complexes).

1.5.1 ELISA

ELISA is a commonly used screening assay for detection of ADA. Compared to cell-based assays ELISA are both easy to design and perform and thus have the advantage of allowing high throughput screening. Different assay formats that may be used include direct, indirect, and bridging ELISA. The bridging assay has high specificity, since the ADA is recognized twice; first by the solid-phase-bound antigen and then by visualization by the labeled antigen (Fig. 17).

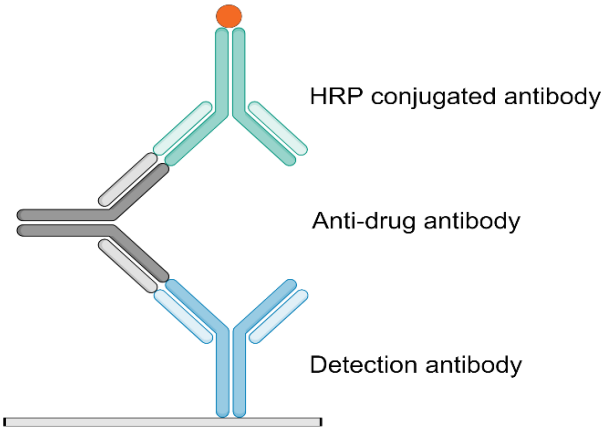


Fig. 17. **Detection of ADA using a bridging ELISA.**

The bridging ELISA is therefore often favored over the other assay formats. However, there are disadvantages with the bridging assay format since it detects disease-specific antibodies, such as RF that can bind to the drug causing interference (275). Direct ELISA, however, is even more prone to RF interference (87). Interference is an issue as it can confounds detection and interpretation of treatment-induced ADA (275). Moreover, the bridging assays cannot identify the IgG4 isotype (e.g. antibodies of the IgG4 subclass undergoing Fab arm exchange) (276), which can lead to false negative results.

1.5.2 Radioimmunoassay

Classically, the assay is performed where the target antigen is radioactively labeled and is based on competitive-binding between the radiolabeled antigen and an unlabeled antigen to a high affinity antibody. The method is more sensitive than the bridging ELISA and has also the advantage of identifying the IgG4 subclass (251). However, since RIA requires use of radioactive materials it is limited to be performed in specialized laboratory facilities.

1.5.3 Cell-based bioassays

Assays used to measure NAb are based on the antibodies ability to antagonize the biopharmaceutical in for example the cellular response of the drug. Numerous assay systems have been developed to measure NAb to IFNβ including the cytopathic effect assay where the cells are challenged with virus or the MxA gene expression assay where the receptor-specific signals are quantified by the reduction of the IFNβ-induced genes such as MxA expression at mRNA or protein level (277-279). The presence of IFNβ strongly and specifically induces the expression of MxA and this response is affected in a NAb titer dependent manner, where high titer NAb abrogate the expression and low titer NAb lower the expression (280, 281). Another readout for NAb detection is the use of cells transfected with a firefly luciferase reporter-gene to quantify drug activity (iLite).

1.5.3.1 NAb positive/negative to IFNβ therapy

In the illustration below (Fig. 18), NAb to IFNβ is analyzed using the iLite IFNβ bioassay. If no NAb are present in the sample, the added IFNβ will stimulate the interferon receptor and thus generate a high luminescence signal (Fig. 18A). If NAb are present in the patient´s sera, they will interfere with the biopharmaceutical, resulting in a low luminescence signal (Fig. 18B). For the calculations of NAb titers the Kawade method has often been used and the titer is defined as the value of the dilution of serum that gives a ten-fold reduction of IFNβ bioactivity and is expressed in ten-fold reduction units (TRU/mL) (282).

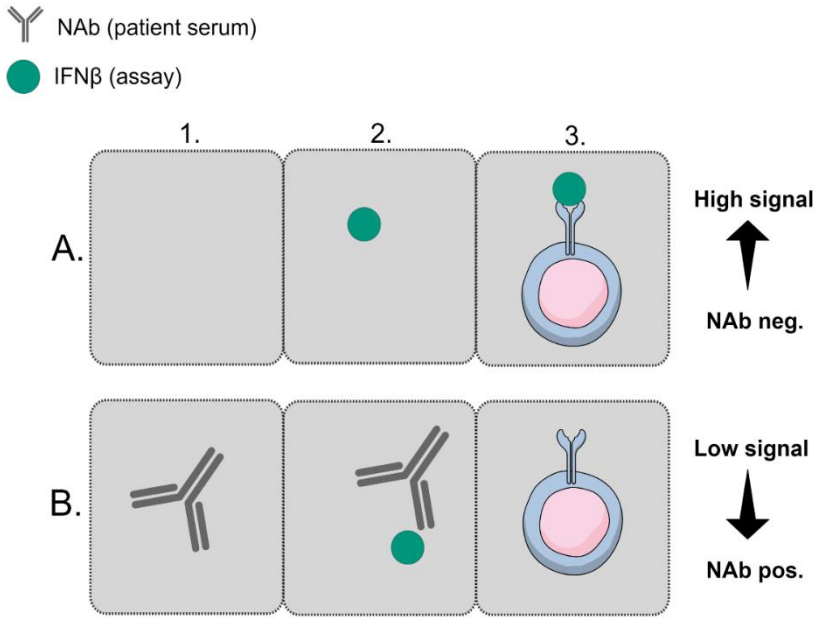


Fig. 18. **Measure NAb to IFNβ in a cell based assay.** (A) No NAb present versus; (B) NAb present in the patient sample.

1.5.3.2 Potential interference in immunoassays

When serum samples are tested for immunogenicity using bioassays, there are potential interference problems that one should be aware of including immune complex formation between the NAb and the drug (273). Thus it is possible to obtain false negative results as many assays cannot measure NAb/drug complexes (283). Immune complexes can for example occur when the patient is treated with mAb drugs such as IFX. In the illustrations below (Fig. 19, 20, 21), NAb to IFX is analyzed using the iLite IFX bioassay.

1.5.3.2.1 NAb negative

For analysis of serum samples that are NAb negative, the added IFX (Fig. 19-1) will be free to bind the added TNF-α (Fig. 19-2). Subsequently the TNF-α will not bind to the TNF-α receptor, resulting in failure to induce transcription and consequently no luminescence signal will be generated (Fig. 19-3). An undetectable signal will result in a NAb negative readout.

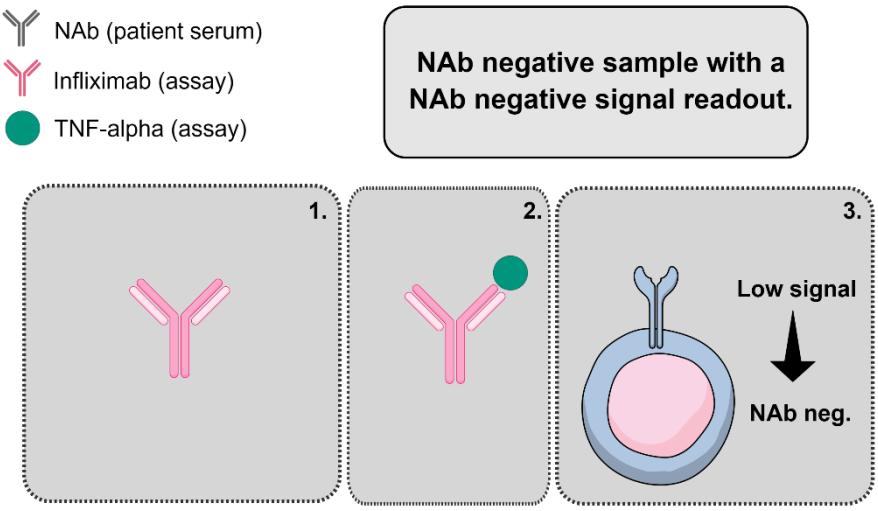


Fig. 19. **Measure NAb to mAb using a cell based assay.**

1.5.3.2.2 NAb positive

The illustration below depicts screening of a NAb positive sample without drug interference (Fig. 20). NAb is present in the serum sample (Fig. 20-1), the added IFX will be bound to the NAb (Fig. 20-2). When TNF-α is added there will be no free IFX available and subsequently TNF-α will bind to the TNF-α receptor resulting in receptor-induced activation of luciferase synthesis and an enhanced luminescence signal (Fig. 20-3). A high signal will indicate the presence of NAb in the patient's serum sample.

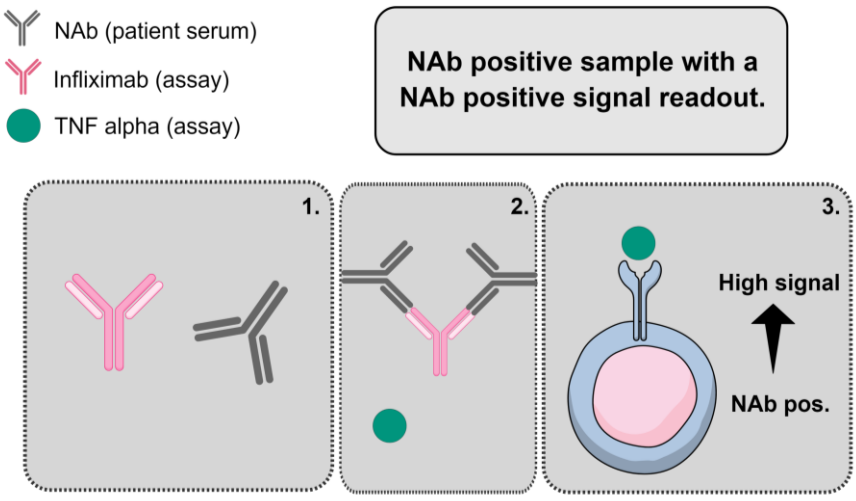


Fig. 20. **Measure NAb to mAb without drug inference using a cell based assay.**

1.5.3.2.3 Drug/NAb immune complexes

The illustration below depicts screening of a NAb positive sample with drug interference (Fig. 21). NAb positivity can be masked because of the presence of drug/NAb immune complexes. If IFX and NAb (both present in the patient´s serum) forms immune complexes the added IFX will be outcompeted (Fig. 21-1). The added IFX is therefore free to attach to the added TNF-α (Fig. 21-2). No TNF-α is available to bind to the cell receptor and thus no luciferase activity is detected and the sample is falsely identified as NAb negative (Fig. 21-3).

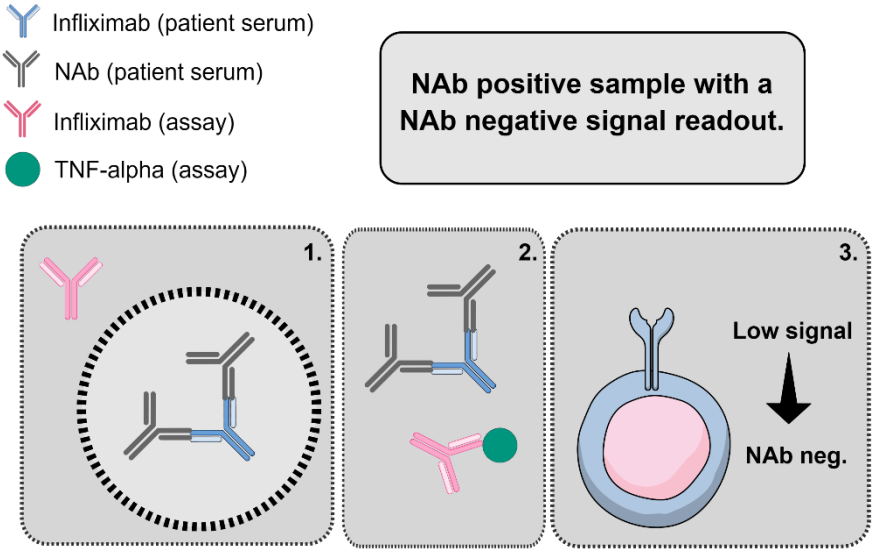


Fig. 21. **Measure NAb to mAb encountering drug inference using a cell based assay.**

1.5.4 ECL assays on the Meso Scale Discovery platform

Both ELISAs and the cell-based assay have the disadvantage of being sensitive to a circulating drug that may interfere with ADA detection and the presence of ADA may interfere with quantification of drug levels. Thus, when assessing ADA, it is essential that the patient sample is retrieved at a time point when the drug level is as low as possible, that is at trough level. A strict sampling window is particularly important when the patient is on mAb treatment as they have a long half-life compared to protein drugs such as IFNβ. Moreover, if the patient has ADA, it could lead to immune complexes between the ADA and the drug, which may interfere with ADA assessment in immunogenicity assays, as already described. Zoghbi and colleagues recently developed a method under the MSD technology platform called Precipitation and Acid dissociation assay (PandA) (Fig. 22), that effectively solves the drug interference problems based on the following four steps (258):

1. Excess of the drug is added to the serum sample to cause saturation of ADA and thereby allows the formation of drug/ADA complexes;
2. Complexes containing ADA are precipitated using polyethylene glycol (PEG);
3. The precipitate is coated in an acidic solution on a high binding carbon plate with the ability to dissolve the immune complexes and prevent reformation of drug/ADA complexes; and

4. Total ADA levels are detected using a SULFO-TAG that is conjugated to the drug allowing an ECL signal that is comparative to the amount of ADA in the serum sample.

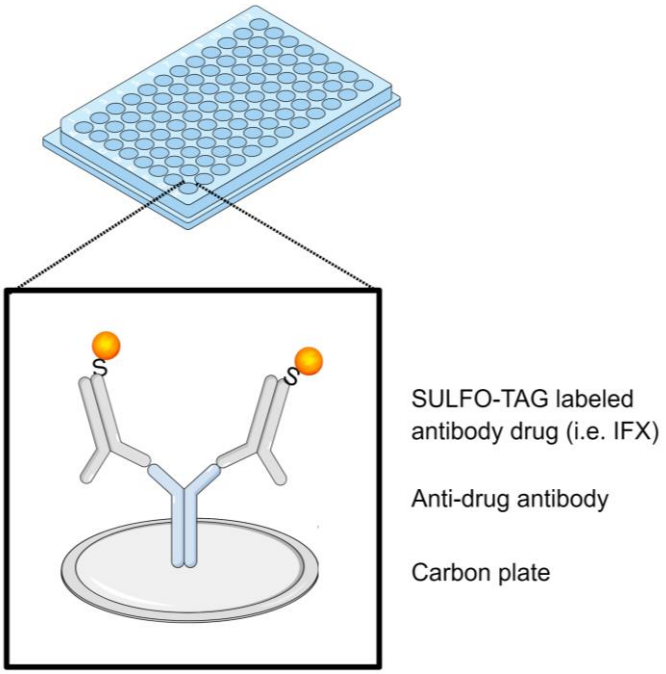


Fig. 22. **Detection of ADA in the ECL assay**.

By performing these four steps, complete recovery of ADA can be obtained regardless of the presence of drug or immune complexes in the sample (258).

1.5.5 Assay validation essential for quality assurance

As there are a range of bioassays available to measure ADA, there can be variations in both the assay used and the method of data reporting between testing laboratories. Immunogenicity of biologic drugs can affect both safety and efficacy of the treatment course, and suitable immunoassays that can measure ADA with a high precision are thus a necessary step to consider even during drug development. The increased use of biopharmaceuticals have resulted in a requirement of drug developers to provide an immunoassay, for every new drug, and measure ADA according to the current regulatory authority guidelines (284, 285). Guidelines on how to perform bioassay immunogenicity testing and data presentation have been published and updated on several occasions (284, 286, 287). Shankar and colleagues suggest that validation of ADA methods according to their recommendations offer the conclusive characterization of samples into ADA-positive versus ADA-negative and that would lead to fewer ADA-inconclusive samples (284). Important factors to consider are serum matrix effects and the use of a statistically based cut-point approach. These recommendations was recently updated by Devanarayan et al. (288). In conclusion, even though the use of validated immunoassays that comply with regulatory authority requirements result in increased assay sensitivity the clinical relevance of these modifications needs to be determined.

2 AIMS OF THESIS

The overall aims of my Ph.D. projects were to characterize the immune response against biopharmaceuticals and evaluate the clinical relevance of drug levels and ADA/NAb in people with MS and RA.

During my Ph.D. the focus of my research has been to:

[Paper 1 and 2] investigate how IFNβ treatment affects endogenous immune processes and how these are blocked by NAb and evaluate laboratory methods used to monitor immunogenicity.

[Paper 3] validate a cell-based bioassay using a cut-point approach to identify NAb to IFNβ.

[Paper 4] monitor TNF-α-inhibitor drug levels and free and bound ADA to TNF-α-inhibitors.

[Paper 5] evaluate immune responses against IFNβ at the injection site.

3 MATERIAL AND METHODS

For a more detailed description of the methodology, see the individual methods section for each research paper.

All studies were approved by the regional ethical board in Stockholm, Sweden.

3.1 STUDY I AND II

Study I - *Interferon beta treatment of multiple sclerosis increases serum interleukin-7.*

Study II - *Anti-interferon beta antibody titers strongly correlate between two bioassays and in vivo biomarker expression, and indicates that a titer of 150 TRU/ml is a biologically functional cut-point.*

3.1.1 Human serum samples

In study I and study II, people with MS on IFNβ treatment previously analyzed for NAb in our routine NAb core at Karolinska Institutet were included. Study I included 184 MS treated patients from all over Sweden. Study II included 44 MS patients treated at four Swedish neurological hospitals including Karolinska Institutet Solna and Huddinge (Stockholm), Danderyds hospital (Stockholm), and Sahlgrenska hospital (Gothenburg). The recruitment lasted for three years (2010-2013) for study II and the inclusion criteria were based on that they should be between 18 to 60 years of age, diagnosed with MS, and being treated with any of the four IFNβ preparations Avonex®, Rebif®, Betaferon®, and Extavia®. All patients signed a consent form. Peripheral blood was collected for gene expression- and NAb analysis.

3.1.2 NAb analysis

To measure the presence and titer levels of NAb, MGA was used in study I, and MGA and iLite were used in parallel in study II. MGA was used in our clinical routine lab for several years and as iLite was newly implemented on the market we wanted to run them in parallel in order to establish whether we could use iLite instead of MGA. iLite has a simplified way to measure NAb with shorter incubation steps and by not requiring cell culture since it has frozen growth-arrested cells ready to use.

In the MGA assay the patient serum was pre-incubated with IFNβ-1a before addition of the suspension to the cell line A549 (human embryonic lung cells). After incubation the cells were lysed and RNA was extracted and converted to cDNA. The ability of the patients' serum to neutralize IFNβ-induced *MX1* was quantified by real-time PCR (TaqMan).

In iLite the protocol was carried out according to the manufacturers' instructions (Biomonitor). In short, division-arrested cells carrying the luciferase reporter gene under the control of an IFN-responsive promoter were used. Transcription of the luciferase gene occurs

when the IFNβ molecule binds to the type I IFN receptor. Luciferase activity was measured using GloMax 96 Microplate Luminometer by relative luminescence units (RLU).

3.1.2.1 Calculations of NAb titers

To calculate the NAb titer the neutralizing activity of the patient's serum sample were adjusted according to the Kawade method using Softmax Pro software for MGA and Microsoft Excel software for iLite. Results from our bioassays were calculated according to the formula:

$$t = f\frac{(n-1)}{(10-1)}$$

Where **t** is the NAb titer, **f** is the dilution of the patient serum at endpoint (1 international unit /mL), and **n** is the amount of added IFNβ. The NAb titers were expressed as 10-fold reduction units per milliliter (TRU/mL) and the patients were classified according to the following four categories (as used in the routine setting):

i. Negative (<10 TRU/mL)
ii. Low positive (10 - 50 TRU/mL)
iii. Medium positive (>50 - 200 TRU/mL)
iv. High positive (>200 TRU/mL)

A titer of >150 TRU/mL was used as a cut-point for a clinically relevant titer (289).

3.1.3 Flow cytometry

For study I, flow cytometry was performed to investigate the cellular expression of IL-7Rα on PBMC incubated with IFNβ and IL-7 or with only IL-7. Cells were kept at 4°C throughout the laboratory procedure supplemented with fetal calf serum during antibody staining.

3.1.4 Gene expression

cDNA was synthesized from RNA, and *IL-7Rα*, *MX1,* and *CXCL10* were quantified by real-time PCR and the expression levels of each gene was normalized to the housekeeping gene *HPRT1* (*GAPDH* – data not shown) using the delta cycle threshold ($^{\Delta}$Ct) formula. Ct values were the mean of technical duplicates and replicates more than 1 Ct cycle apart were excluded as a technical outlier. Each normalized $^{\Delta}$Ct was calibrated against the $^{\Delta}$Ct value from a healthy control. Relative gene expression was calculated using the $2^{\Delta\Delta}$Ct method.

3.1.5 ELISA

Serum IL-7 and CXCL10 levels were detected using commercially available ELISA kits. The sensitivity of the IL-7 and CXCL10/IP-10 ELISA´s were 0.1 pg/mL and 1.67 pg/mL respectively. All samples were run in duplicates.

3.1.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6. For study I, p-values were calculated by unpaired two-sided *t*-tests. The Shapiro-Wilk test was used to verify normal distribution of the data. As the data for study II was not normally distributed, we used a Spearman non-parametric correlation test to determine the relationship between two variables. To establish differences between unpaired and paired groups we used non-parametric Mann-Whitney and Wilcoxon matched paired test. Statistical significance was defined as a p-value below 0.05.

3.2 STUDY III

Development and validation of cell-based luciferase reporter gene assays for measuring neutralizing anti-drug antibodies against interferon beta.

3.2.1 Pre-validation laboratory work

Pre-validation work of iLite was performed as follows:

3.2.1.1 Selection of the optimal stimulation concentration of IFNβ

To establish half of the maximal effective concentration (EC) 50, i.e. 50% of IFNβ maximum effect on the cells, dose-response curves were generated (Fig. 23). The dose-response curves were defined by four established parameters; first - the baseline response (bottom), secondly - the maximum response (top), third - the slope (steepness), and lastly - the drug concentration that provokes a response halfway between the baseline and maximum. The dose-response curves needed to reach a lower and upper plateau. To determine the EC50, two operators ran three independent dose-response curves on three plates, on three different days. In sum, this created 18 independent dose-response curves. EC50 was calculated using a 4 parametric logistic model (4PL) fit.

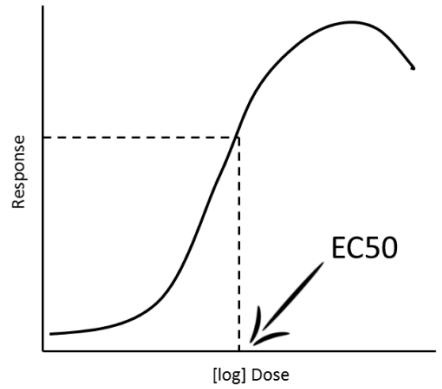


Fig. 23. **Dose response curves to select the EC50.**

3.2.1.2 Selection of an HPC and LPC

The selected stimulation concentration of IFNβ (EC50) was used to perform inhibition curves with a positive control for IFNβ that reached an upper and lower plateau (Fig. 24). Two operators ran three independent inhibition curves per plate on three different days. In sum, this created 18 independent inhibition curves that were fitted with 4PL. A preliminary high positive control (HPC) and low positive control (LPC) were selected in the upper and lower linear area of the inhibition curve respectively.

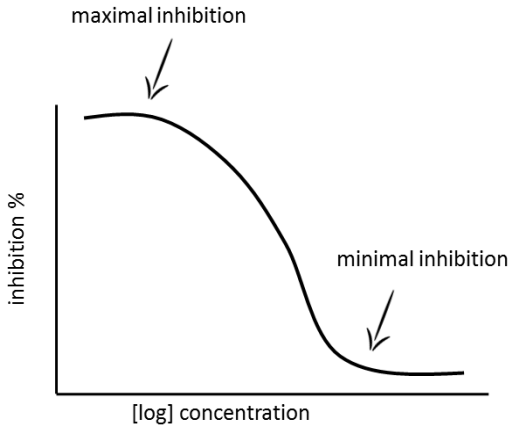


Fig. 24. **Inhibition curves to select the HPC and LPC.**

3.2.1.3 Minimum required dilution

The selected EC50 and high and low positive controls were used to perform the third step; to establish the minimum required dilution i.e. the Z-factor (Fig. 25) (290). The Z-factor reflects the difference in the size of the signals and the signal variation within both the sample and the control i.e. how well the positive signal resolves from the negative signal. On the x-axis the sample signal is lower than the control signal since it is an inhibition assay. In the assay, assuming the blank has a fixed signal intensity and spread, the Z-factor will increase as the sample becomes more NAb positive (i.e. the separation between the sample and blank is increased). Z values of 1 are ideal as the separation between the signals is very good. A Z-factor of between 0.5 and 1 is excellent. If the Z-factor is 0 there is no difference between the sample and the blank (290). To calculate the Z-factor dilutions of 6 human serum samples and control samples (assay media) were prepared. The samples were spiked with IFNβ alone or with IFNβ plus anti-IFNβ to give the selected EC50 IFNβ concentration and anti-IFNβ concentrations corresponding to the HPC and LPC.

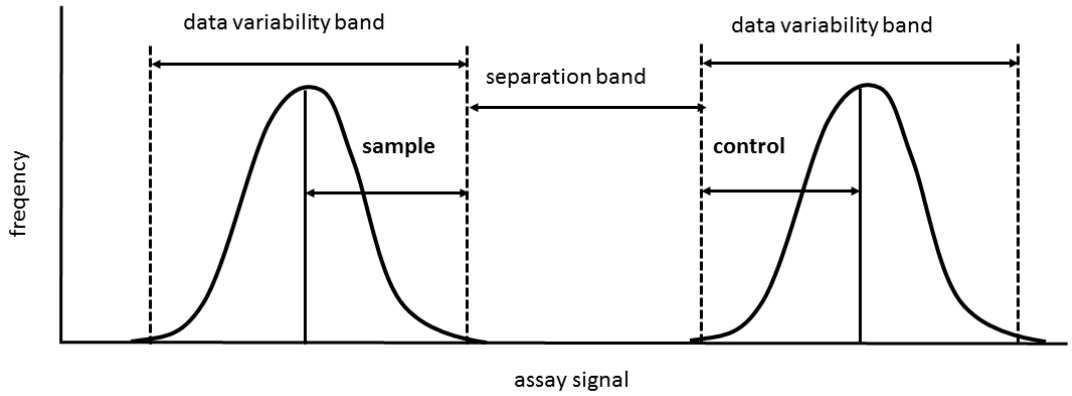


Fig. 25. **The Z-factor score assess the quality of the assay.**

3.2.2 Validation laboratory work

3.2.2.1 Serum samples

To establish the minimum required dilution (MRD), cut-points, and sensitivity, serum samples from treatment-naïve MS patients are preferred since patients with autoimmune diseases may have higher immune reactivate components in their serum than the overall healthy population. Consequently, disease state samples may have components that can cause the background signal to vary if compared to healthy serum, and thus the use of healthy serum may generate a different cut-point than if the disease state serum samples are used. However, we could not retrieve enough disease state samples in this experiment and therefore used 54 serum samples from healthy individuals.

3.2.2.2 Cut-point

To determine the cut-point for iLite and the LUC assay, 54 serum samples from healthy controls were tested in duplicates, on three separate days by two operators generating 6 independent tests for each serum sample. All plates included positive and negative controls and the serum samples were subjected to IgG immunodepletion to establish a confirmatory cut-point. The linear scale versus the logarithmic scale was evaluated for distribution and symmetry for the values generated for each assay run. Next we checked for biological outliers using box-plots. Removal of biological outliers is necessary when determining a cut-point since these values are not representative of samples from the patient population. If such values are not removed they could potentially lead to a very high or very low cut-point and thus lead to either false-negative or false-positive samples. Next, analytical outliers were removed using box-plots and the samples were characterized by the 25th, 50th and 60th percentiles. Analytical outlier refers to when serum from one donor generates significantly higher or lower values in one of the runs compared to the other donor samples in the same run. Distribution and skewness were assessed for each run to know what method to use to calculate the cut-point and specificity cut-point.

3.2.3 Statistical analysis

Assay data reflecting variability were expressed in terms of the mean, the sample standard deviation, and the coefficient of variation. Curve fitting and statistical analysis were performed using Excel software (Microsoft®), XLfit (ID Business Solutions), GraphPad Prism version 6 (La Jolla), JMP (SAS Institute Inc.), and SPSS (IBM). For validation work, Shapiro–Wilk, one-way ANOVA and Levene's test were used for the cut-point assessment. Spearman correlation test was used to assess the relationship between NAb titers.

3.3 STUDY IV

Measurement of serum infliximab levels and detection of free and bound anti-infliximab antibodies in patients with rheumatoid arthritis

3.3.1 Study samples

This study included RA patients from three cohorts in Sweden; 101 RA patients from the SWEFOT cohort (Karolinska University Hospital), and REALlife including 272 patients from Stockholm (Karolinska University Hospital) and 42 patients from Gothenburg (Sahlgrenska University Hospital).

3.3.2 ELISA

The serum level of TNF-α inhibitors and ADA to TNF-α inhibitors were measured with two in-house developed and validated ELISA (291) used in clinical routine at Karolinska University Hospital and at Sahlgrenska University Hospital.

3.3.2.1 Detection of IFX serum levels

The level of IFX in a patient´s serum sample is based on the binding of added patient serum (containing IFX) to TNF-α-coated ELISA plates. Alkaline phosphatase (ALP)-conjugated IgG (Fc-specific) antibodies thereafter bind to IFX (if present in the patient's serum sample). The signal generated by the detection antibody reflects the concentration of IFX present in the patients´ sample (Fig. 26).

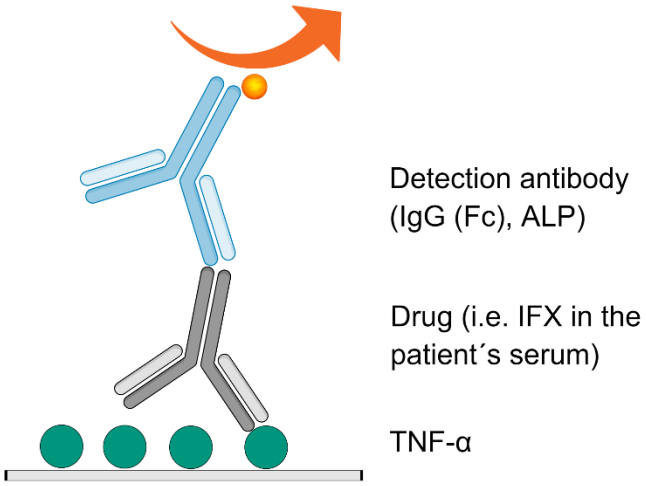


Fig. 26. **Direct ELISA.**

3.3.2.2 Detection of antibodies to infliximab

Inhibition ELISA, also known as competitive ELISA, was used for quantification of ADA levels to IFX (Fig. 27). ADA detection is based on the inhibition of binding ALP labeled IFX to TNF-α-coated ELISA plates. A signal will be generated if no ADA is present in the serum sample (A), while a lower signal or no signal will be generated if ADA is present (B).

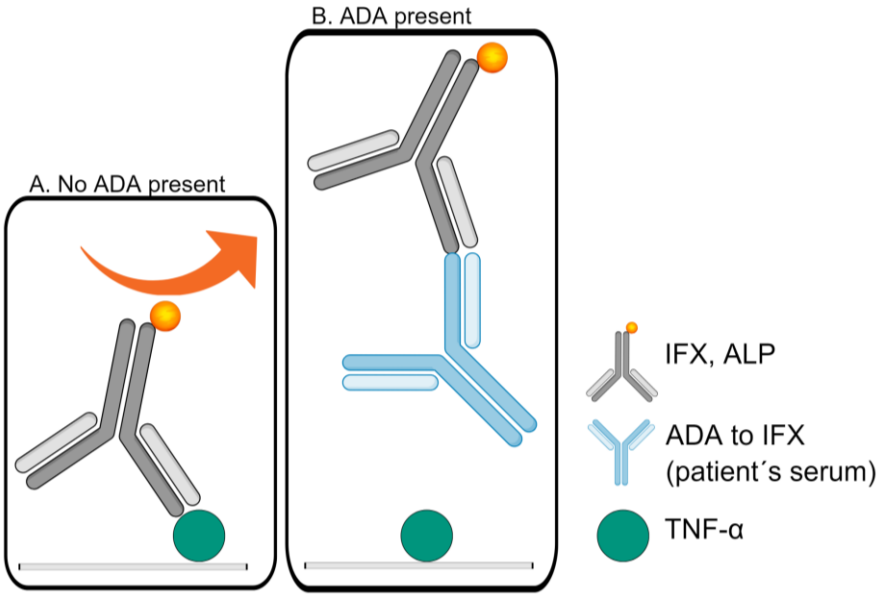


Fig. 27. **Inhibition ELISA**

3.3.2.3 Free and bound ADA detection with PandA

Presence of free and bound IFX antibodies was assessed using an in house validated PandA method on the Meso Scale Discovery platform. In the assay, added excess of IFX to the serum sample cause saturation of ADA and thereby allowing the formation of drug/ADA complexes. The complexes containing ADA are precipitated using PEG. Thereafter the precipitate is coated in an acidic solution on a high binding carbon plate (high coating capacity), which prevents reformation of drug/ADA complexes. The total ADA levels are detected using a SULFO-TAG that is conjugated to the drug (IFX) allowing an ECL output.

3.3.3 Measurement of neutralizing ADA

The neutralizing capacity of ADA positive serum samples were analyzed using the iLite™ IFX NAb bioassay (Biomonitor). The protocol was carried out according to the manufacturers' instructions. The assay uses cells that are sensitive to TNF-α and can thus measure TNF-α bioactivity. In the assay format NAb positivity will be indicated when added TNF-α binds to the TNF-α receptor. Luciferase activity was measured using GloMax Luminometer (Promega) and the antibodies neutralizing activity was normalized to Renilla.

3.3.4 Statistics

Assay data reflecting variability were expressed in terms of the mean, sample standard deviation, and the coefficient of variation. Statistical calculations were performed using Prism software (GraphPad Inc. version 6). The data was calculated by linear regression analysis and statistical significance was defined as a p-value below 0.05.

3.4 STUDY V

Different interferon beta preparations induce the same qualitative immune response in human skin.

3.4.1 Human skin model

3.4.1.1 Ex vivo skin model

Skin was retrieved from patients who underwent abdominal plastic surgery. The laboratory procedure went as follows: First, the skin was surgically removed from the patient and the skin was stored at 4°C. The skin tissue was shortly thereafter transported on ice to Karolinska Institutet. The laboratory work included removal of the adipose tissue with a scalpel, then intradermal injections were performed with IFNβ diluted in PBS and with PBS alone (Fig. 28). The syringe was placed at a 5 to 15 degree angle and the technique was applied to get a bollows in the epidermis. The formation of a bollow allows easy control that the drug was correctly injected into the dermis. Right after the injection a biopsy was sampled and transferred to a tissue plate with cell media. Biopsies for the different experiments were collected as follows (Fig. 29);

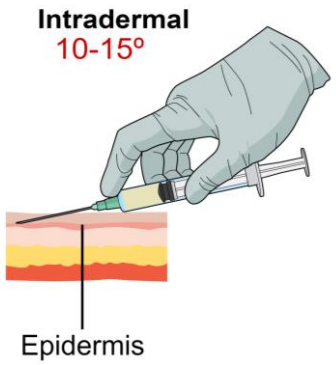


Fig. 28. **Injection technique used in the *ex vivo* skin model**.

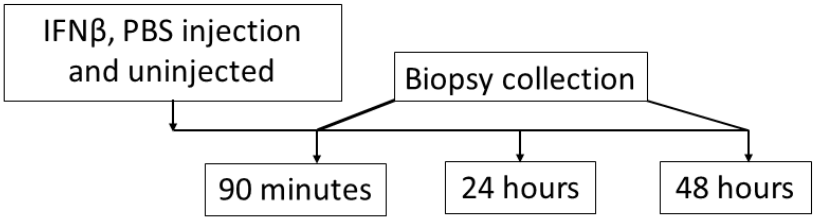


Fig. 29. **Timeline for the *ex vivo* biopsy collection.**

From the skin piece, the injection and biopsy collection were performed with a drug concentration gradient in mind meaning that the uninjected biopsies were collected first, followed by PBS injection and collection. Lastly the lower concentration of IFNβ was injected and biopsies collected, followed by the high dose IFNβ. Skin sites that had stretchmarks or tattoos were not used. The edge of the skin piece was avoided, allowing at least 2 centimeters from the edge to be disregarded.

3.4.1.2 In vivo skin model

MS patient biopsies were collected after s.c. IFNβ injection into the abdomen or thigh. A control biopsy was collected simultaneously on the opposite side of the body (Fig. 30), and the timeline illustrates when the biopsies were sampled (Fig. 31).

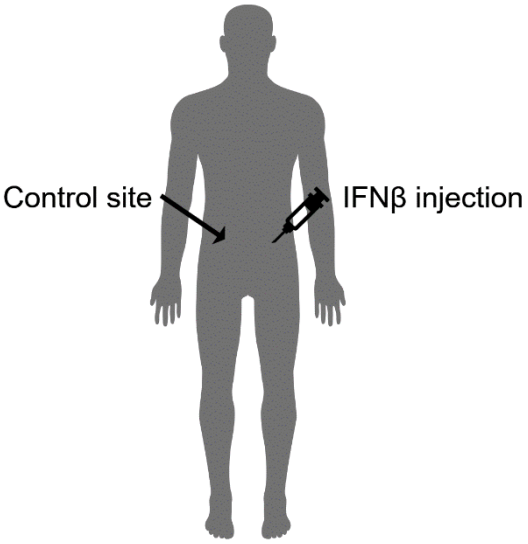


Fig. 30. **An example of injection/biopsy sampling sites.**

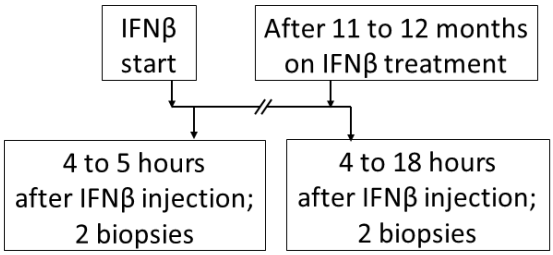


Fig. 31. **Timeline for the *in vivo* biopsy collection.**

3.4.3 DC phenotyping

For phenotyping, flow cytometry was performed on migrated skin cells retrieved from abdominal reconstructive surgery using the antibodies listed in Table II. Cells were kept at 4°C throughout the laboratory procedure and supplemented with fetal calf serum during antibody staining.

Table II. **Antibodies used to phenotype the dendritic cells by flow cytometry.**

Product name	anti-HLADR	anti-CD11c	anti-CD1a	anti-CD14	anti-CD207	anti-CD86
Catalog nr	347402	25-0116-42	559775	345784	IM3577	555665
Isotype	IgG2a, κ	IgG1, κ	IgG1, κ	IgG2b, κ	IgG1, κ	IgG2b, κ
Clone	L243	3.9	HI149	MϕP9	HI149	IT2.2

3.4.4 CD4⁺ T cells

Although the cells had been subjected to a one-step purification by bead separation, it was not sufficient to achieve a pure naïve CD4⁺ T cell population. As a result, the cells were subjected to a second step purification using flow cytometry sorting in order to obtain a highly purified naïve CD4⁺ T cell population (Fig. 32). To identify the population of naïve CD4⁺ T cells, we examined the expression of CD4⁺ and CD45RA.

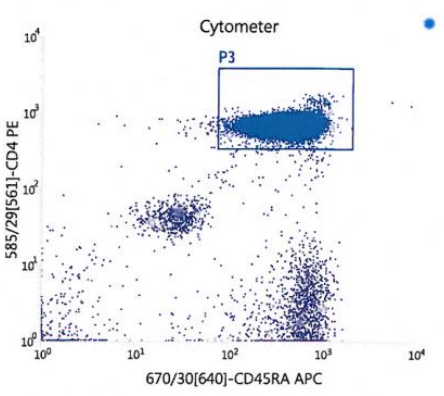


Fig. 32. **Naïve CD4⁺ cell sorting.**

3.4.5 ATP*lite*

To evaluate CD4⁺ T cell proliferation, the ATP*lite* luminescence assay (PerkinElmer) was used. In short, migrated DC were irradiated and cultured with naïve CD4⁺ T cells and proliferation was measured after 5-6 days. As controls, naïve CD4⁺ T cells and DC were cultured separately.

3.4.6 Immunohistochemistry

Immunohistochemistry staining was performed, using the antibodies listed in Table III, on OCT embedded skin tissues from abdominal reconstructive surgery or biopsies from MS patient's on IFNβ treatment. Diaminobensidine and hematoxylin were used for staining and counterstaining respectively. Appropriate isotype controls were included to determine nonspecific binding. The sections were prepared in a way so that the testing condition were on the same slide, i.e. for each antibody testing condition the biopsies from the uninjected site, PBS injection, and the IFNβ injection (from one donor) were on the same slide. Thus, the same condition was implemented for the biopsies and thereby more reliably comparison of staining intensity.

Table III. **Antibodies used during immunohistochemistry.**

Product name	anti-HMGB1	anti-IL-1 beta	anti-IL-6	anti-COX2	anti-PGE2	anti-MXA	anti-HLADR	anti-TLR4
Host	rabbit	rabbit	rabbit	rabbit	rabbit	mouse	mouse	mouse
Catalog nr	ab79823	ab2105	ab6672	ab15191	ab2318	n/a	L243	ab22048
Description	mono	poly	poly	poly	poly	mono	mono	mono
Isotype	IgG	IgG	IgG	IgG	IgG	IgG2a	IgG2a	IgG2b
Serum block	goat	goat	goat	goat	goat	rabbit	rabbit	rabbit
Secondary antibody	goat anti-rabbit					rabbit anti-mouse		

3.4.7 Statistical analysis

Data are presented as the mean, standard deviation, and coefficient of variation. The data was not always normally distributed and was therefore analyzed using non-parametric paired Wilcoxon test. Statistical calculations were performed with GraphPad Prism 6. Significance was defined as a p-value < 0.05.

4 RESULTS AND DISCUSSION

4.1 STUDY I AND STUDY II

Study I

Interferon beta treatment of multiple sclerosis increases serum interleukin-7.

Hypothesis

IFNβ therapy has immunomodulatory properties on the endogenous levels of IL-7 and this effect is important for the treatment efficacy and reduced by NAb.

Reflections

Study I provided laboratory experience including establishing cell culture conditions, setting up flow cytometry and ELISA. In addition, I learned the statistics program GraphPad and other relevant statistical tools.

Study II

Anti-interferon beta antibody titers strongly correlate between two bioassays and in vivo biomarker expression, and indicates that a titer of 150 TRU/ml is a biologically functional cut-point.

Hypothesis

NAb titers measured with two bioassays, MGA and iLite, give comparable titers and the gene expression of IFNβ inducible genes *MX1* and *CXCL10* are significantly reduced in NAb positive patients.

Reflections

Study II introduced me to gene expression analysis, how to use reference genes, and the delta-delta CT analysis method. In addition to this biological threshold value for NAb positivity was thoroughly explored.

4.1.1 Background study I

Outside the HLA region, the *IL-7R* was the first confirmed gene associated with MS (53-55), and a GWAS study identified *IL7* as a MS susceptibility gene (50). IL-7 is a non-redundant survival cytokine essential for T and B cell development and T cell homeostasis (292). IL-7 is mainly produced by stromal and epithelial cells localized in the peripheral tissues (293) including the lymph node, skin, and intestine. IL-7 is particularly essential for T cell biology and plays a crucial role during the maturation of T cells in the thymus, CD4/CD8 lineage choice

during positive selection, and maintains naïve and memory T cell survival and homeostasis in the periphery (293-295). Generally, IL-7 is constitutively produced and its levels are unaffected by external signals (296). However, since IL-7 concentration in serum is elevated in lymphopenic humans (297), the expression can be modulated. A paper published by Lee and colleagues suggested that high endogenous serum IL-7 levels before IFNβ treatment onset, were a predictor of a good response (157). Consequently, we decided to investigate the relationship between IL-7 (genetically associated) and IFNβ (first-line treatment). A year later IL-7 levels could not be confirmed as a prediction of response status using a validation cohort (158), but using our already collected data we found that the serum samples from IFNβ treated MS patients had elevated IL-7 levels.

4.1.2 Aim study I

Determine if IL-7 homeostasis is affected by IFNβ and NAb.

4.1.3 Elevated IL-7 levels as a consequence of IFNβ treatment

For study I, our first finding was that IL-7 levels were elevated in MS serum after IFNβ administration. Whether the dosing schedule had an effect on the IL-7 levels was not initially a research question for us but was later found to be highly relevant for the project. We found that serum samples retrieved shortly after IFNβ administration had elevated IL-7 levels compared to samples collected more than 48 hours after the latest injection, suggesting that IFNβ somehow affected the IL-7 levels as measured by ELISA. Our conclusion was strengthened by the finding that the presence of high NAb titers (>1280 TRU/mL) to IFNβ resulted in significantly lower serum IL-7 levels compared to patients with NAb negative status. With the study II cohort we had the opportunity to validate our finding whether the serum IL-7 levels were affected by IFNβ in 20 MS patients using a paired t-test (not published data) (Fig. 33). The patients had left two serum samples at different time points. Our findings, illustrated in the graph below, show that the overall levels of IL-7 are significantly decreased

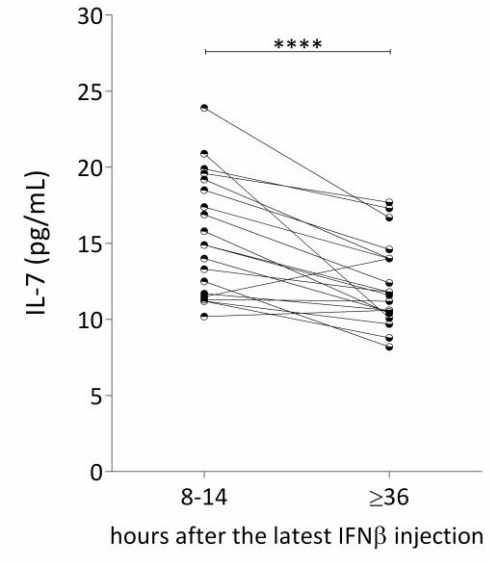


Fig. 33. **Serum IL-7 levels were significantly higher ≤14 hours, compared to ≥36 hours, since**

in patient's serum 36 hours after the IFNβ injection compared to samples retrieved less than 14 hours after the IFNβ injection. Except for two individuals the IL-7 levels went down between the two time points.

Continuing using the study II cohort we could further validate our results showing that the NAb negative patients had elevated IL-7 levels compared to NAb positive patients (NAb titers above 200 TRU/mL) in serum samples retrieved ≥36 hours after the latest IFNβ injection (Fig. 34).

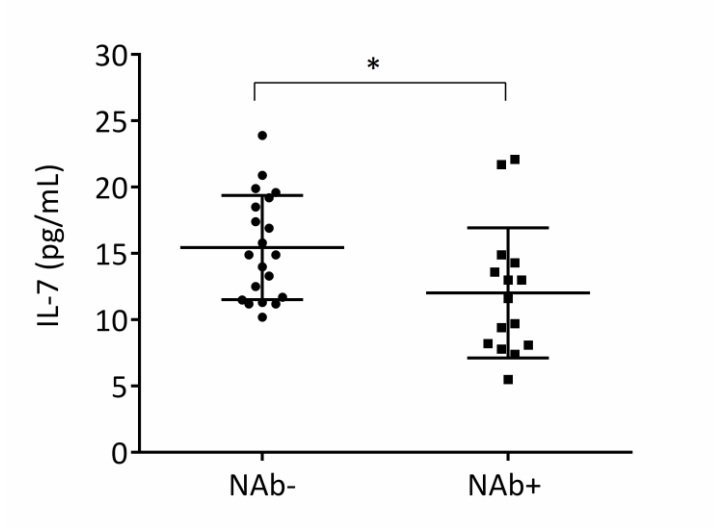


Fig. 34. **NAb positive patients had significantly lower serum IL-7 levels than NAb negative patients.**

In study I, we also had IL-7 data (as measured by ELISA) for some of the patients after they had switched treatment to natalizumab. We concluded that it was the switching of treatment that resulted in decreased IL-7 levels, suggesting it was not the disease itself but rather the treatment that caused the change in concentration. Consistent with our result, Villani and colleagues recently published a paper showing that natalizumab treatment led to significantly reduced serum IL-7 levels in RRMS patients (298).

Based on these observations we speculated that the elevation of serum IL-7 in IFNβ treated MS patients could be due to 1) increased production of IL-7 by stromal cells and/or 2) reduced expression of IL-7Rα on the cell surface. Since IL-7 is known to be constitutively secreted by stromal cells we hypothesized that the elevated IL-7 levels were caused by down-regulation of IL-7Rα rather than that the stromal cells started to produce IL-7 in excess.

4.1.4 Reduced IL-7 consumption of cells exposed to IFNβ

To explore what caused the changes in IL-7 levels, an *in vitro* model was used to study whether the PBMCs were affected by co-culture with IL-7 and IFNβ or IL-7 alone. Measured by ELISA, cells cultured with IL-7 and IFNβ resulted in lower uptake of IL-7 compared to the culture with IL-7 alone; suggesting that the receptor expression was altered on the PBMC when co-cultured with IFNβ.

Using the IL-7 and IFNβ or IL-7 alone as culture settings we could see, by flow cytometry, a dose-dependent decrease in the IL-7Rα expression amongst $CD14^+$ gated monocytes in response to IFNβ compared to cells cultured with IL-7 alone (Fig. 35).

We further showed that MS patients initiated on IFNβ treatment, when followed for up to 13 weeks, had an increase in plasma IL-7 levels, which inversely correlated with a down regulated IL-7Rα expression on their $CD4^+$ T cells. These data collectively support our hypothesis that IFNβ treatment does in fact affect the expression of IL-7Rα.

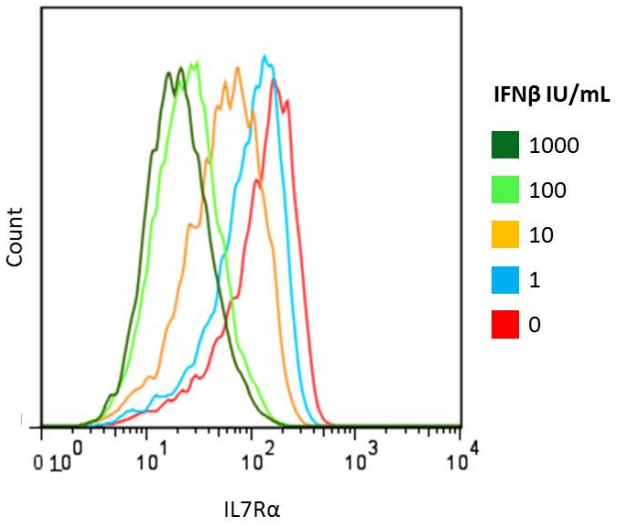


Fig. 35. **IL-7Rα expression was down-regulated on $CD14^+$ monocytes in a dose dependent manner.**

Our findings were further supported by samples from study II, which showed a similar pattern. We could, for example, see that *IL-7Rα* expression (amplification of total cDNA) was affected by NAb and that the NAb negative patients had significantly lower *IL-7Rα* than the ones with NAb and in particular with titers over 200 TRU/mL (Fig. 36).

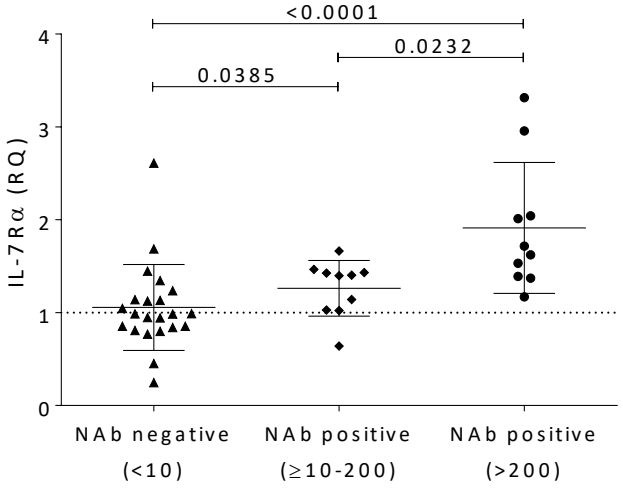


Fig. 36. ***IL-7Rα* expression (total cDNA) was down-regulated in the presence of high NAb titers to IFNβ.**

MxA has an inverse correlation to NAb and the graph below which includes study II samples show that high *IL-7Rα* gene expression levels yield low *MX1* gene expression (r = -0.6029, p = <0.0001) (Fig. 37).

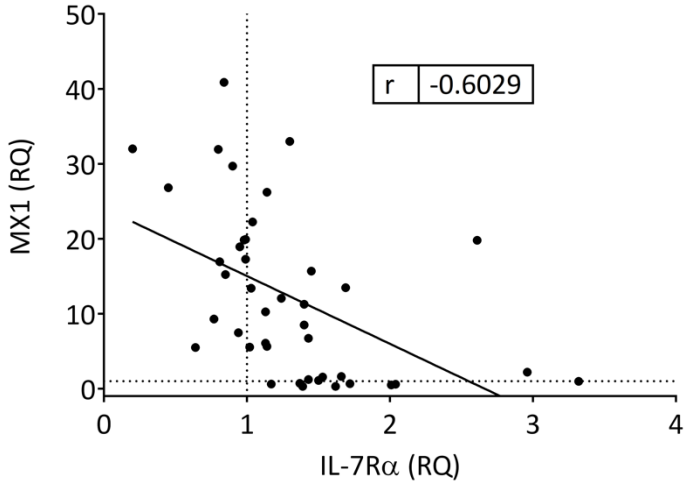


Fig. 37. ***MX1* and *IL-7Rα* expression inversely correlated.**

Furthermore, we could also see that low *IL-7Rα* expression significantly correlated with high serum IL-7 levels (r = -0.3735, p = 0.0209) (Fig. 38).

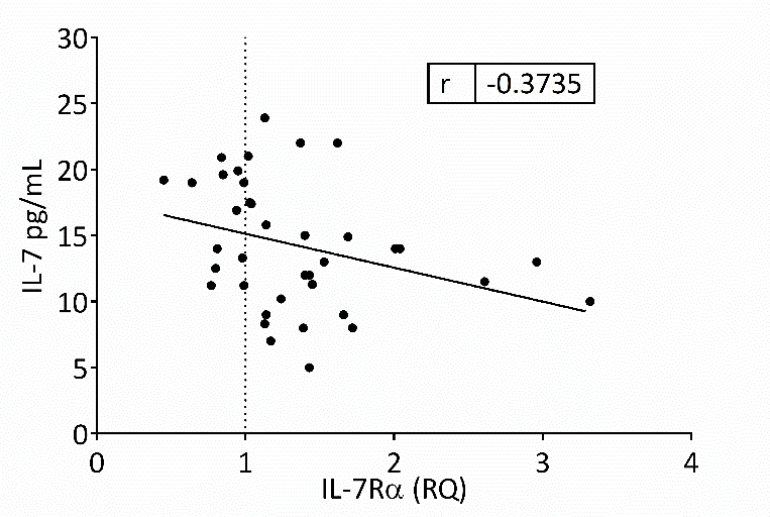


Fig. 38. **IL-7 levels and *IL-7Rα* expression inversely correlated.**

4.1.5 Conclusions for study I

We found a 3-fold increase of serum IL-7 levels in IFNβ treated MS patients compared to natalizumab treated MS patients and healthy controls. Increased IL-7 levels were related to decreased IL-7 consumption, as shown by the lowered *IL-7Rα* expression on cell surfaces. Furthermore, the study provides a biological readout of the impact of NAb on IFNβ treatment and these results highlight the potential importance of a cytokine (IL-7), a receptor (IL-7Rα) genetically associated with MS, and the widely used RRMS treatment (IFNβ). The clinical effect of the elevated IL-7 levels should be further studied since it could potentially provide a

pro-inflammatory stimulus. An interesting follow-up question to address is whether it is the decreased lymphocyte count, the reduced *IL-7Rα* expression, or the combined role that drives up IL-7 levels.

4.1.6 Background study II

Due to the occurrence of NAb to IFNβ it is important to establish reliable methods for NAb detection and to establish at what titer the biological activity of IFNβ is impaired. At the time of study II initiation MGA (278) was used in our laboratory to measure NAb to IFNβ, but were cumbersome and thus we wanted to find another assay that required less maintenance while maintaining reliable results. Therefore we compared MGA and iLite back to back to measure anti-IFNβ-antibody titers in order to evaluate the correlation of titers between these assays and further to estimate a biologically functional cut-point. A study published from our group had previously addressed the biologically functional cut-point and estimated it to be 150 TRU/mL (198).

4.1.7 Aim study II

Compare two bioassays, iLite and MGA, to measure IFNβ specific NAb and to evaluate the NAb titer threshold that abrogates the biological activity of IFNβ.

4.1.8 MGA and iLite gave similar NAb titers

NAb titers measured with the MGA and iLite correlated (Fig. 39). There were some differences between the number of patients classified as NAb positive between the two assays. In the MGA, 64% (28 patients of 44) were recognized as NAb positive whereas iLite identified 48% (21 patients of 44) as NAb positive. Thus there was a discrepancy of 16% (7 patients), but these samples were, however, all classified with low NAb titers in the MGA assay. One thing we discussed during this project was that the serum concentration (MRD) might affect the readout and sensitivity of the assay (289). The MGA dilute the serum 1 to 10 (10%), whereas iLite diluted 1 to 20 (5%) and iLite thereby has a lower serum concentration in the assay. When validating the iLite method in study III we discovered that adding serum to the assay media increased the cells sensitivity to respond to IFNβ (299). I therefore assume that if we would

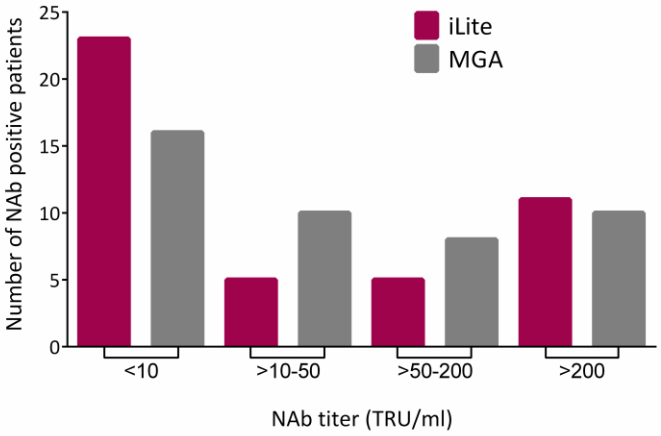


Fig. 39. **Comparison of IFNβ NAb titers using iLite and MGA.**

reanalyze these 44 serum samples with the validated iLite IFNβ assay (study III) the seven samples that tested NAb negative might have turned out low positive.

4.1.9 NAb titers above 150 TRU/mL block the effect of IFNβ

To estimate a biologically functional cut-point we estimated the gene expression of two IFN-induced genes, *MX1* and *CXCL10,* following IFNβ injection and correlated with the NAb titer. We found that IFNβ administration caused upregulation of *MX1* and *CXCL10* (Fig. 40). IFNβ upregulated the expression of the two studied genes in NAb negative subjects and NAb positive individuals had a titer-dependent blockage of the IFNβ effect.

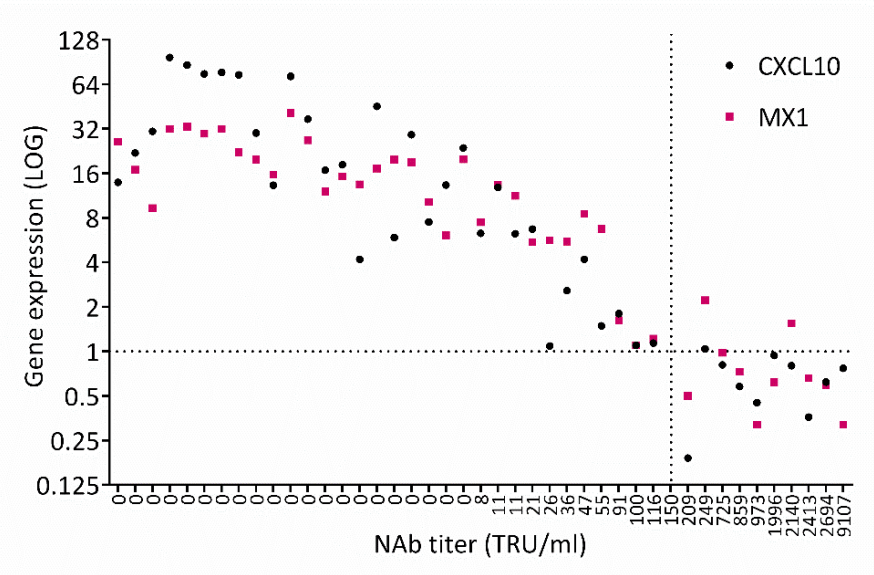


Fig. 40. **Correlation of the relative gene expression of *CXCL10* and *MX1* with NAb titers (measured by iLite).**

This effect was also seen at protein level where NAb positive patients had significantly lower levels of protein CXCL10 in the blood compared with NAb negative patients, as measured by ELISA (Fig. 41).

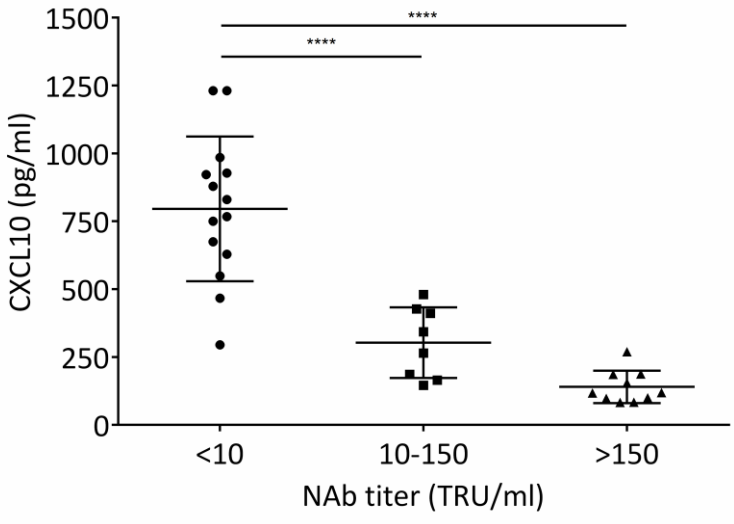


Fig. 41. **Protein CXCL10 levels were down-regulated in the**

4.1.10 Conclusions for study II

The traditional cell based inhibition assay (MGA) and the luciferase reporter gene assay (iLite) were directly comparable when measuring IFNβ-specific NAb titers and could therefore replace MGA in our routine setting. We further showed that both assays identified a medium titer of 150 TRU/mL as a biologically threshold for significant neutralization as previously shown (198). Our results also show that lower NAb titers may impact the effect of IFNβ, although to a lesser extent. We therefore believe that it would be of biologically relevance to set a new evidence-based threshold for both assays. We also believe that the NAb levels should be monitored regularly during treatment with IFNβ as NAb clearly affect IFNβ bioavailability. The activity of IFNβ is already affected at titers between 10 and 150 TRU/mL and treatment should be monitored, and discontinued, if treatment effect decreases. In the case of confirmed NAb titers above 150 TRU/ml switching drugs should be considered even before breakthrough of disease is evident.

KEY POINTS

- IFNβ treatment leads to elevated serum IL-7 levels.
- Cells exposed to IFNβ *in vitro* have reduced IL-7 consumption.
- MGA and iLite were directely comparable to measure IFNβ-specific NAb titers.
- NAb titers above 150 TRU/mL block the effect of IFNβ *in vivo*.

4.2 STUDY III

Development and validation of cell-based luciferase reporter gene assays for measuring neutralizing anti-drug antibodies against interferon beta.

Hypothesis

A validated bioassay, according to industry recommendations, will provide a sensitive and reproducible assay for quantification of NAb to IFNβ.

Reflections

Initiated at the outset of my Ph.D. research, this study afforded me greater experience of how to ensure quality assay design – a skill highly relevant for my other studies during my Ph.D. Moreover, this project gave me the opportunity to learn advanced statistical concepts, calculations, and SOP documentation.

4.2.1 Background

The majority of routinely used cell-based IFNβ NAb assays utilize the Kawade principle to calculate NAb titers (including MGA and iLite). The Kawade method has statistical limitations as it assumes that NAb only are relevant if they can neutralize IFNβ by at least 90%. The cut-point approach is designed to be more sensitive than the Kawade method since it relays on a more robust statistical evaluation using a group of individuals (around 50 is recommended) to identify a cut-point where a signal above is recognized as NAb positive.

This work was part of the "Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK" (ABIRISK) consortium. All work with the iLite assay was performed at Karolinska Institutet, Stockholm and the LUC assay was validated at two sites including Innsbruck Medical University and Rigshospitalet Copenhagen. Since my responsibility was to address re-development and validation of iLite, the data presented here describes mainly the work with iLite.

4.2.2 Aim

The aim of this study was to re-develop and validate the LUC and iLite cell-based assays using a cut-point design, instead of the Kawade principle, to identify NAb positive samples to IFNβ.

4.2.3 Pre-validation

The pre-validation was performed using three steps. In short, as a first step IFNβ dose response curves were run to determine the EC50 (50% of IFNβ maximum effect on the cells). As a second step, the selected stimulation concentration of IFNβ (EC50) was used to perform

anti-IFNβ antibody inhibition curves with the aim to select a low- and a high IFNβ positive control. In a final step, the selected EC50, HPC and LPC were tested, using the Z-factor score, to ensure quality of the assay.

4.2.3.1 Serum increased the assay sensitivity

During our extensive pre-validation work, we noticed that the addition of human serum (1 in 20 to 1 in 50) to the assay media increased the sensitivity and recovery of the assay (Fig. 42). After running several anti-IFNβ antibody dose response curves, we chose to include 2.5% (1 in 40) human serum to the assay media, demonstrating the importance of addressing the matrix effect.

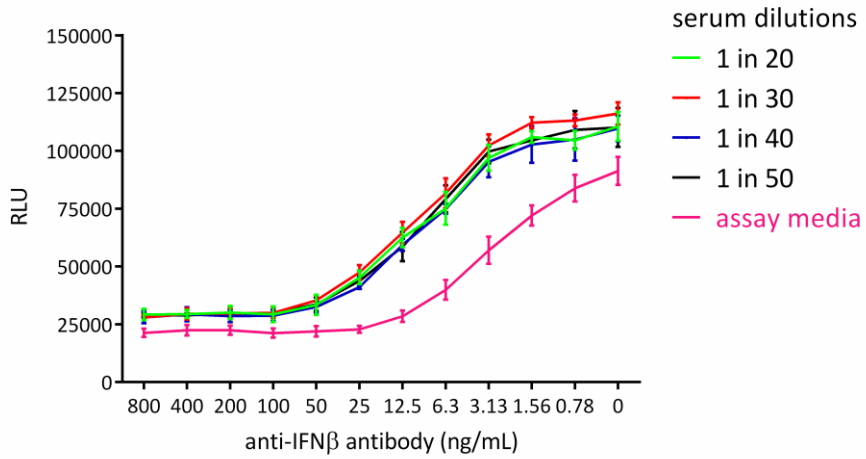


Fig. 42. **Added serum to the assay media made the cells more receptive to IFNβ stimulation.**

4.2.3.2 The EC50

The first step in the pre-validation work was to establish an EC50 i.e. the IFNβ concentration required to stimulate the cells by 50% of their maximum. For iLite, this was calculated to be 1.3 international unit/mL (Fig. 43). No significant variations were found between the EC50 values using the different serum dilutions (1 in 20 to 1 in 50) used for the dose-response curve experiment.

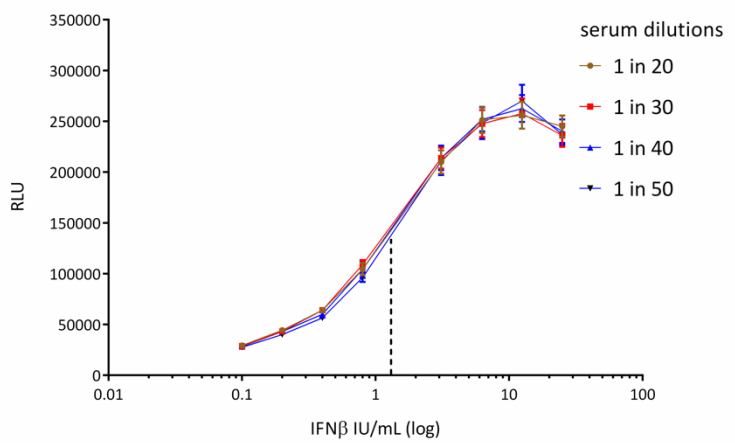


Fig. 43. **Calculation of the EC50 using dose-response curves.**

4.2.3.3 High and low positive controls

We then used the selected EC50 concentration of IFNβ to perform anti-IFNβ inhibition curves to define the high and low positive control. The HPC and LPC were defined using 11 concentrations of the anti-IFNβ antibody that was diluted 2-fold from 800 ng/mL to 0.78 ng/mL in assay media containing human pooled serum at concentrations 1 in 30, 1 in 40, 1 in 50, 1 in 60 and assay media only (Fig. 44). The concentrations of anti-IFNβ antibody that inhibited the maximum EC50 signal by 25% (LPC) and 60% (HPC) was calculated to correspond to 480 ng/mL and 800 ng/mL respectively in neat serum.

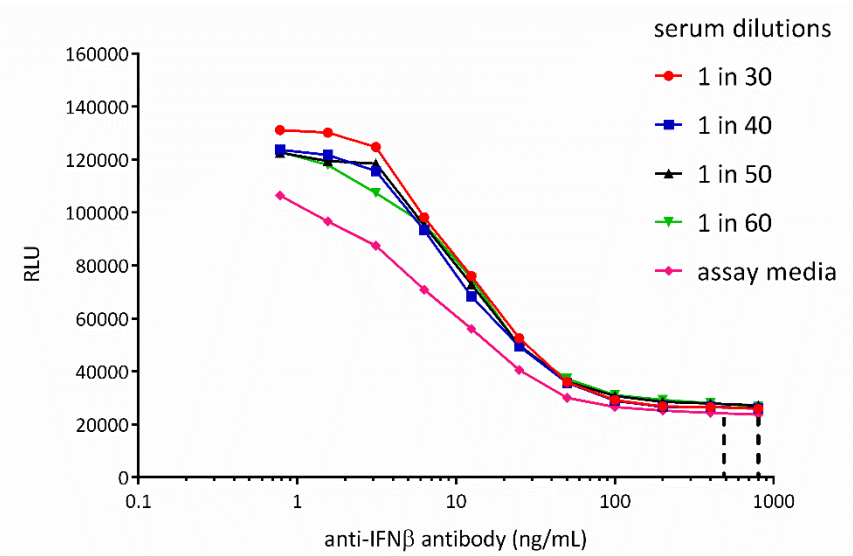


Fig. 44. **Selection of a high and low positive IFNβ control.**

4.2.3.4 Z-factor

The quality of the assay was assessed by calculating the Z-factor score. We found that the HPC and LPC had a Z factor of 0.76 and 0.65 (separation score from the negative signal) respectively when using assay matrix 1 in 40. Since a score between 0.5 and 1 is considered to reflect an excellent separation of the positive and negative signal, the pre-validation criteria´s for iLite were therefore accepted and allowed us to start the validation work (Table IV) using 1 to 40 as dilution factor.

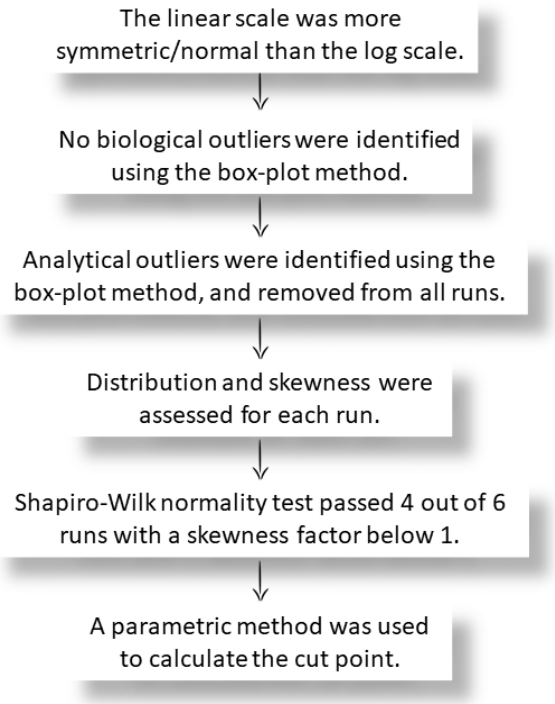
Table IV.

Serum dilution	**Z factor**	
	LPC	HPC
1:30	0,83	0,77
1:40	**0,76**	**0,65**
1:50	0,75	0,70
1:60	0,76	0,76

4.2.4 Validation

4.2.4.1 A floating cut-point was applied

The assay cut-point is the response level that defines if a sample response is positive or negative. The cut-point was retrieved as summarized in the flowchart below.



In short, the healthy control serum samples were more normally distributed and symmetrical using the linear scale (W = 0.92, skewness = 0.39) compared to log-transformed data (W = 0.91, skewness = 0.32) as indicated by that the W factor and skewness factor were bigger for the linear distribution (Fig. 45).

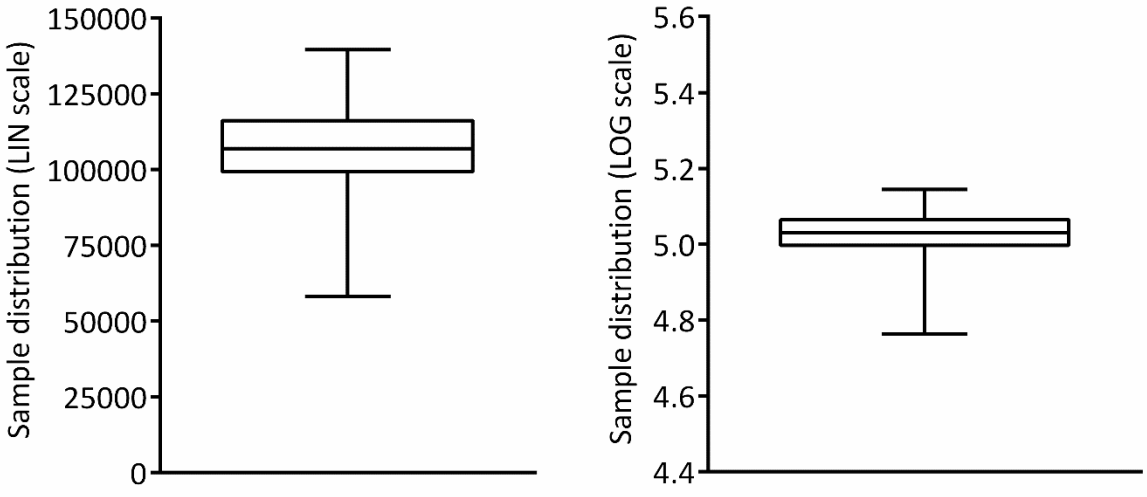


Fig. 45. **Evaluation of the sample distribution using the linear scale versus the logarithmic scale.**

Therefore, we did not need to transform the data for continued work. The box plot method was used to illustrate the differences and spread of the samples and no biological outliers were identified. However, two analytical outliers were identified and these were removed from the individual runs. Biological outliers refer to the removal of samples that stands out from a response rate from the whole population tested. Analytical outliers refer to samples that are outliers, compared to the other samples, in one individual run. For the majority of runs, Shapiro-Wilk test indicated normal distribution since the p-value was higher than 0.05, and a skewness coefficient below 1. The healthy control samples used to establish the cut-point was confirmed to be directly proportional to the response of the negative control, which could be used for normalizing of the cut-point (Fig. 46).

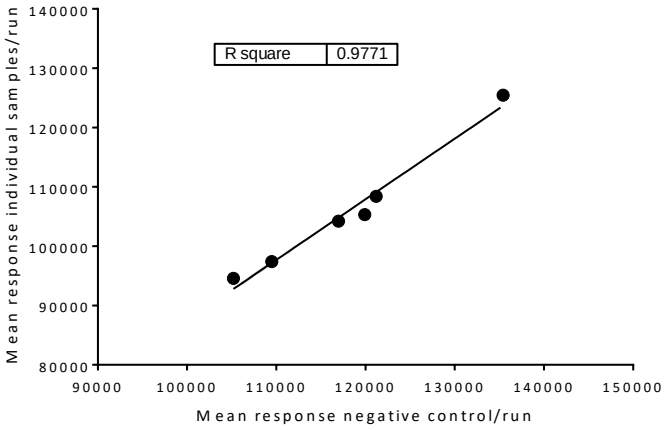


Fig. 46. **The response values of the negative control was directly proportional to the healthy control samples.**

The parametric approach was used to calculate the cut-point (284). The majority of immunogenicity assays use a floating cut-point, which has the advantage of not being sensitive to variation in signal between plates. In our assay, a floating cut-point was used since the variance around the mean was constant but mean response of negative control varied between plates and analysts.

4.2.4.2 Limit of detection

Next, we assessed assay sensitivity by running antibody dilution curves. The anti-IFNβ antibody was titrated spanning the cut-point. The sensitivity of the assay gives an indication of the lowest concentration analyte (NAb antibodies) that can be statistically distinguished from the background signal, and at the same time adequately precise to be quantified. The iLite assay was shown to have a very high sensitivity with a limit of detection (LOD) of 320 ng/mL.

4.2.4.3 iLite lot variation stopped further validation work

In the end of the validation work, we had to switch iLite batch due to that the iLite kits we started the validation process with were used up. To our surprise, the cells from the three new batches responded differently to IFNβ stimulation. As illustrated in the graph below the new cell lot´s responded significantly less than the originally used lot that the cut-point was based upon (Fig. 47).

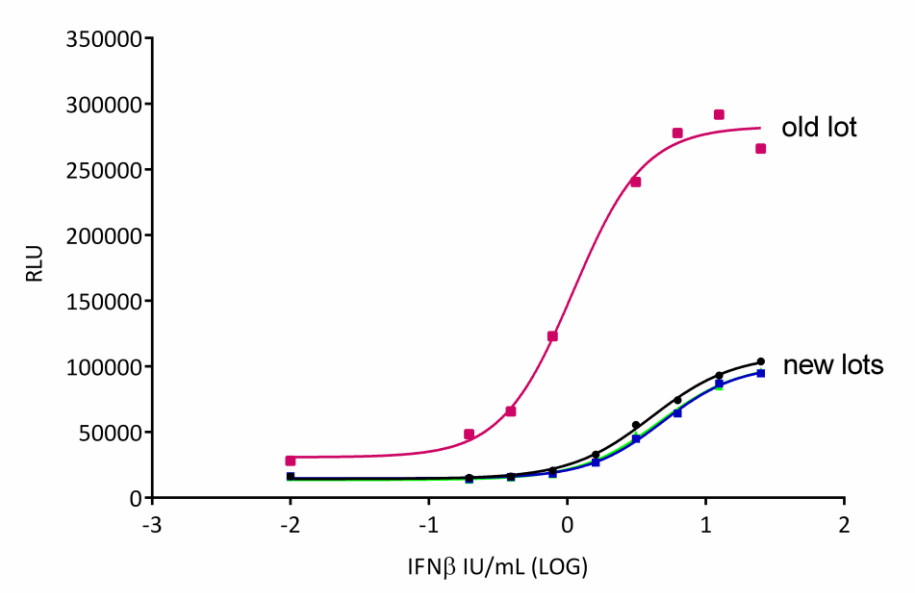


Fig. 47. **Cell batch variations were identified between lots.**

The batch differences turned out to be a major problem for us since it affected our cut-point based method and resulted in that the established EC50 could not be applied to the new cell batches, and that the cut-point needed to be re-established.

4.2.5 The importance of a validated bioassay

I believe that all ADA bioassays must be validated by using the same internationally agreed standard to ensure quality and reliability of the data generated. If we compare ADA frequency between different laboratories there is a huge discrepancy in the percentage being positive. The differences can, of course, be based on many factors but I believe that the assay sensitivity is one major issue. Validation will provide proof that what the assays detect is actually what you want it to detect and that it does not suffer from artifacts. The long-term goal was to design an assay that was sensitive enough to find all patient samples that were reactive (NAb) towards the drug (IFNβ). Thereafter we would investigate the specificity of the reactivity to establish a clinically relevant threshold for when the NAb would affect the drug in a way that it becomes relevant for the patient, i.e. when the patient loses the effect of their treatment.

4.2.6 Conclusions

This work provides a detailed description of re-development and validation of two luciferase reporter gene bioassays, LUC and iLite, using a cut-point approach to identify NAb positive samples in IFNβ treated MS patients. The validation was conducted according to the latest assay guidelines preferred by the pharmaceutical industry and governmental regulatory agencies. Due to the batch differences using the iLite assay, this assay could not be used for NAb testing. Instead, the LUC (Copenhagen) assay was chosen.

The use of the cut-point increased the assays sensitivity for NAb detection. Using the validated LUC (Copenhagen) cut-point assay, 12% more NAb positive samples were identified when compared with the Kawade method. We could further show that discrepant samples (NAb negative with Kawade and NAb positive with cut-point) had an average relative gene expression level that was two-fold (*MX1*) and four-fold (*CXCL10*) lower than the samples that were NAb negative with both assay designs (Fig. 48). This reduction indicates a possible biological effect even for low NAb titers that could be of clinical importance, although the relevance needs to be further evaluated.

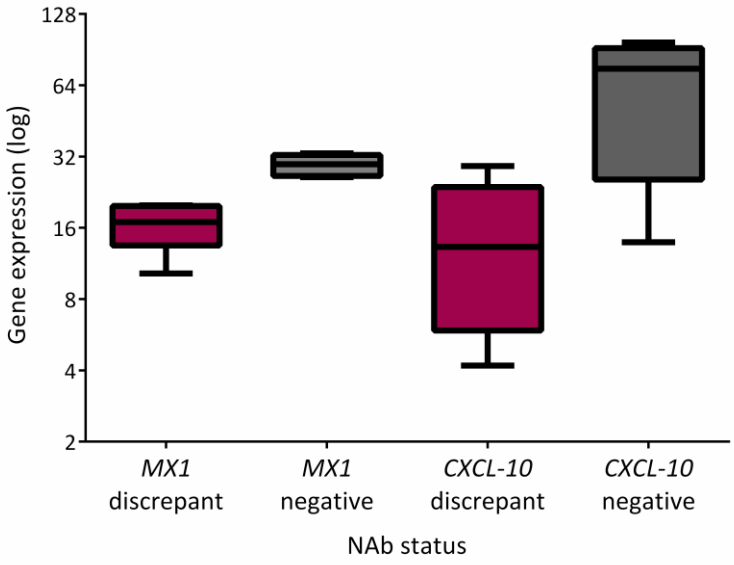


Fig. 48. **NAb discrepant samples had an average relative gene expression that was two-fold (*MX1*) and four-fold (*CXCL10*) lower than the samples that were NAb negative.**

KEY POINTS

- Validation was performed following the latest assay guidelines accounting for matrix effects and using cut-point design.
- iLite showed high sensitivity and specificity.
- The validation work resulted in increased sensitivity to detect NAb.

4.3 STUDY IV

Measurement of serum infliximab levels and detection of free and bound anti-infliximab antibodies in patients with rheumatoid arthritis

Hypothesis

Monitoring of serum IFX levels and ADA in RA patients will enable identification of patients with optimal treatment response, give an indication to why some patients not benefit from this type of treatment and what treatment alternatives that instead should be considered.

Reflections

Because my previous work has primarily been focused on IFNβ (cytokine) and MS, this project afforded me new insight into the complexity of autoimmune diseases and mAb therapy.

4.3.1 Background

For RA patients on TNF-α inhibitor treatment, serum drug trough levels are not routinely monitored in the clinics. As of today, there is no consensus on how often IFX levels should be measured or the optimal drug trough level. For the optimal patient benefit, individualize therapeutic regimens should be implemented. A study by Casteele and colleagues showed that an IFX serum trough concentration between 3 to 7 μg/mL was optimal dosing for patients with inflammatory bowel disease (300). Another study showed that IFX treated patients with inflammatory bowel disease with a serum trough level below 6.2 μg/mL were more likely to experience loss of response (301). Since no published guidelines are available for the optimal trough IFX concentration in RA patients, we chose to implement the recommendation suggested by Casteele for our study. A drug level below 3 μg/mL was regarded as too low, a level above 7 μg/mL as too high, and a level of 3-7 μg/mL as optimal. These recommendations must, however, be correlated to the patient´s clinical parameters to assess whether this interval is the correct dosing regimens for our cohorts. This will be performed when we receive data from the Swedish Rheumatology Quality register (SRQ).

4.3.2 Aim

The aim and long-term goal of this study was to combine pharmacokinetic and immunogenicity results with the patients' clinical response to propose an algorithm for monitoring and interpreting the treatment response to IFX. Moreover, we investigated what type of assay that should be used for monitoring of the IFX immunogenicity.

4.3.3 Few patients have an optimal TNF-α inhibitor drug level

One part of the project was to study the serum TNF-α inhibitor drug levels in people with RA. Overall, in both the SWEFOT and REALlife cohorts we observed that only a quarter of the patients had an optimal drug concentration (Fig. 49). In the SWEFOT cohort, only 24% of the patients had a drug concentration within the optimal range of 3-7 µg/mL after 21 months on TNF-α inhibitor treatment. Too low drug levels (<3 µg/mL) were found in 46%, and too high drug levels (>7 µg/mL) were found in 31%. In the REALlife Stockholm cohort (either new to treatment or >2 years on treatment), 70% had an IFX level below 3 µg/mL and only 6% above 7 µg/mL. Only 24% had a drug level of 3-7 µg/mL. Despite differences in the number of individuals with either too low or too high drug levels, the two cohorts exhibited the same fraction of patients with optimal dosing interval (24%). Thus, the majority of patients appear not to have the ideal drug concentration and our data, therefore, reinforce the importance of "drug level test adjusted treatment strategies" at the clinic to allow for an optimal dosing plan.

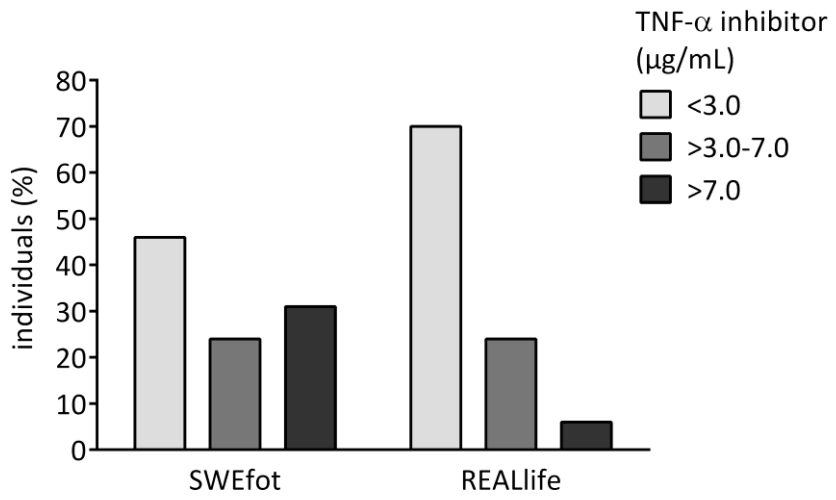


Fig. 49. **TNF-α inhibitor drug levels in two RA cohorts.**

4.3.4 PandA could detect ADA in samples with detectable drug levels

4.3.4.1 The pattern for ADA positivity differed between the ELISA and PandA

ADA could be detected in the majority of serum samples with a drug level below 0.2 µg/mL, as measured by ELISA. What was unexpected in the SWEFOT cohort was that the ADA results, if categorized for the individual patient over time, could be classified into seven groups based on the different patterns for ADA positivity (Table I, manuscript nr. IV). It should be noted that patient serum samples with a drug level above 0.2 µg/mL are not screened for ADA since the ELISA is drug sensitive. Therefore, a sample with a drug level above 0.2 µg/mL is automatically regarded as ADA negative without actually being screened for ADA. However, we had the advantage of having the PandA method (acid dissociation) that allowed samples to be screened for ADA regardless of the drug concentration. Thus, we analyzed some of the

patient's three consecutive samples at 3, 9 and 21 months post-treatment initiation that had not previously been screened for ADA. When the patients were classified according to the ADA results retrieved with the PandA, the pattern for ADA positivity changed (Table II, manuscript nr. IV), and we found that ADA could be detected earlier with the PandA assay than with the ELISA. Moreover, the number of patients regarded as ADA transient reduced from fifteen (as measured by ELISA) to two with the PandA method. Though the clinical importance of these findings need to be further evaluated, it gives us the opportunity to analyze some of the samples with unexpected results in the ELISA. As an example, one patient sample tested ADA positive after 3 months post-treatment initiation (drug level below 0.2 µg/mL) using the ELISA. At 9 months post-treatment initiation, the same patient left a sample with a drug level of 1.8 µg/mL and were thus categorized as ADA negative or rather undetermined as ADA could not be measured by ELISA. At 21 months post-treatment initiation, the patient tested ADA positive again (drug level below 0.2 µg/mL). The sample at 9 months post-treatment initiation was therefore tested with PandA and tested ADA positive (with a high titer). This is an example when the PandA method is useful as a complement to the ELISA method.

4.3.4.2 Undetectable drug levels and ADA negative

All patients in the REALlife cohort with a drug level below 0.2 µg/mL tested ADA positive with ELISA, whereas in the SWEFOT study 14% with a drug level below 0.2 µg/mL did not have detectable ADA with ELISA. We speculated that the samples had drug/ADA immune complex formations that could be separated using the PandA assay. However, only one sample showed moderate ADA reactivity when measured with PandA. In the REALlife cohort (which includes more patients than SWEFOT), we could not find any samples showing the same pattern of having low drug levels and at the same time be ADA negative. One difference between the two cohorts is that all patients in the REALlife study are treated with IFX, which is administrated intravenously at the clinic. In the SWEFOT cohort, the patients could have switched treatment to adalimumab or etanercept, both drugs are administrated s.c. at home. Although we have no evidence of this, one should be aware of the possibility that the undetectable drug levels could be due to poor treatment compliance.

4.3.4.3 Up to what drug trough level should ADA be monitored?

As ADA is only measured in serum samples with a drug concentration below 0.2 µg/mL we wanted to investigate if we could detect ADA in serum samples with a drug concentration between 0.2 µg/mL to 7 µg/mL. Interestingly, ADA reactivity could indeed be detected in many of these samples using the PandA method (Fig. 50). In the SWEFOT cohort, we found more ADA reactive samples than in the REALlife cohort (Stockholm). My interpretation of the difference seen between cohorts is that the SWEFOT cohort included patients from treatment initiation for up to 21 months, whereas the REALlife cohort were either new to treatment or

had been treated for more than two years. Therefore, the samples in the SWEFOT cohort were retrieved in a timeframe when most patients would develop ADA and identification of ADA reactive samples are thus expected. In contrast, the REALlife study included patients who had been treated long term and therefore likely to respond well compared to the ADA positive patients who may already have switched to other treatments. Furthermore, the newly treated patients included in this cohort may not yet have developed ADA. In the graph below (Fig. 50), the dotted line on the y-axis represents the cut-point for ADA reactivity using the PandA method. As previously mentioned the significance of these findings needs to be further addressed and coupled to clinical data (SRQ registry). As of now we speculate that a recommendation for ADA screening should be implemented for serum samples with a drug level below 3 µg/mL.

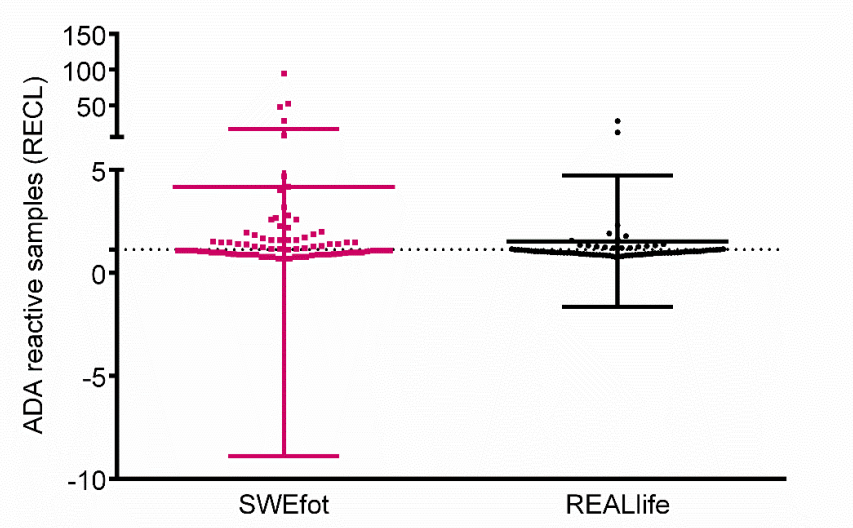


Fig. 50. **PandA identified ADA reactivity in serum samples with detectable drug levels.**

4.3.5 Neutralizing ADA (iLite) correlates to a certain extent with % inhibition (ELISA)

Serum samples from the REALlife (Stockholm) cohort that tested ADA positive with the ELISA were further tested for neutralizing ADA using the iLite bioassay. iLite characterized 66% of the patients (19 of 29 patients) or 71% of the samples (25 out of 35 samples tested from the 29 patients) as positive for neutralizing ADA. We then decided to correlate the RLU signal generated in iLite with the % inhibition (to the blank) in the ELISA measurement to investigate if the % inhibition was predictive of the ADA neutralizing capacity (Fig. 51). The dotted line on the y-axis represent the cut-point for when the sample is regarded as NAb positive (iLite). The dotted line on the x-axis represents the % inhibition in the ELISA where we suggest could be a hallmark for when a serum sample should be regarded as neutralizing. Overall the correlation of the assays were good. The samples that tested NAb negative in the iLite assay clustered together with the samples that had a low % inhibition in the ELISA, with the exception of two samples (samples are marked in blue and green). The samples that had ≈100% inhibition (ELISA) clustered with the highest RLU values (iLite), with the exception of

one sample (marked in green). However, as many as ten samples tested NAb negative with the iLite assay that tested ADA positive with the ELISA. Since the iLite assay have the potential of drug/ADA interference, we wanted to run these samples on PandA.

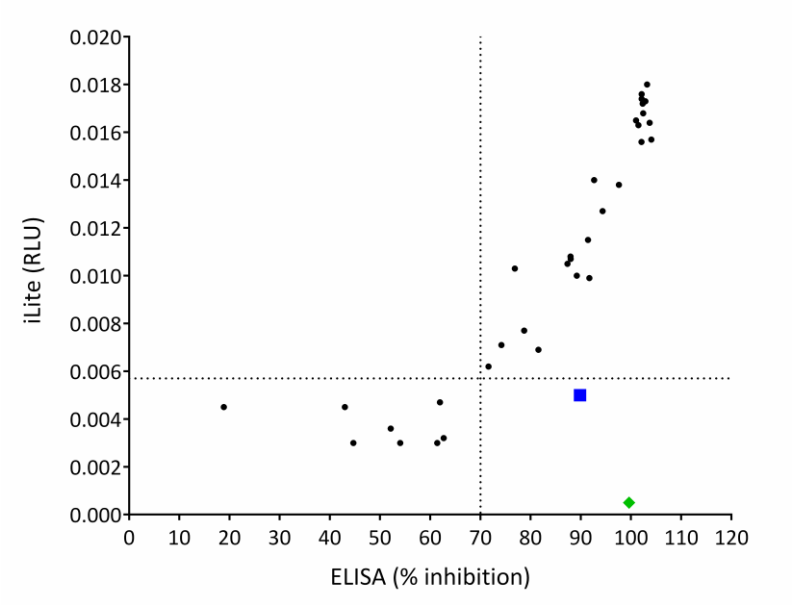


Fig. 51. **Correlation between the % inhibition (ELISA) versus NAb (iLite).**

The PandA assay identified 92% (36 out of 39 samples) as ADA reactive (not confirmed ADA positive) (Fig. 52). The dotted line on the y-axis represent the cut-point for when the sample is regarded as ADA positive (PandA). The dotted line on the x-axis represents the cut-off for the % inhibition that suggest neutralizing ADA as measured by the ELISA. Out of the ten samples that tested NAb negative in the iLite, only three samples tested ADA negative with the PandA but the seven samples that tested positive had a very low ADA reactivity (<1.5 Relative ECL) and would most likely turn ADA negative when titrated in the confirmatory step.

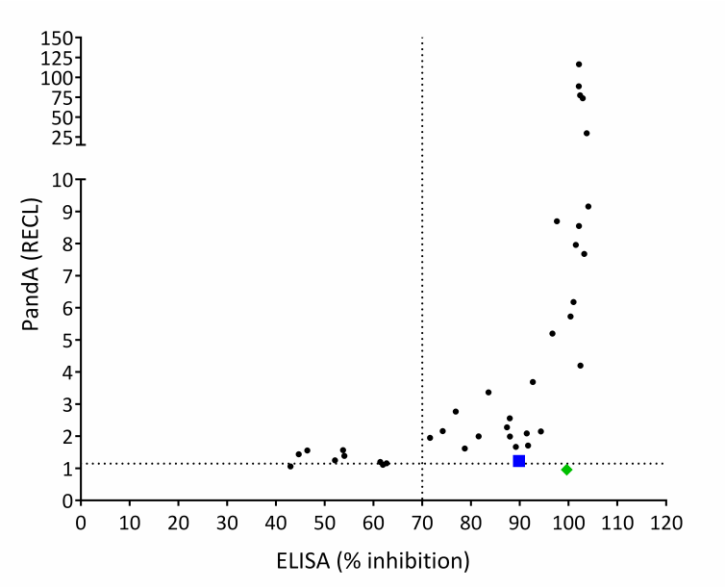


Fig. 52. **Correlation between the % inhibition (ELISA) versus ADA (PandA).**

The results from the iLite and PandA have thus a good correlation (Fig. 53). The dotted line on the y-axis represent the cut-point for when the sample is regarded as ADA positive (PandA). The dotted line on the x-axis represents the cut-point for when the sample is regarded as NAb positive (iLite).

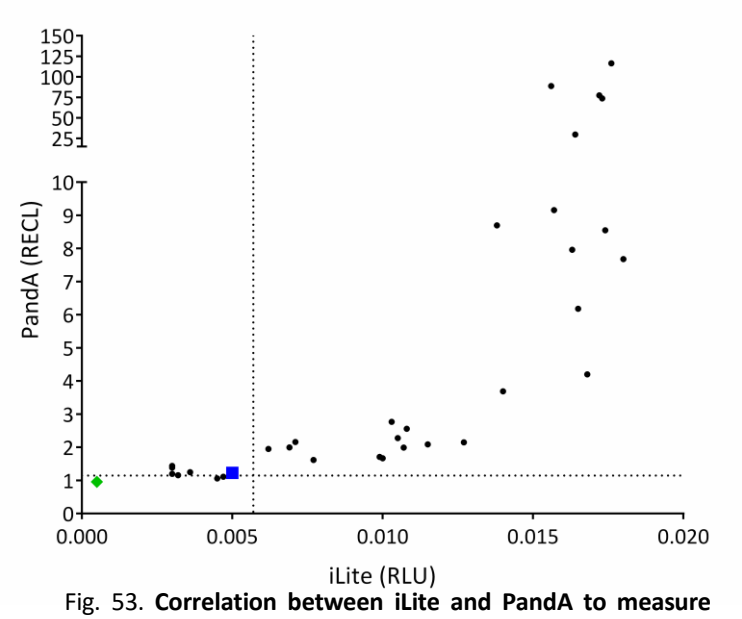


Fig. 53. **Correlation between iLite and PandA to measure NAb and ADA respectively.**

In summary, out of the 40 samples that tested positive in the ELISA, 93% (36 out of 39) tested positive for ADA reactivity in the PandA, and 71% (25 out of 35) tested NAb positive in the iLite assay (Table V).

Table V. *Comparison of frequency of ADA/NAbs to IFN β*

	Positive	Negative	Samples tested
ELISA (ADA)	40	n/a	40
iLite (NAb)	25	10	35
PandA (not confirmed) (ADA)	36	3	39

A study by Schie and colleagues showed that the majority of ADA to IFX (>90%) were neutralizing (87), but we identified fewer patients with neutralizing ADA (66%). Our data was strengthened by that samples that tested NAb negative with the iLite assay also tested ADA negative or had very low ADA reactivity in the PandA assay. Thus, the ELISA is more sensitive, but might lack in specificity.

Collectively, our data suggest that serum samples with an inhibition below 70% should not immediately be regarded as neutralizing without further confirmatory NAb/ADA testing. Since the PandA and iLite assays correlate well, any of these two assay could be an option to confirm ADA/NAb positivity. If no NAb or high titer ADA can be detected with the iLite or PandA, dose escalation and/or increased dosing intervals might be beneficial.

4.3.6 Proposed treatment algorithm

Based on the results of the IFX concentration and ADA measurements retrieved so far we suggest the following preliminary algorithm for monitoring of IFX. These recommendations will, however, be adjusted when the clinical data are analyzed from the SRQ registry. Treatment decisions should be adjusted depending on the patient's disease state.

As a pilot algorithm, we suggest that the patient's drug level is monitored with ELISA every 3 months during their first treatment year (Fig. 54). Patients with either a very low (<0.2 µg/mL) or low (0.2-3 µg/mL) drug level should be analyzed for ADA with ELISA or PandA. If the patient is ADA positive, it would suggest that the patient should change treatment plan if they have an active disease. Patients in remission could stop their current treatment and await a new treatment plan. If the patient is ADA negative they should change treatment if active disease, since this indicates that they probably need a drug with another mode of action. Patients in remission should continue treatment since they most likely have benefit of their treatment. If the patient has high drug levels (>7 µg/mL), dose tapering might be beneficial. By combining monitoring of drug levels and ADA with clinical data, the clinician will be able to optimize the treatment of individual patients in a more efficient way and thus both reduce patient disease management and healthcare costs. As of today, the red numbers are still unknown.

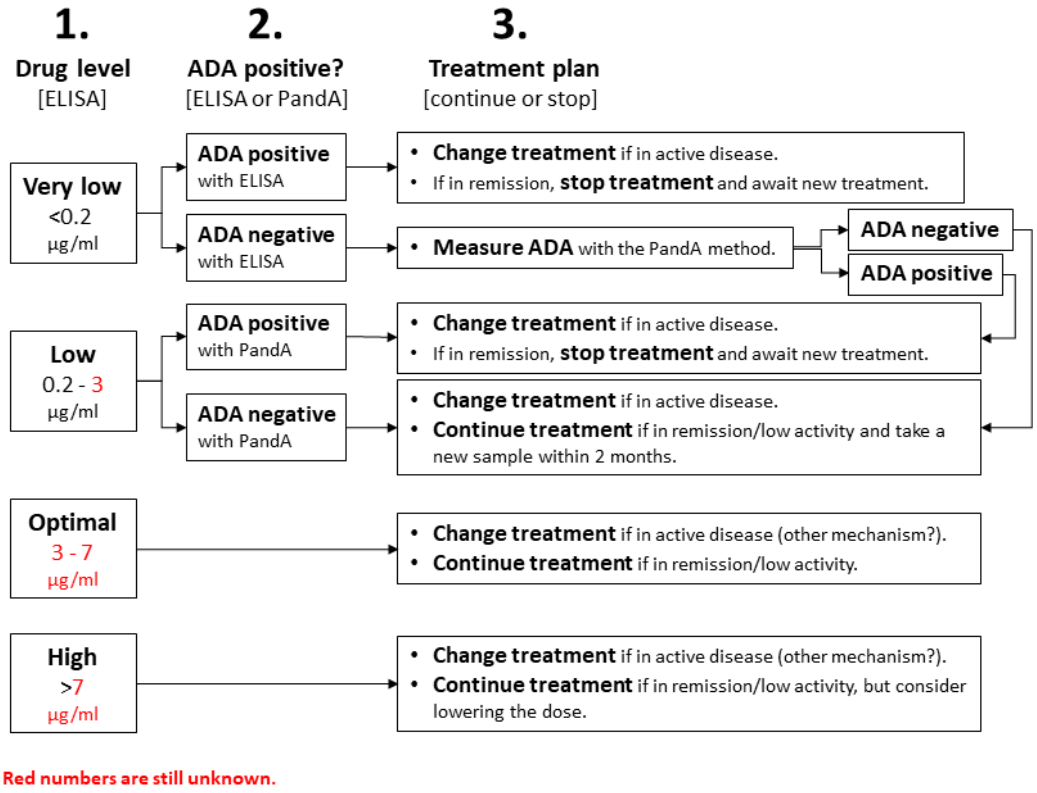


Fig. 54. **Pilot algorithm to monitor IFX treatment.**

4.3.7 Conclusions

A substantial proportion of RA patients are considered non-responders to their TNF-α inhibitor treatment. Not only is it a problem for the patients that are on a treatment plan that is ineffective and therefore not actually giving the intended treatment benefits, but it is also a problem from a health-economical perspective. In Sweden alone, if we estimate that around 30% of the treated patients with the top 10 most sold drugs in Sweden are ADA positive, then ≈815 million Swedish kronor are spent on drugs that are ineffective yearly.

We as researchers have the responsibility to translate our findings to clinicians in an effective way in order to facilitate implementation of translational research. Rogers model of diffusion of innovation describes how new knowledge can be distributed efficiently and are categorized into four groups: (1) innovation (2) communicated through certain channels (3) over time (4) among the members of a social system (302). With the data generated summarized in the treatment algorithm, we hope to channel out our experience and contribute to easier treatment decision making.

KEY POINTS

- Only 24% of the patients had optimal drug levels after 21 months of treatment.
- Serum samples with a drug level above 0.2 µg/mL were screened for ADA with PandA and up to 51% tested positive for ADA reactivity.
- Neutralizing ADA was found in 66% of the patients tested.

4.4 STUDY V

Different interferon beta preparations induce the same qualitative immune response in human skin

Hypothesis

Repetitive IFNβ injections activate skin dendritic cells and promote the dynamic recruitment of other cells to the injection site that over time correlate with risk of ADA development.

Reflections

I found this study particularly challenging due to the laboratory skills required while it led to personal development as a researcher. In this project I worked independently with the laboratory experiments and solved problems as they came along. This led, among other things, to me spending two months in Amsterdam to learn the skin model method.

4.4.1 Background

IFNβ is a widely used therapy for RRMS and administration of IFNβ can result in the development of NAb in as many as 47% and negatively affects the treatment response. S.c. administration is suggested to be more immunogenic than the i.m. route (206). The mechanisms of induction of immunological responses against biological drugs administered through the skin are still relatively unknown. Buttmann and colleagues investigated the impact of s.c. IFNβ administration on cell infiltration to the site of injection (167). They found chemokines expression and T cell and macrophage infiltrates at the injection site suggestive to cause inflammatory skin reactions.

The skin is an important immunological barrier (303) and to the best of our knowledge, this is the first study investigating the local biological role of IFNβ using a human *ex vivo* skin model. The model had previously only been used in vaccination studies (304, 305). Additionally, we have studied the impact of IFNβ by sampling skin biopsies after IFNβ injections in MS patients.

4.4.2 Aim

The aim of this study is to monitor the migration status of skin cells and surrounding matrix after administration of three different IFNβ preparations. This was studied in an *ex vivo* skin model as well as *in vivo* to determine if the different immunogenicities of the IFNβ preparations were evident at the injection site.

4.4.3 Intradermal IFNβ injection led to decreased cell migration but increased the percentage of CD86 expression.

Initially, we thought that IFNβ treatment would promote cell migration from the tissue. Unexpectedly it was the opposite. The higher concentration of IFNβ led to lower cell migration than the low dose IFNβ and the PBS control. I believe this could partly be due to the saturation of IFNβ and that cells potentially may have been killed. This is supported by that increased staining of high mobility group box (HMGB) 1 during immunohistochemistry indicating cell death (306). However, I did not use a cell death marker during the immunohistochemistry procedure to confirm this. If the high dose IFNβ led to cell death, it could explain the event of fewer DC in the tissue media (cell counting) and lowered frequency detected during flow cytometry analysis compared to the low dose IFNβ and PBS. Interestingly, the high dose IFNβ induced a higher percentage of CD86 positive $CD1a^+$ and $CD14^+$ DC suggesting that IFNβ induced cell activation of the skin cells that migrated from the skin site. So despite the fewer cells migrating, the ones that did had an expression of CD86, which suggests enhanced activation.

4.4.4 Decreased $CD4^+$ T cell proliferation

Increased naïve $CD4^+$ T cell proliferation was not observed after co-culture with IFNβ treated DC. Intradermal administration of IFNβ hampered the ability of the crawl-out DC to promote proliferation of allogenic naïve $CD4^+$ T cells compared to PBS treated DC when using luminescence detection of intracellular ATP as a measure of cell numbers. These data seem to conflict with our results showing enhanced expression of costimulatory molecules HLA-DR and CD86, which indicates a mature phenotype. However, several studies have shown that IFNβ alters the function of antigen presenting cells by downregulating their antigen presentation ability and thus their ability to stimulated T cell responses.

4.4.5 IFNβ injection led to proinflammatory cytokine production

Microscopic examination was performed to evaluate the impact of IFNβ injection after immunohistochemical staining with different inflammatory markers. The biopsies were collected 90 minutes and 24 hours after sampling. After 90 minutes, we could see increased staining of the epidermis and dermis of HMGB1, IL-1β, IL-6, and Cyclooxygenase-2 (COX-2) in IFNβ-injected skin compared to the saline control and the uninjected biopsies sampled. Prostaglandin E2 (PGE2) and toll-like receptor (TLR) 4 had similar staining intensity in IFNβ-injected skin and the saline control site. After 24 hours, the saline injected biopsies showed overall similar staining intensity as the IFNβ-injected skin sites, and the uninjected sites showed weak to negative staining. MxA was used as a positive control for IFNβ and was increased first after 24 hours after the IFNβ injection compared to the saline and uninjected biopsies. We believe that when the cells are subjected to stress, such as the event of IFNβ

injection or at some instances the injection with PBS, this will contribute to cell death and the release of HMGB1 (307, 308) as illustrated in the simplified picture below (Fig. 55).

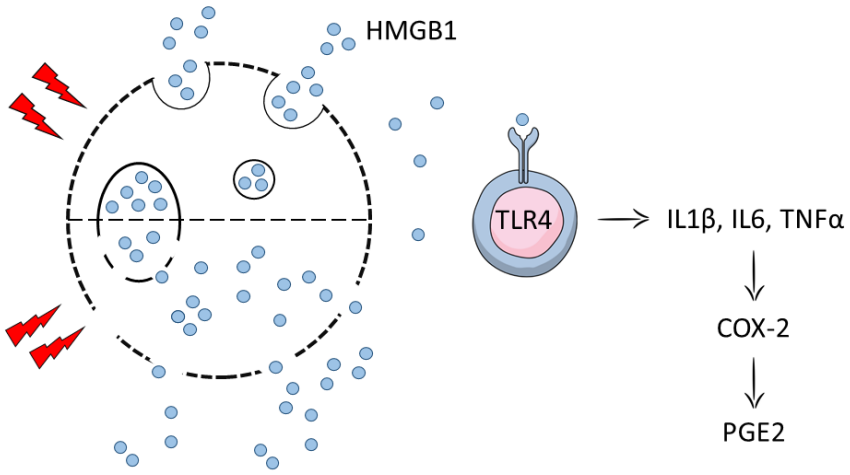


Fig. 55. **Proinflammatory cytokine release initiated by HMGB1.**

HMGB1 is localized in the cell nucleus in all nucleated cells. Upon danger signals, as a result of cellular stress and cell damage, HMGB1 is released to activate an immune response (309). When released, HMGB1 induce secretion of proinflammatory cytokines by inducing nuclear factor kappa B signaling downstream of TLR2 and TLR4 (310). Thus, the induced secretion of proinflammatory cytokines including IL-1β and IL-6 (311) could be due to HMGB1 release contributing to local inflammation in the tissue.

4.4.6 Different IFNβ preparations - same immune response?

Evidence suggests that the three different IFNβ preparations elicits different immunogenicity. While IFNβ-1b gives low titer NAb often of IgM subtype, IFNβ-1a (i.m.) and (s.c) give rise to persistent high titer NAb often of IgG subtype. In our *ex vivo* assay, we found that when the IFNβ preparations were normalized in regards to units and site of injection (intradermal) there were no significant changes in the way the dendritic skin cells responded. Depending on the IFNβ preparation used for treatment there is a substantial difference in how many units of IFNβ patient receive over one year. IFNβ-1b and IFNβ-1a (s.c.) are known to be most immunogenic and over a year they are received at 1460 million units and 1872 million units respectively in contrast to IFNβ-1a (i.m.) that is considered to be of lower immunogenicity and is received with 312 million units during one year.

4.4.7 Are the skin reactions important for the ADA formation?

In the vaccine field, human skin explant models are used to investigate the impact on skin-resident cells due to their immune responsiveness and high accessibility (312). These types of studies have not yet been implemented for biological drugs. Since biological drugs often are administrated either s.c. or i.m., thus through the skin route, I found it highly relevant to investigate the local immune effect. A majority of people treated with IFNβ are suffering from

injection site reactions (313) and the cause and effect of these reactions have not been extensively studied. Our data suggests the release of inflammatory cytokines which, at least partly, could explain the reactions seen at the skin site after drug administration. To minimize the risk of immune activation at the injection site the use of a topical anti-inflammatory cream such as hydrocortisone could be applied before IFNβ administration (314).

Since IFNβ is administrated with repetitive injections it is probable that the skin DC at the injection site are triggered to become activated (Fig. 56). A possible chain of events could be that after the IFNβ injection the skin resident DC engulf and process IFNβ to peptides, migrate to the lymph node where the peptides will be presented to T cell clones, that in turn become activated. The T cells will further activate B cells resulting in anti-IFNβ antibody producing plasma cells.

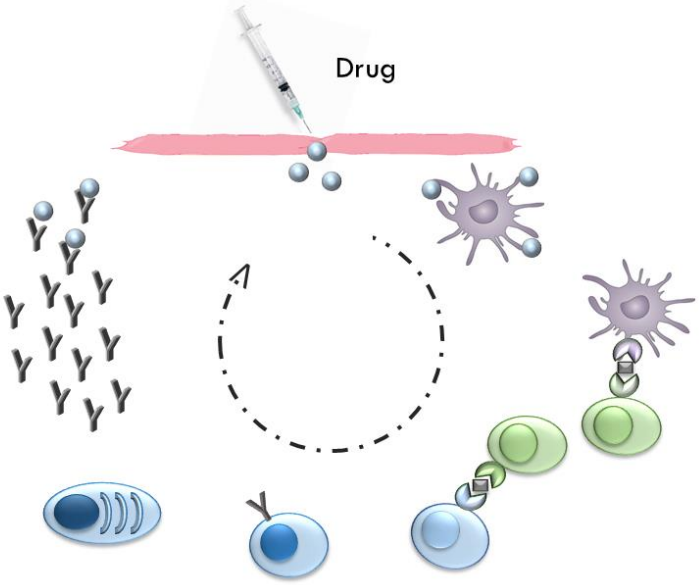


Fig. 56. **Immunity of the skin.**

4.4.8 Conclusions

In our *ex vivo* skin model, we found that injection with IFNβ significantly enhanced maturation of skin DC. We could further demonstrate that IFNβ injection elevated the expression of alarmin and several inflammatory cytokines at the skin site. These findings were further validated in the MS biopsies, where similar results were observed. We could also show that when three different IFNβ preparations were normalized, with regards to dose and injection site, the immune responses to the drug gave similar results. This indicates that the differences in immunogenicity between the different IFNβ preparations are more likely due to the route and frequency of administration, rather than the formula of the preparations. Our findings suggest that IFNβ administration triggers an immune response at the injection site through local cytokine release and cell maturation. We hypothesize that this may be the first in a series of events that lead to ADA formation. A better understanding of these molecular and cellular events could be explored to modify immunogenicity of drugs.

KEY POINTS

- Both intradermal and subcutaneous administered IFNβ initiated a pro-inflammatory cytokine release at the site of injection.
- Skin-resident cells had increased expression of costimulatory molecules after IFNβ injection.
- When three different IFNβ preparations were injected at the same skin site with the same dose there were no qualitative differences in the immune response.

5 REFLECTIONS AND THESIS SUMMARY

5.1 BIOPHARMACEUTICALS AND THE IMMUNOGENICITY ISSUE - STRENGTHS, LIMITATIONS AND FUTURE DIRECTIONS

5.1.1 Strengths

The introduction of biological therapeutics has significantly improved the daily function and quality of life for a substantial number of individuals with chronic inflammatory diseases. Treatment efficacy can be clinically monitored by slowed disease progression and significant reductions of disease symptoms. The expanding knowledge of biopharmaceuticals aids in both the development of improved therapeutics as well as providing a broader panel of therapeutics available. Making alternative drugs available has the advantage of allowing the replacement of one drug with another to increase treatment efficacy.

5.1.2 Limitations

The overall aim of my thesis was to characterize one obvious limitation with biopharmaceuticals: the development of ADA/NAb. In study I – IV we have shown that the presence of ADA/NAb have a negative impact on the serum drug concentration. For example, in study IV, we showed that only a quarter of the patients had optimal drug levels after 21 months of treatment and that ADA was detected in the majority of serum samples with a drug level below 0.2 µg/mL. In study I – III, the impact of NAb was also shown on the gene expression level where the biological response to IFNβ was abrogated in presence of high titer NAb. Thus, ADA is a major concern to achive a safe and efficient treatment course. The long-term safety of biologicals developed to mimic human endogenously produced proteins are crucial to discuss already during drug design and development. For example, ADA to therapeutically administered IFNβ has been shown to also interfere with endogenous IFNβ (198). The long-term consequences of ADA development have not yet been scientifically shown but it is, of course, important to follow these patients to evaluate any negative impact on aspects such as for example combating virus infections. With continuous releases of new biological therapies it is now more than ever important to strive for less immunogenic drug compounds.

Another limitation of drug therapy is injection site reactions after drug administration which can bring discomfort and pain to the treated patients. In study V, by immunohistochemistry, we identified several inflammatory cytokines at the injection site after IFNβ injection, both *in vivo* and *ex vivo* that potentially could trigger the swelling and irritation. Furthermore, since skin cells sometimes are targeted for vaccine delivery to induce a strong immune response it is tempting to visualize a link between the local inflammation triggered by IFNβ and the potential to induce ADA/NAb development.

5.1.3 Future directions

Future research should strive to overcome the limitations outlined above. This aim is achievable with the immunological knowledge and immunoassays that we have today, and can develop in the future, to measure immunogenicity with high specificity. This was addressed in study III were we showed that when validation was performed following the latest assay guidelines, increased sensitivity to detect NAb was achieved. When reliable NAb testing strategies are available the results could be merged with clinical data to allow the structuring of algorithms used as treatment outcome predictors. Higher accuracy would allow meaningful use in clinical practice as discussed in study IV.

Effective and safe treatment could be achieved by collaboration and bridging the knowledge gap between researches, clinicians, and biopharmaceutical companies. Moreover, a structured monitoring of immunogenicity in clinical routine can stimulate and reward future development of improved therapeutics that are less immunogenic and have a prolonged serum half-life. Below are some good examples of how this could be accomplished.

5.1.3.1 Prediction of T cell mediated immune responses

To engineer less immunogenic therapeutics is scientifically challenging. Since ADA mostly are of IgG isotype, it is suggested to be a T cell-mediated response. Different screening methods can be used to localize regions within the drug protein sequence that contribute to immunogenicity, and measure T cell dependent immune responses to therapeutics to predict immunogenicity. Deletion of T cell epitopes or amino acid substitutions have the potential to reduce immunogenicity. Methods used for predicting T cell responses include cell epitope-screening (*in silico*), where the regions of interest can further be validated in an HLA binding assays (*in vitro*) to measure affinity (315). However, as with all predictive models, they must be validated in a clinical testing to confirm improved properties of the de-immunized therapeutic.

5.1.3.2 PEGylated therapeutics

Since the early 1990s, there is a growing use of PEGylated therapeutics that allow an extended serum half-life and thereby limit the frequency of administration, particularly important in chronic diseases. PEGylation is the attachment of PEG to lysine residues on the protein surface. More than 10 PEGylated therapeutics are currently used in the clinic, of which one is used for the treatment of MS (PEG-IFN). Interestingly, a study by the ADVANCE trial showed a frequency of NAb to PEG-IFN in less than 1% of the treated MS patients that were followed for two years (240). However, there are some difficulties using the PEGylation technology to improve protein therapeutics. For example, the attachment of PEG to the drug molecule allows binding around the active sites of the molecule and could reduce the drugs activity and hence efficacy. However, anti-PEG antibodies can also form and we do not yet know what impact they will have on treatment and patients.

5.1.3.3 Affibody molecules

Lastly, an interesting candidate for the treatment of chronic inflammatory diseases is Affibody molecules (316). Due to its small size (around 6 kDa) the molecules can easily penetrate the tissue and compared to larger proteins the same volume would allow a higher molar dose. Comparatively, IgG antibodies have a size of 150 kDa. Affibody molecules could also, potentially, be administrated via alternative routes to the skin site. Since the skin is a major immunological barrier and, potentially, a sub-optimal administration route with regards to ADA development (as addressed in study V), the next generation of biopharmaceuticals might have to be designed in a way to overcome such problems. These include, for example, finding ways to survive an acidic environment when drug administration is via the oral route.

5.1.3.4 Conclusions

ADA need not to be a limitation of biological treatments if they are taken into account during drug development, managed well in the clinics, and recorded in patient registries for monitoring of future issues, should they manifest.

6 ACKNOWLEDGEMENTS

First and foremost I want to express my heartfelt gratitude to the patients, donors, hospital staff and collaborating clinics, for selfless and persistent contributions that made this all possible.

To my wonderful main supervisor **Anna Fogdell-Hahn** I would like to convey my sincerest appreciation for everything you have taught me. You allowed me to grow as a person, build confidence as an academic and gain independence as a scientist. Thank you for all your support and for keeping me on track while being so much more than a mentor.

To my co-supervisors **Saedis Saevarsdottir** and **Jan Hillert. Saedis** it has been so much fun working with you on the RA project. Thank you for your invaluable comments and insights. I wish you the best of luck on your new endeavors in Iceland. **Jan** thank you for your continued support throughout the project and in particular with the IL-7-study.

To former and present colleagues in the **Neuroimmunology group**. **Malin** and **Elin** thank you for your generous, continuous, and unwavering friendship and for giving me constant support throughout my Ph.D. work. It has been a pleasure working with you both in the lab. Let us keep up with the wine dates. **Nicky** for reminding us that nothing is impossible and for proofreading this thesis. **Anna M** and **Ingegerd** for being the heart and soul of the lab and ensuring everything runs smoothly. **Rasmus** for being a great college. **Yousra** and **Nick** for enthusiastically supporting our research projects.

To former and present colleagues in the **Hillert group**, thank you for all these years! **Clemens** for your exceptional knowledge in the MS field; **Sahl** for being a great office buddy; **Ali** for great journal club discussions and for your suggestions on the MS section; **Helga** for good fika times; **Wangko** for sharing lab skills during study I; **Virginija** for collaborations with project V and for good times running in Hagaparken. It was really fun being toastmadame at your dissertation; **Jenny** for helping me with GraphPad analysis; **Stefanie** for your positive energy; **Katharina** for contributing with clinical knowledge and for your suggestions on the MS section; **Ryan** for interesting discussion about life and science; **Ingrid** for your knowledge in statistics; **Andrius** for your friendly spirit; **Kerstin** for addressing tough research questions; **Iza** for your positive outlook; **Anna G** for arranging fun group events.

I would also like to thank the co-authors and collaborators on my different projects. To the **ADAMS family**: **Daniel**, **Elisa**, **Paul**, **Poul Erik**, **Dorothea**, **Florian**, **Michael**, **Thomas**, and **Kathleen**. Thank you for our weekly telcos, continued support, insight and tips leading up to paper III & paper V. **Marc** and **Sebastian** for all your exellent work with ABIRISK. To the **RA team**: **Saedis**, **Per**, **Karen**, **Inger**, **Nancy**, and **Rille** for generously sharing your knowledge with me. I very much look forward to continued work with you in the future. **Esther** for generously

accepting me into your lab and **Toni** for teaching me the skin model and that cycling was an integral part of the Amsterdam life. **Bibbi** for providing me with samples to study V, and always with a smile. Without your help, this study would not have been possible. **Susanna** for great collaborations with study I. **Annika** for all your help with flow cytometry. **Gustaf**, **Emily** and **Frank** for sharing knowledge and protocols within immunohistochemistry.

Patrizia for overhearing me talking Swedish on the Navy Yard bus and later becoming my mentor. **Bob** for being an excellent director of studies. **Gullan** for your kindness and attentive help with administrative work.

Kristin & **Maria** for adding glitz to my workdays and for being such ridiculously cool and inspirational friends.

Floor **00(7)** for making this workplace a little bit more awesome. Taking a fika at this floor can turn any day into a good one ☺.

Göran for believing in me as a researcher and for giving me the post-it that led me to Boston. **David** for letting me join your Harvard lab and sponsoring my work visa. **Christene** I will forever be grateful for your guidance and kindness. **Zhirui** your knowledge in molecular biology is inspirational. Thank you for being my teacher and sharing your knowledge of laboratory techniques.

Some people are just born with glitter in their veins, and I have had the great privilege to cross paths with the following incredible women. Thank you **Yohanna** for letting me see life through your glittery fairy filter - love you; **CeCe** for finding me in Boston and for sharing the philosophy of life with me; **Ann** for being an excellent study buddy; **Frida** for knowing everything about me and never judge; **Sofia** for saving me in Uppsala. To flickorna i Småland that have known me since I was a little girl! What would I ever do without you? **Jane**, **Joanna** & **Cia** I love you girls. We´ll be best friends forever - you know too much! **Angela** & **Johanna** my partners in wine.

To my extended family. Thank you **Bengt** for over-the-top support throughout my entire education and for welcoming me to Boston with open arms. **Sonja** for embracing a positive attitude to life and **Emilia** for being the animals voice and for knowing the importance of a perfect cup of coffee (what would we do w/o iKaffe?). To my **Assyrian family** for being amazing survivors and for showing me what family is all about.

To my big, loud, opinionated, caring family. I love you so! Darling sisters, **Cecilia** (en sekund i taget), **Angelica** (systrarna P) and **Josefine** (fuldansarnas Queen) thank you for being my best friends. It has been a strength throughout life to have these three musketeers watching my back. To my dearest youngsters of the family - I get comforted by the fact that you are part of the future. **Susanne** and **Sven** for making my parents happy.

Mamma and **Pappa** thank you for being fantastic and loving parents. **Mamma** if I'm ever given only one chance to make a phone call it would be to you. You are always there for me no matter what. Thank you for all the unconditional love you give me and for being a constant source of support. Jag älskar dig. **Pappa** you were born in the mountains of Turkey. Still you managed to build a beautiful life for your family here in Sweden. That is truly inspirational. The kindness within you is unique. Seni seviyorum babacim.

My family of three. I love you endlessly. ♥ **Kristian** my soulmate and my best friend. Together we can achieve anything. Thank you for your unconditional support and for your trust in me. I remember the rainy evening when we walked on the cobblestone streets on Beacon Hill, and you listened to my thoughts of pursuing a PhD and you lifted me when I needed it most. Your support during my research journey and thesis writing has been invaluable. Now I've done it - the book is written! I also would like to thank you a little extra for sharing your DNA with me and for being the world's best dad to our little peanut. ♥ **August** you are the only one who has heard the sound of my heartbeat from the inside and the day you were born you took my heart with you into the world to hold in infinity. Thank you for coming into my life and making me your mother. You make me a better person and my love for you is endless. You are without a doubt the most beautiful thing that ever happened to me.

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