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STRATEGIES TO ASSESS AND IMPROVE PROGNOSTICATION OF PLASMA CELL DISORDERS

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STRATEGIES TO ASSESS AND IMPROVE PROGNOSTICATION OF PLASMA CELL DISORDERS
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Till Johan, Alexandra och Cornelia – bryggan väntar på oss

POPULAR SCIENCE SUMMARY OF THE THESIS

Plasmacellssjukdomar (PCD) är en grupp av olika blodsjukdomar som uppstår i våra plasmaceller och beror på förändringar i plasmacellernas gennivå. Dessa sjukdomar blir mer vanligt förekommande med stigande ålder och kan därför komma att bli ett växande problem med en åldrande befolkning. Karaktäristiskt för PCD är den stora variationen av olika sjukdomsformer som kräver olika uppföljning och behandling. Detta variera från att kontinuerliga kontroller men utan behandling till att behandla med exempelvis cellgifter och byte av stamceller i benmärgen. Det är viktigt att i ett tidigt skede av sjukdomsförloppen kunna förutse vilka individer som löper en stor risk att utveckla en av de allvarligare och mer behandlingskrävande sjukdomsformerna. En tidigare upptäckt skulle kunna minska risken för framtida komplikationer av sjukdomarna.

Gemensamt för majoriteten av PCD är att de sjuka plasmacellerna har förmåga att föröka sig och börja producera antikroppar, s.k. M-komponent. Antikroppar är ett protein som normalt hjälper immunförsvaret att skydda kroppen mot skadliga ämnen, exempelvis bakterier och virus. Antikropparna, som produceras vid PCD, är tyvärr av en och samma typ och kan därför inte bidra till att stärka immunförsvaret. I stället kan de stora mängderna av likartade antikroppar skada kroppen, till exempel genom att ansamlas i njurarna och därmed försämra njurarnas funktion. Den ökande mängden av plasmaceller kan också skada benmärgen, vilket leder till blodbrist, men även skada på det skelettet som omger benmärgen kan uppstå. Dessa antikroppar eller M-komponenten går oftast att mäta i både blodet och urinen vilket gör att dessa sjukdomar lätt kan spåras genom relativt enkel provtagning. Dessutom kan man även mäta mängden av fria lätta kedjor (FLC), som är en del av M-komponenten, i blodet. Oftast används en kombination av M-komponent och FLC mätningar för att identifiera och följa upp de olika sjukdomsformerna.

Det övergripande syftet med detta doktorandprojekt var att utvärdera dels riskfaktorer, dels nivåskillnader i biomarkörer över tid för att förbättra diagnostik samt uppföljning av patienter med PCD. Tre sjukdomsformer av PCD är fokus för doktorandprojektet. Två av dessa, monoklonal gammopati av oklar signifikans (MGUS) och asymtomatisk multipel myeloma (SMM), behöver inte läkemedelsbehandling men i de flesta fall krävs en livslång uppföljning på grund av risken att bli behandlingskrävande. Den tredje sjukdomsformen är multipelt myelom (MM) som är den näst vanligaste blodcancern i världen. Denna blodcancer kräver i regel omgående läkemedelsbehandling. Forskning har visat att de patienter som utvecklar MM med stor sannolikhet tidigare har haft MGUS eller SMM. Det finns därför ett stort intresse av att under uppföljningen av dessa tillstånd tidigt kunna urskilja riskfaktorer för utveckling av MM. Utöver detta har tidigare studier visat på en ökad dödlighet hos personer med MGUS, men det saknas alltjämt kunskap om vilka riskfaktorer som är kopplade till denna ökade dödlighet.

Denna avhandling fokuserar därför på tre huvudsakliga frågeställningar: Den första frågan är om man kan förutse risken för att utveckla behandlingskrävande sjukdom hos individer med MGUS och SMM genom att mäta skillnader i biomarkörer över tid (**studie I och II**). Den andra frågan är om man antingen genom att mäta M-komponent eller FLC snabbare kan påvisa att patienter med MM svarar på sin behandling samt får återfall i sin sjukdom (**studie**

III). Den tredje frågan slutligen är att identifiera vilka riskfaktorer som är förknippade med en ökad dödlighet hos individer med MGUS (**studie IV**). Studierna är godkända av svenska etikprövningsnämnden och baserar sig på historiska data.

I **studie I**, undersökte vi hur olika metoder för att mäta M-komponenten över tid kunde indikera vilka personer med MGUS som riskerade att bli behandlingskrävande genom att studera ökningar av M-komponenten, efter det att en person fått en MGUS diagnos, och fram till dess att de utvecklade MM. Resultatet jämfördes mot en grupp av personer med MGUS som inte utvecklade MM. I denna studie kunde vi peka på att personer där FLC ökade med 100mg/L eller mer under uppföljningen löpte större risk att bli behandlingskrävande än de vars FLC-nivåer låg stilla eller ökade med mindre än 100mg/L. Vi kunde även påvisa att flera andra riskfaktorer, en ålder överstigande 65 år, en M-komponent >15g/L och/eller FLC >100mg/L. var betydelsefulla för att lättare kunna identifiera individer som riskerade att bli behandlingskrävande. Detta visar på att det är viktigt att inte bara bedöma riskfaktorer vid första diagnostillfället utan även under uppföljningen av en individ med MGUS.

I **studie II** fokuserade vi på vilka faktorer, hos individer med SMM, som pekade på en ökad risk att bli behandlingskrävande. Våra resultat visar att ökningar över tid både av M-komponent och FLC är viktiga riskfaktorer att upptäcka personer med risk för att få MM. Intressant var att våra forskningsresultat även pekade på att patienter med ökad FLC-kvot vid diagnostisering inte hade en ökad risk för att utveckla MM. Men om FLC-kvoten ökade över tid under uppföljning förelåg risk för att utveckla MM. Dessa resultat visar på vikten av att kontinuerligt förnya bedömningen av risken att utveckla en behandlingskrävande sjukdom under uppföljningen av individer med SMM.

I **studie III**, undersökte vi patienter med MM för att granska potentiella tidsskillnader mellan nivåerna av M-komponent och FLC för att bedömning av svar på behandling, minskning av nivåerna, och återfall, ökning av nivåerna. Vi fann att svar på behandling, dvs minskning av nivåer, sågs lika snabbt eller snabbare med FLC som med mätning av M-komponent. Analys av FLC var även likvärdigt gentemot M-komponent, i de flesta fall, för att kunna bedöma när patienter fick ett återfall. Undantaget var hos personer med väldigt låga eller omätbara nivåer av FLC vid start av behandling. Detta visar att mätningar av FLC skulle kunna komplettera eller i vissa fall ersätta av M-komponent vid bedömning av svar på behandling och återfall för patienter med MM

I **studie IV**, vars syfte var att identifiera riskfaktorer för en ökad dödlighet hos individer med MGUS som inte hade utvecklat en behandlingskrävande blodsjukdom eller MM. Vi fann ett flertal faktorer som var sammanlänkade med ökad dödlighet, däribland en viss typ av MGUS, lättkedje MGUS, nedsatt njurfunktion och låga albuminnivåer. I gruppen av lättkejde MGUS, dog en stor andel av personerna av hjärtsjukdom. Lättkedje MGUS kännetecknas av produktion av endast en liten del av M-komponenten, den s.k. lätta kedjan: Hos vissa individer kan denna lätta kedja ansamlas i hjärtat vilket leder till nedsatt funktion. Då vi kunde visa på ett samband mellan lättkedje MGUS och dödlighet samt att individer med lättkedje MGUS dog av hjärtsjukdom i relativ utsträckning kan det vara möjligt att personer hade denna form av inlagringssjukdom, AL amyloidos. Våra rön indikerar att individer med lättkedje MGUS bör undersökas och följas upp avseende hjärtåkommor.

De fyra studierna i denna avhandling har resulterat i ökade kunskaper om hur utredning med FLC kompletterar utvärdering av M-komponent vid MGUS, SMM och MM. En förbättrad uppföljning av individer med MGUS och SMM skulle potentiellt kunna leda till mindre lidande för patienten och lägre kostnader för sjukvårdande behandlingar genom att tidigare kunna identifiera individer med behov av behandling. För patienter med MM kan en förbättrad uppföljning leda till en möjlighet att tidigare kunde ändra pågående eller sätta in nya behandlingar. Vidare kan denna avhandling öppna upp för ytterligare forskningsinsatser framför allt inom området med dödlighet hos individer med lättkedje MGUS.

ABSTRACT

Plasma cell dyscrasias (PCD) are a group of disorders, most of which have the overproduction of monoclonal immunoglobulins (M-protein) in common. Included in the group of PCDs are both benign and treatment demanding disorders. Multiple myeloma (MM) is one of the treatment demanding PCDs and also the second most common hematological malignancy. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) are both PCD disorders currently not thought to require treatment. Although, in the case of SMM, recent trials have shown the time to progress to symptomatic MM was prolonged with treatment.

Biomarkers are an essential key for diagnosing, risk prediction, and monitoring diseases. An essential biomarker in PCDs is the assessment of M-protein in serum/plasma and urine used to detect and monitor. In the past decades, new research findings and technical development of biomarkers have improved the ability to diagnose and monitor PCDs. Particularly, serum free light chain (FLC) assessment has shown an important role in diagnosing and risk prediction in MGUS, SMM, and MM and for assigning stringent complete response in MM. Research has shown that MGUS or SMM consistently precedes MM. This observation has lead to a growing interest in the dynamic evaluation of biomarkers to predict patients at risk. However, the kinetics of biomarkers in PCDs is still a developing research field.

In **study I**, we aimed to identify dynamic changes of M-protein and FLC associated with increased risk of progression from MGUS to symptomatic MM. We observed that dynamic increases of involved FLC (iFLC) above 100mg/L were a consistent risk factor during follow-up, while M-protein elevations above 5g/L were associated with increased risk of progression at only a few time-points. Furthermore, we identified several independent predictors of progression at the time of MGUS diagnosis, age >65 years, M-protein >15g/L, and iFLC >100mg/L. We observed that the 5-year cumulative probability of progression was higher in patients with two or three risk factors at diagnosis (31%) than patients with no risk factors (2%).

In **study II**, we attempt to define cut-offs for temporal biomarkers in individuals with SMM, associated with progression to symptomatic MM. We found that increases of M-protein and iFLC ratio (iFLCr) were significant predictors of progression, with the optimal cut-offs at >5g/L and >4.5, respectively. Moreover, we could confirm that clonal bone marrow plasma cells >20% and M-protein >20g/L, at diagnosis, were independent risk factors of progression. Interestingly, while increases in iFLCr during follow-up were associated with increased risk of progression, iFLCr at diagnosis were not an independent risk factor.

In **study III**, we investigated whether response and progression in patients with MM were detected earlier by iFLC or M-protein. We observed that at least partial response, or better, was overall observed significantly earlier when assessed with iFLC than M-protein, while no overall significant differences were detected between the two biomarkers when detecting biochemical progression. Subgroup analysis included heavy chain type, measurable disease

groups, and early and late progression. In these subgroup analyses, iFLC appears to be non-inferior in response detection compared to M-protein. The subgroup analyses of the time to progression showed that M-protein detected biochemical progression significantly earlier than iFLC in patients with iFLC <100mg/L and a detectable M-protein, >10g/L.

In **study IV**, we investigated the causes of death and risk factors for overall survival in patients with MGUS that had not progressed to hematological malignancy. Light chain MGUS (versus IgG MGUS) was associated with inferior survival. Additionally, independent predictors for overall survival were male gender, hypoalbuminemia, and renal insufficiency.

In conclusion, with these studies, we have increased the knowledge of temporal FLC and M-protein assessments in MGUS, SMM, and MM, potentially improving these patients' follow-up. Furthermore, this dissertation may open up further research efforts, especially in excess mortality in individuals with light chain MGUS.

LIST OF SCIENTIFIC PAPERS

I. **Gran C,** Liwing J, Wagner AK, Verhoek A, Gezin A, Alici E, Nahi H. Comparative evaluation of involved free light chain and monoclonal spike as markers for progression from monoclonal gammopathy of undetermined significance to multiple myeloma. Am J Hematol. 2021; 96:23-30. doi: 10.1002/ajh.25999.

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III. **Gran C,** Afram G, Liwing J, Verhoek A, Nahi H. Involved free light chain, an early independent predictor of response and progression in multiple myeloma. *Manuscript under revision in Leukemia and Lymphoma*

IV. **Gran C,** Wersäll J, Susek K H, Wagner A K, Afram G, Nahi H. Light chain subtype and severe inflammation predicts worse overall survival in Monoclonal gammopathy of undetermined significance. *Manuscript*

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CONTENTS

LIST OF ABBREVIATIONS

AGA	High-resolution agarose gel electrophoresis
BM	Bone marrow
BMPC	Clonal bone marrow plasma cells
CA	Chromosomal Aberration
CE	Capillary electrophoresis
CI	Confidence Interval
CRAB	Criteria for symptomatic MM, hyperCalcemia, Renal impairment, Anemia, Bone lesions
CV	Coefficient of Variation
eGFR	Estimated glomerular filtration rate
FISH	Fluorescent in situ hybridization
FLC	Free light chain in serum
FLCr	Free light chain ratio in serum
GPS	Glasgow Prognostic Score
HR	Hazard Ratio
IFE	Immunofixation
iFLC	Involved free light chain in serum
iFLCr	Involved free light chain ratio in serum
IMWG	International Myeloma Working Group
IQR	Interquartile Range
ISS	International Staging System
ISUB	Immunosubtraction
KM	Kaplan-Meier plot
KUL	Karolinska University Laboratory
LC-MGUS	Light chain monoclonal gammopathy of undetermined significance
LIS	Laboratory Information System
MDE	Myeloma Defining Event
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
M-protein	Monoclonal immunoglobulin in serum/plasma

OS	Overall Survival
PC	Plasma cell
PCD	Plasma cell dyscrasia
PEP	Protein electrophoresis
RCV	Reference change value
R-ISS	Revised - International Staging System
ROC	Receiver operator characteristic
ROC AUC	Area under the ROC-curve
SHM	Somatic hypermutation
SMM	Smoldering multiple myeloma
TTP	Time to progression
TTR	Time to response
uIFE	Immunofixation of urine
uM-protein	Monoclonal immunoglobulin in urine
uPEP	Protein electrophoresis of urine

1 BACKGROUND

Plasma cell dyscrasias (PCD) refers to a group of disorders characterized by the clonal proliferation of plasma cells (PC). This proliferation of malignant plasma cells can result in monoclonal protein (M-protein) production and ultimately end-organ damage. However, the diseases have a heterogeneous spectrum of severity and outcomes. PCD's can present as benign forms, monoclonal gammopathy of unknown significance (MGUS), and smoldering multiple myeloma (SMM), which still require lifelong follow-up in the majority of cases. Nevertheless, many patients are identified only when the end-organ damage is apparent, such as multiple myeloma (MM) and plasma cell leukemia (PCL). The PCD's are disorders of the elderly. With the increased incidence observed in elderly patients, an increased prevalence of PCDs could be anticipated with a growling elderly population.

The acts of diagnosing, monitoring progression from benign to symptomatic disease, response to treatment, and relapse are essentially dependent on accurate laboratory testing. Assessment of end-organ damage is included in guidelines for diagnosing in PCDs as well as management of MM. However, the fundament for MGUS, SMM, and MM related laboratory investigation is the detection, quantitation, and typing of the M-protein produced by the clonal PCs. Several traditional chemistry laboratory assays, such as electrophoresis (PEP) and immunofixation (IFE) of serum and urine, are vital for assessing the M-protein. In recent years, assays detection free light chains (FLC) in serum have further increased the ability to detect and monitor the PCDs.

1.1 A BRIEF HISTORY OF DIAGNOSTICS IN PLASMA CELL DYSCRASIAS

Since the early days of humanity, attempts to explain and treat diseases have been abundant. These attempts have included trying to identify a disease or condition. One of the earliest descriptions of a test for a condition is found in the Berlin papyrus dating back to 1350 BC (1). "*Another test for a woman who will bear or a woman that will not bear. Wheat and spelt; let the woman water them daily with her urine, like dates and like Sh'at seeds in two bags. If they both grow she will'bear: if the wheat grows it will be a boy; if the spelt grows, it will be a girl. If neither grow, she will not bear.*" (Verse 2, 1-2. Translated by Dawson W.R (2)). This diagnostic assessment has since been shown to identify pregnancy with a 70% sensitivity (3).

The history of biomarkers in PCD is not as long as that of pregnancy. However, already in 1847, dr Bence Jones published the first record of a PCD assessment (4). Although the terms PCD and MM had not been introduced, dr Bence Jones gave a detailed description of how to treat urine samples to identify the compound he called hydrated deutoxide albumin, which we now know as immunoglobulin light chains (5).

Von Rustizky first proposed MM as a term in 1873, when he observed several separate tumors in the bone marrow (BM) of a patient during an autopsy (6). The tumor cells were described, a round cell with a nucleus in the periphery, similar in size to leucocytes. PCs were not yet defined; however, von Rustizky's description would suggest these were the cells identified. Once Marschalkó described PCs' characteristics in 1895 (7), and BM evaluation

from sternum aspiration was introduced in 1929, the possibility to identify patients pre-mortem increased (8).

The great work by Tiselus in the late 1930s led to the advent of electrophoresis (PEP), which enabled the separation of proteins (9, 10). Using PEP, Longsworth et al. could identify the distinct pattern of a narrow peak that we now know represents the M-protein (11). Immunofixation (IFE), where the heavy and light chains of the M-protein are identified, further refined the PEP diagnostics of PCDs (12).

Korngold and Lipari were able to show that the proteins in urine described by Dr. Bence Jones were, in fact, the light chains of a monoclonal IgG (13). Their observations were honored by naming the detected light chains kappa and lambda. Several methods to detect the free light chain (FLC) of the M-protein in serum have been attempted. However, the early assays could not distinguish between FLC and the light chains of intact antibodies. This problem was solved in 2001 with the introduction of Freelite, a nephelometric assay using polyclonal antibodies, that allowed for detection of the FLC (14). Currently, methods using both mono and polyclonal antibodies are available in the diagnostic repertoire.

1.2 PLASMA CELL EVOLUTION AND MONOCLONAL PROTEIN

A broad spectrum of terminally differentiated plasma cells are developed from B-cells in the healthy immune system. These specialized plasma cells each secrete a unique immunoglobulin (Ig), an antibody. The antibodies are composed of two identical heavy chain classes (IgM, IgG, IgA, IgD, or IgE), and two identical light chain classes (kappa κ or lambda λ) (Figure 1).

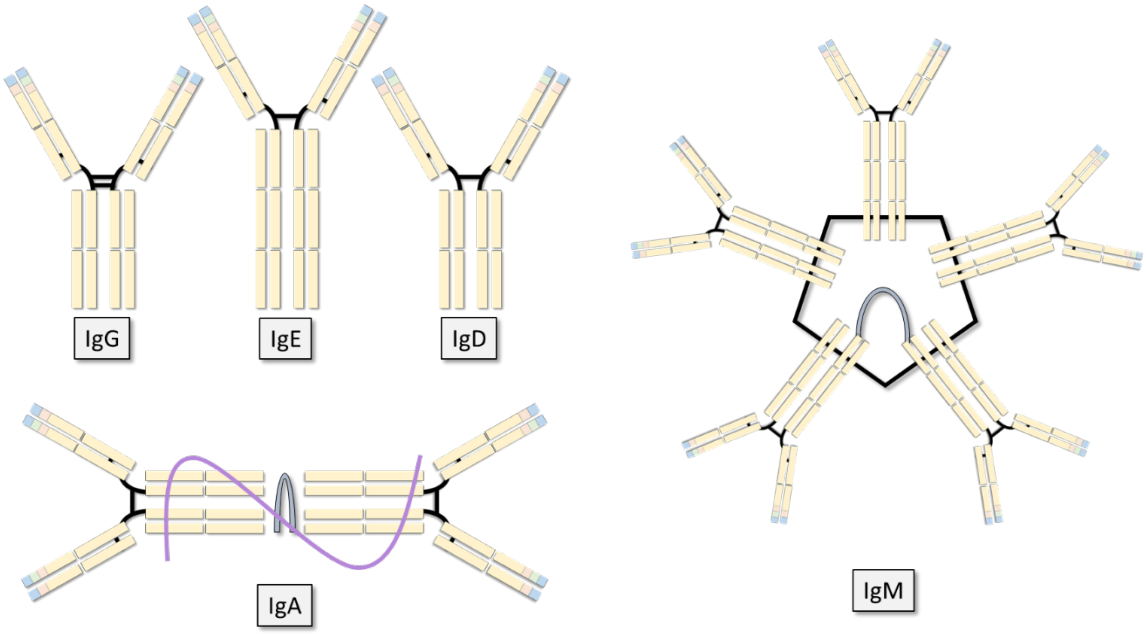


Figure 1. The five immunoglobulin classes

The differentiated plasma cells develop from the hematopoietic stem cells that have undergone a V(D)J recombination, somatic hypermutation (SHM), and isotype switch to produce a large variety of Igs, each with a different variable region. The V(D)J recombination starts in the Pro-B-cells in the BM. During the V(D)J recombination, the heavy and light chain loci DNA are recombined. In the heavy chain loci, a D (diverse) segment and a J (joining) segment are first combined and then joined to a V(variable) segment. During the recombination, the V(D)J segments not used are discarded. A similar process occurs for light chains, except that only V and J segments are recombined. The constant region of the antibody is later spliced during RNA processing (Figure 2) (15).

The immature B-cells migrate from the BM to the lymphoid tissue, undergoing further activation and maturation. This maturation includes the SHM and the isotype switching. Once activated, the B-cells can differentiate into memory B-cells or plasma cells that will secrete a unique Ig. With this differentiation utilizing V(D)J recombination, SHM, and the isotype switching recombination, a theoretical range of $3x10^{11}$ possible combinations for the variable region could be produced.

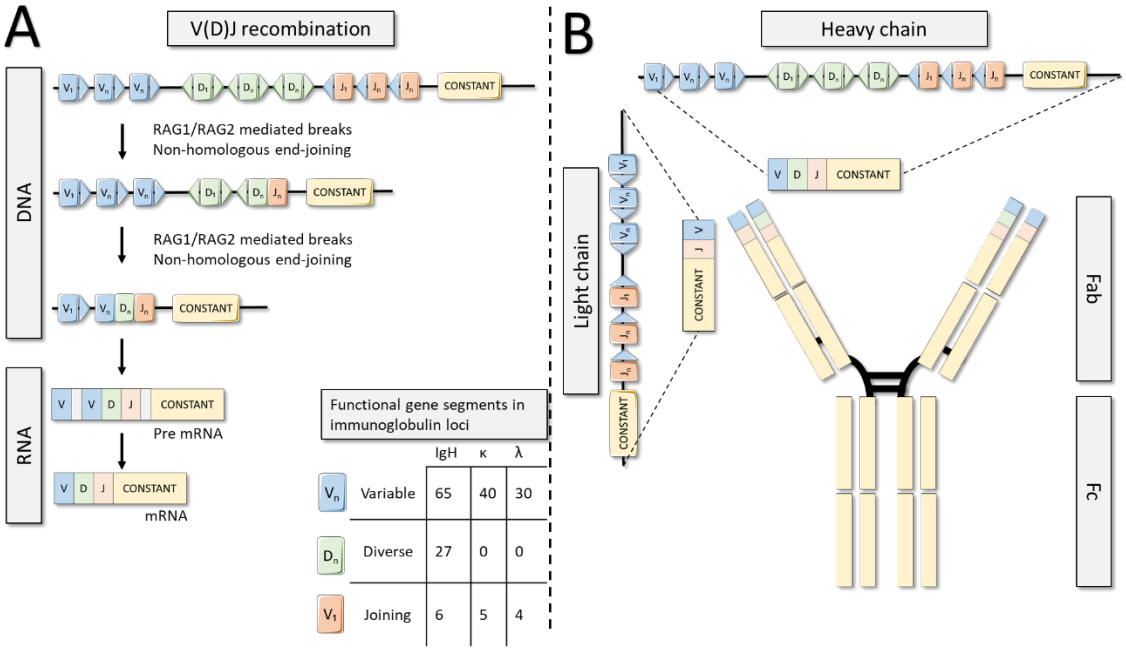


Figure 2. A. Overview of V(D)J recombination process on DNA and RNA levels. B. The structure of the antibody with the heavy and light chains

The various subclasses of immunoglobulins exhibit different half-times. The intact Igs have a relatively long-half-life due to their size, 146-970 kDa, making them too large to pass through glomerular filtration and degradation. For IgA and IgM, the half-life is approximately 6 and 10 days, respectively, while concentration-dependent recycling in IgG increases the half-life to approximately 21 days (15). On the other hand, the light chains, κ or λ, which are produced in excess, can pass through glomerular filtration on account of their smaller size, 22.5kDA and 45kDa for κ or λ respectively. The light chains are then degraded in the proximal tubuli. This rapid metabolism by the kidneys can be seen in the short half-life of the light chains, 2-4 hours for κ and 3-6 hours for λ. In cases of renal impairment, the light chains are degraded by the same system as intact Igs, pinocytosis, which leads to an increased half-life of approximately 2-3 days.

During the PC differentiation, genetic/chromosomal aberrations can be introduced into the PC's genome. A gene associated with cell growth or survival can potentially recombine with the promoter regions of the heavy or light chain locus causing a malignant transformation of the PC. Together with microenvironmental changes, these genetic changes can lead to the expansion of a single malignant PC clone (16). Once the PC's malignant transformation has occurred, the single clone can have the potential to invade the BM and expand. This clonal expansion can ultimately cause end-organ damage, such as lytic bone lesions, affecting the normal hematopoiesis, which causes anemia.

In the majority of cases, these malignant plasma cells will secrete an M-protein. The M-protein secreted by the PCs is usually detected in the serum or urine of the patients. An M-proteins discovery can often be an early observation, in many cases, preceding the presentation of symptomatic MM (17, 18). While the most common M-protein is an intact

M-protein, consisting of both heavy and light chains, they can also be composed of a light chain only (κ or λ) and, in very rare cases, only of a heavy chain.

1.3 BIOMARKERS

The FDA-NIH biomarkers working group defined biomarkers in 2016 as "*a defining characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions.*" (19)

A clinically useful biomarker should be specific, distinguish between healthy and diseased states, and be easy to measure in routine clinical samples with well-defined reference ranges. Furthermore, successful biomarkers must be evaluated with validated additional value to established biomarkers (19, 20). It is also preferable if the biomarkers are cost-effective.

The validation of biomarkers is dependent on the biomarkers' intended use, for example, diagnosing, screening, risk modeling/staging, monitoring, or predicting the disease. Once the difference in the biomarker's expression in healthy and diseased individuals has been established, the biomarkers' relationship to the disease's outcome must be validated (19-22). A biomarker's performance can be evaluated by sensitivity, specificity, positive and negative predictive value, where sensitivity and specificity can assess and visualize the discriminatory ability by receiver operation characteristic (ROC) curves. While 100% sensitivity and specificity of a biomarker are sought after, a compromise between sensitivity and specificity must often be made in reality. When diagnosing, it is essential to confirm and rule out healthy individuals, therefore opting for high specificity, while in screening situations, the biomarkers' ability to identify true positive is often preferred, thus requiring high sensitivity (23). Moreover, the reference change value (RCV), i.e., the difference attributed to a true change in clinical status and not due to either biological or analytical variation, should be considered when evaluating a biomarker (24).

A two-fold problem exists when introducing new tumor surrogate biomarkers. First, heterogeneity of tumor cells can result in a need to combine multiple biomarkers to provide adequate sensitivity and specificity in staging and monitoring the disease. Thus, new biomarkers should be evaluated both alone and together with established biomarkers. Second, biomarkers are often evaluated only at diagnosis or another single point in time, thus providing only a cross-section evaluation of the disease and not considering its evolution. The latter is essential when evaluating disorders with heterogenous outcomes and those where there is a need to distinguish between premalignant and treatment demanding stages.

1.3.1 General aspects of biomarkers for Plasma Cell Disorders

There are many biomarkers for PCD either in use or suggested in studies. Most can be classified as surrogate tumor markers or end-organ-specific markers. M-protein and FLC assays detecting the secreted M-protein levels are used as surrogate markers of malignant PCs' proliferation and decline. End-organ damage markers include hemoglobin, creatinine, and calcium. The essential surrogate tumor biomarkers used to diagnose and monitor PCD are mentioned below. However, in literature, there are many more to be found.

1.3.2 Plasma Cells

As both lymphoproliferative disorders and PCDs can give rise to an M-protein, a distinction between these disorders is critically important due to the different treatment regimens used for the diseases. BM assessment can differentiate between a clonal plasma or lymphoid cell proliferation by morphology, immunohistochemistry, or flow cytometry. The BM assessment also evaluates the fraction of clonal PCs. The fraction of clonal bone marrow plasma cells (BMPC) is vital to differentiate between the subtypes of PCDs. In current diagnosis criteria, patients with BMPC% <10% are considered MGUS, while patients with BMPC ≥10% are either SMM or MM depending on the presence of end-organ damage (25). Conventional BM aspirate or biopsy examination is typically performed at diagnosis to assess BMPC, with the higher of the two values used in case of discrepancies (26).

As BM sampling is an invasive test, it can be questioned if all suspected PCDs patients need to endure it. In patients with low-risk MGUS (IgG-MGUS and M-protein<1.5g/dL), the probability of finding BMPC ≥ 10% is 4.7%, leading to a missed SMM or MM diagnosis in less than 1% of low-risk MGUS patients (27).

1.3.3 Protein Electrophoresis and Immunofixation

M-protein is a primary biomarker when diagnosing PCD, risk stratification in premalignant stages, and monitoring response and relapse in symptomatic MM (25, 28-31). Protein electrophoresis (PEP) is the primary laboratory assay for detection and quantitation, where the M-protein can be visualized as a distinct band in gel PEP, or peak, in capillary zone PEP, in the normal migration pattern. Observation of a new band or peak requires confirmation with immunofixation (IFE) or immunosubtraction (ISUB). Both assays, PEP and IFE/ISUB can be performed in serum/plasma and urine.

Assays for protein electrophoresis

Currently, the two primary assays used for PEP are high-resolution agarose gel (AGA) and capillary (CE) (figure 3), where AGA is the most commonly used of the assays (32). Both assays separate proteins depending on their electrophoretic migration. Identification of the M-proteins's heavy and light chains is performed with IFE in AGA, and the corresponding assay in CE is called ISUB (figure 3).

In AGA, detection and quantitation of the M-proteins are facilitated by staining the gels with a protein-binding dye that also has a sensitivity for M-proteins. This assay is susceptible to the differences in protein bindings with the dye. In addition, larger M-proteins can affect dye saturation, which can interfere with quantitation (33). Additionally, gel PEP requires manual handling of the samples and assessing the gels to detect and quantify the M-protein.

In contrast, in CE and ISUB, the sample processing can be automated. In this type of PEP, the sample migrates in a silica capillary and is detected with UV. A more throughout detection compared to protein biding dyes is enabled with the UV-detection. This can be observed with the higher sensitivity of CE, 95%, compared to AGA, 91%, while the specificity is similar for the two methods, 99% (34). However, it has been suggested that ISUB is less sensitive compared to IFE when assessing small M-protein, including light chain

M-proteins (35, 36). Comparisons between these assays have shown a lack of concordance in detection and quantitation of the M-protein (37-41)

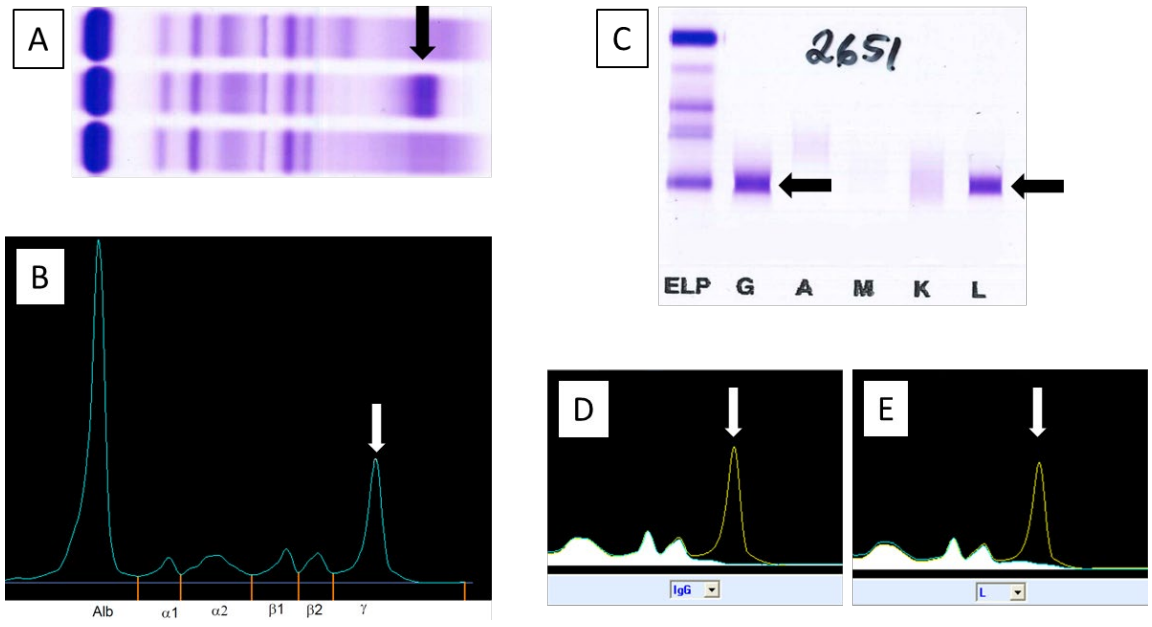


Figure 3. Detection (A and B) and class identification (C-E) of an IgG-λ M-protein with protein electrophoresis. A. High-resolution agarose gel electrophoresis B. Capillary electrophoresis. C. Immunofixation on agarose gel. D. IgG immunosubtraction in capillary electrophoresis, E. Lambda immunosubtraction in capillary electrophoresis. The arrows (black and white) points at the M-protein in each image.

Several challenges exist concerning the evaluation of PEP. The quantitation of M-protein is influenced by the chosen technique to integrate the M-protein against the polyclonal background. Both overestimation, with a perpendicular drop gating, or underestimation, with tangent skimming gating, of the M-protein, have been observed (33, 41). The quantitation can also be influenced by increased variability in smaller M-protein (<10g/L), comigration of M-protein in the fraction other than gamma, and the co-calculation of a polyclonal background with the M-protein (33, 35, 36, 41). Interferences such as hemoglobin-haptoglobin complexes in hemolysis and cryoglobulins, where the first can give the appearance of an M-protein, and the latter can precipitate as a band or a peak when the temperature drops below 37°C, can also affect detection. A significant interference is fibrinogen, that will produce a distinct band below C3 in the upper part of the gamma zone that can be difficult to distinguish from an M-protein. The use of serum instead of plasma will easily circumvent this issue. More recently, residues of monoclonal immunoglobulin-based treatments, where the occurrence of a new IgG-κ in a known MM patient, should raise concerns (42-44). While sPEP and IFE each have an overall relatively high sensitivity in PCDs, 79% and 87%, respectively, this is dependent on the subtype of PCD (28). The combination of both assays further enhances the sensitivity. Up to 94.4% of MM patients with complete M-protein, i.e., both heavy and light chain, are detected with a combination of sPEP and IFE (28). In addition to the challenges mentioned above, the detection limit is approximately 0.5g-1/L for sPEP and 0.1g/L for IFE. Thus, residues of an M-protein could

still be present with negative IFE, potentially affecting the accuracy of response classification that is based on sPEP and IFE levels (30). RCV for M-protein has been determined in stable MGUS patients. A change ranging from 25% up 39.6% in M-protein levels could be attributed to biological and analytical CV (45-47). This RCV can be contrasted to the IMWG relapse criteria, where a 25% increase of M-protein is classified as a biochemical progression.

Urine PEP (uPEP) is recommended in the work-up of suspected PCD to identify a possible light chain M-protein. For adequate sensitivity, uPEP and urine IFE (uIFE) should preferably be performed on the 24-h collection. However, a 24-h urine collection can be challenging to adhere to by patients. Morning samples can detect an M-protein with adequate sensitivity (48, 49), while random samples are unsuitable when assessing uPEP and uIFE (48). UPEP is affected by similar challenges as sPEP, including the detection limit.

1.3.4 Free Light Chains

The light chain part of an immunoglobulin, κ and λ, is produced in excess compared to the heavy chain. This excess production can be detected with FLC assays. These assays use mono or polyclonal antibodies targeting the light chain's hidden epitope to quantify the produced surplus. To assess the FLC, one has to account for the difference in production, where κ is produced in approximately 1.8:1.0 ratio compared to λ (15), and the renal clearance, where the monomer κ is cleared at approximately twice the rate of the dimer λ. Evaluation with the κ/λ ratio can indicate the presence of an M-protein when abnormal FLC ratios are observed. The ratio is reported either as κ/λ ratio (FLCr) or as involved/uninvolved ratio (iFLCr). However, it is important to note that not all patients with a PCDs will have an abnormal FLC ratio. Abnormal FLC ratios can be observed in 30-49% of MGUS cases and 74-90% in SMM compared to 95% of MM (18, 28, 50-52).

IMWG guidelines recommend assessing FLC in combination with serum IFE when screening for PCDs, except when AL-amyloidosis is suspected (25). FLC evaluation is also included in the risk stratification of MGUS and SMM (29, 53, 54). Assessment of FLC is incorporated in the stringent complete response (sCR) (30). However, the IMWG guidelines only support FLC assessment in patients with unmeasurable M-protein in serum and urine for stages other than sCR when assessing response and progression (55, 56). These recommendations were based on studies utilizing the Freelite assay. Currently, FLC measurements are not included in the recommendations for monitoring either MGUS, SMM, or MM (29). The combination of FLC and M-protein has repetitively demonstrated high sensitivity in MM diagnostics (28, 57). The high sensitivity has led to suggestions that response classification could be carried out with FLC rather than uM-protein (58). Additionally, as FLC serum dynamics are more rapid than immunoglobulins, an earlier prediction of response (including progression) could be anticipated in patients with MM (59). However, FLC's role compared to M-protein in assessing time to response or biochemical progression has not been thoroughly investigated. When evaluating FLC changes over time, similar to M-protein, the RCV of FLC should be considered (45, 47).

Assays for FLC detection

In 2001, a novel assay was released, Freelite, that detected the FLC (figure 4) (14). The Freelite assay utilizes polyclonal antibodies together with a turbidimetric platform to detect FLC (14). The advantages of the polyclonal antibodies are the ability to detect multiple epitopes and the relative in-expensive. Several assays have been developed after the introduction of Freelite. These assays have used both mono and polyclonal antibodies together with either nephelometric or ELISA based methods. Of the second-generation assays, N-Latex FLC utilizes monoclonal antibodies (60), for more reproducible detection, together with a nephelometric assay, to increase sensitivity and precision. One potential issue with a monoclonal-based assay is that the specificity for an antibody could be too specific. Thus, not detecting the whole spectrum of potential targets. Comparative studies between Freelite and N-Latex FLC have shown discrepancies in the absolute FLC values and iFLCr, with N-Latex FLC consistently having lower levels (61-64). However, no study has shown the superiority of either assay.

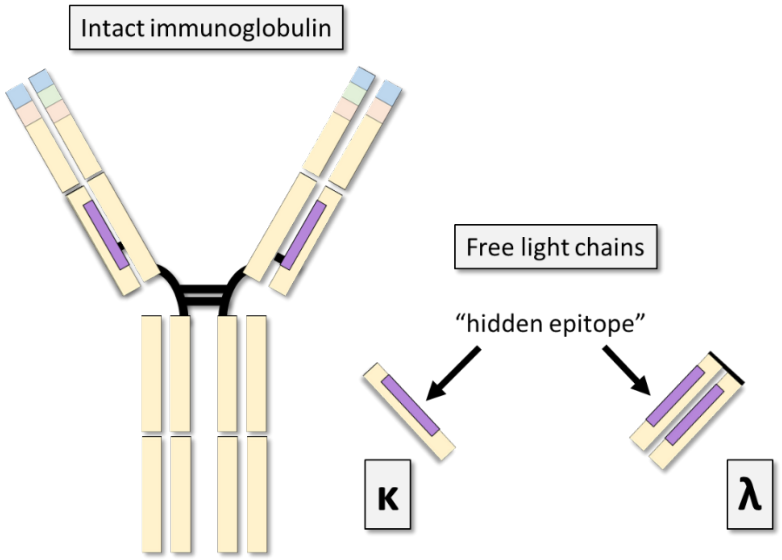


Figure 4. The hidden epitope. Serum free light chain assays utilize antibodies that target an epitope on the κ and λ chain. In intact immunoglobulins, this epitope is located between the heavy and light chain and therefore not accessible for detection.

1.3.5 Immunoglobulin assessment

Assays to quantify the Ig levels is a complement to M-protein assessment. A wide range of nephelometric, turbidimetric, and ELISA methods are available, however as these assays cannot distinguish between poly- and monoclonal Igs, they lack adequate sensitivity to enable complete detection of M-proteins as a stand-alone assay. With increased infiltration of malignant PCs in the bone marrow, a reduction of uninvolved Igs can be observed. Immunoparesis, the reduction of one or more of the uninvolved Igs below the lower limit of normal, is a suggested risk factor for progression in both MGUS and SMM (65-71).

1.4 PLASMA CELL DYSCRASIAS

1.4.1 Epidemiology

MM is the second most common hematological malignancy (72), with a median age at diagnosis of 66-70 years. The incidence of multiple myeloma worldwide was 2.1 per 100 000 in 2018 (73). However, the incidence rate varies considerably, with the higher rates seen in North America, northern Europe, and Australia (Figure 5). In Sweden, the incidence is approximately 6.7 cases per 100 000 (crude rate) (74). Within the group of PCDs, MGUS, rather than MM, is the most common presentation. The incidence of MGUS increases with age, affecting 3% of individuals 50 years or older and 10% of individuals over 70 years (75-78). SMM occupies the space between MGUS and MM, but the incidence is difficult to ascertain due to the lack of an ICD code for SMM. Estimates from studies indicate that 8-20% of patients diagnosed with MM were SMM, with an estimated incidence of 0,9 per 100 000 (79, 80). Similarly, the Swedish myeloma registry reported approximately 19% of MM cases from 2014 to 2018 as SMM (81).

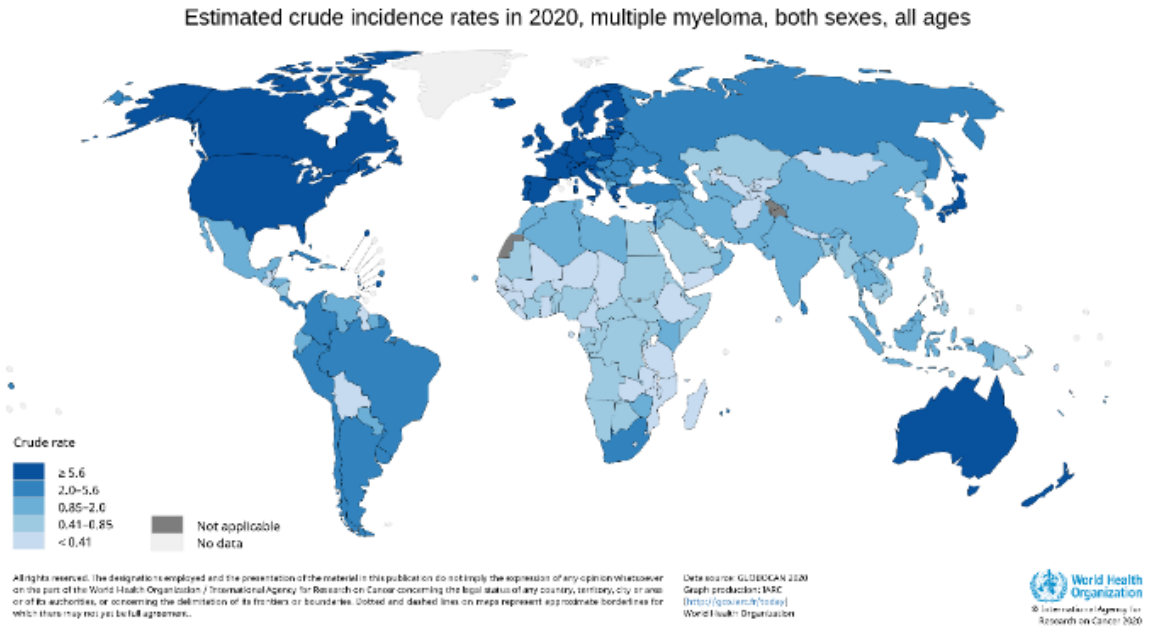


Figure 5 The crude incidence rate of multiple myeloma, source; the International Agency for Research on Cancer. (82)

MM and MGUS are more common in men than women (83-87). In addition to differences between genders, there is a difference in prevalence by ethnicity. Both MGUS and MM are more common in African Americans than Whites, while Asians exhibit the lowest prevalence (88-91).

PCD, like many hematological disorders, is a disease of the elderly. An increasingly elderly population together with diagnostics advantages will possibly lead to an increased prevalence of PCD. As MGUS is often an incidental finding in routine laboratory testing (78), more sensitive laboratory assays could increase incidence in the future. One indication of this possible increased prevalence is the extensive studies of the Olmsted county cohort. In this cohort the prevalence was estimated to 3.2% when assessed with PEP and IFE (78), 4.2% when the assessment of FLC was added (92), and 5.1% with the introduction of mass-spectrometry based evaluation (93).

1.4.2 Diagnosis and disease evolution

Diagnosis of MGUS, SMM, and MM is based on BMPC evaluation and M-protein assessment together with end-organ damage appraisal (Figure 6) (25). The CRAB criteria (increased serum calcium level, renal dysfunction, anemia, and destructive bone lesions) define end-organ damage (Table 1). Anemia is the most common, of the CRAB criteria, in symptomatic MM, observed in 73% of patients at diagnosis(94). As the anemia in MM is due to decreased production, it is typically normocytic. In contrast, hypercalcemia is the least common of the CRAB symptoms in MM(94).

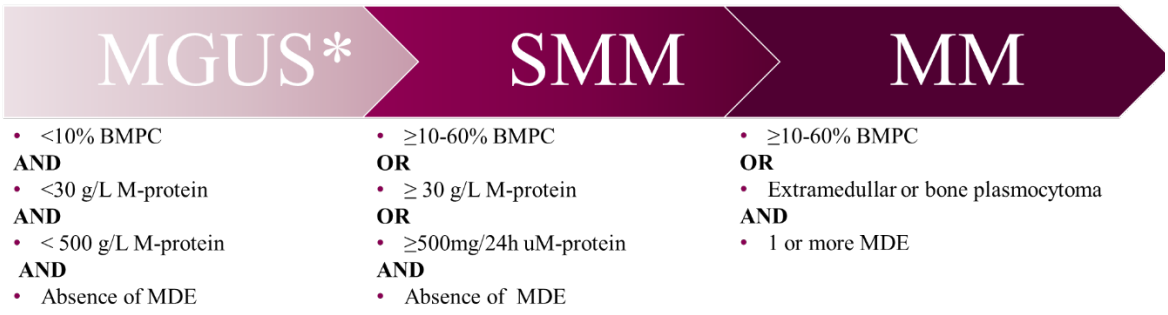


Figure 6 The diagnosis criteria of plasma cell disorders. MGUS= monoclonal gammopathy of undetermined significance, SMM=smoldering multiple myeloma, MM= multiple myeloma, BMPC=clonal bone marrow plasma cells, M-protein=Monoclonal protein in serum/plasma, MDE=Myeloma defining event including CRAB criteria *Non-IgM and IgM MGUS.

Table 1. Definitions of CRAB and MDE

CRAB	Hypercalcemia	Serum calcium level >2.75mmol/L or >0.25mml/L above upper limit of normal
	Renal insufficiency	creatinine clearance <40 ml/minute or serum creatinine > 177µmol/L
	Anemia	hemoglobin value of >20g/L below the lowest limit of normal or below 100 g/L
	Bone Lesion	≥ one osteolytic lesion on skeletal X-ray, CT, or PET-CT
MDE	BMPC	≥60%
	iFLCr	≥100 and involved FLC ≥100 mg/L
	Focal lesion	>one lesion, at least 5mm in size, on MRI

MDE=myeloma defining events, BMPC = clonal bone marrow plasma cells, iFLCr= involved serum free light chain ratio, CT=computed tomography, PET-CT=positron tomography-CT, MRI=magnetic resonance imaging

It is currently considered that symptomatic MM evolves from a premalignant stage, MGUS, and SMM (17, 18, 86). This risk of evolution often leads to a life-long follow-up of patients with MGUS and SMM. The overall progression rate to symptomatic MM is higher in patients with SMM than MGUS (65, 68, 95, 96). However, the risk of progression in SMM is not stable over time. Instead, the highest risk of progression is observed in the first five years (10% annual risk), and after ten years, the risk or progression is similar to that of MGUS (1% annually) (95)

Molecular heterogeneity

Characterization of the genetic landscape of MM and its more benign precursor stages have . shown a vast genetic heterogeneity and complexity where the tumor cell genome can have multiple structural variants and copy number variations in addition to a multitude of point mutations. Two major subgroups of primary chromosomal aberrations have been identified in MM: hyperdiploidy and non-hyperdiploidy (97-99). The former is characterized by trisomy's of odd number chromosomes and the latter by translocations affecting mainly the Ig heavy (IGH) chains locus. Two translocations, t(4;14)(p16;q32) and t(14;16)(q32;q23) are associated a shorter OS (100), while role of the most common IgH translocation t(11;14)(q13;32) is unclear (101-104).

The gain of mutations required for the plasma cells to evolve from the premalignant stages of MGUS and SMM to symptomatic MM have previously been studied, where clonal heterogeneity is already present at MGUS and SMM (105). Two different patterns, static and evolutionary, have been observed in SMM progression to MM (106). Similar is seen in multiple myeloma where branching, linear and stable evolution patterns of chromosomal aberrations can be seen during the treatment of MM (107-110). Therefore, biomarker expression could be anticipated to show different patterns before, during, and after treatment.

1.4.3 Monoclonal Gammopathy of Undetermined Significance

Three different types of MGUS, non-IgM MGUS, IgM MGUS, and light-chain MGUS (LC-MGUS), have been described (Table 2). MM progression patterns differ between these subtypes, ranging from 0.5 to 2% of annual risk of progression (50, 65, 92, 96, 111). Additionally, progression to other PCDs such as AL-amyloidosis can be observed (65, 68, 96, 112). Due to the risk of progression, a life-long follow-up is recommended, albeit with longer intervals between assessment for patients with a low risk of progression (29). However, as not all MGUS patients will progress to a treatment demanding MM, this lifelong monitoring of patients could be questioned. Particularly in those with unmeasurable M-proteins (<10g/L) (112), a disappearance of the M-proteins has been observed in 2-5% of MGUS patients over time (65, 112). Furthermore, recently, it has been debated whether LC-MGUS progresses to active MM at all (111).

Table 2. Monoclonal gammopathy of undetermined significance subtypes

	Diagnose criteria	Yearly risk of progression	Progression to
Non-IgM	• <30g/L M-protein • <10% BMPCs • No MDE or CRAB	Approximately 1%	MM, Plasmacytoma, AL amyloidosis
LC-MGUS	• Abnormal FLCr • Increased level of iFLC • No heavy chain on IFE • <500mg/24h in uM-protein • <10% BMPCs • No MDE or CRAB	Approximately 0,3%	Light chain MM, Al Amyloidosis
IgM	• <30g/L M-protein • <10% BMPCs • No MDE or CRAB • No symptoms attributed to lymphoproliferative disorder	Approximately 0,5%	Waldenstrom's, AL amyloidosis, in rare cases IgM MM

BMPCs=clonal bone marrow plasma cells, M-protein=monoclonal protein in plasma/serum, uM-protein=monoclonal protein in urine, MDE=myeloma defining events, CRAB=hypercalcemia, renal dysfunction, anemia, bone lesions, MM=multiple myeloma, MGUS=monoclonal gammopathy of undetermined significance, FLCr=serum free light chain ratio, iFLC=involved serum free light chain, IFE=immunofixation

While MGUS is often considered a relatively benign disorder due to the observed low risk of progression to a malignant disorder, excess mortality, unrelated to malignant progression, has been observed in individuals with MGUS (67, 112-114). An increased risk of death by disorders such as cardiovascular, liver, and renal disease and infections add to the excess mortality observed in individuals with MGUS (67, 113, 114). As MGUS is a condition generally detected during the clinical work-up of an unrelated disorder, it is possible that the excess mortality observed is related to the underlying comorbidities. Thus, the correlation

observed between MGUS and excess mortality might be without causation. However, identifying risk factors associated with decreased overall survival in MGUS patients would be interesting to expand the knowledge within to possible causes for the excess mortality.

Risk prediction in MGUS

The risk of MGUS progression has been evaluated in numerous prediction models (Table 3). The current IMWG guidelines have incorporated the Mayo Clinic model (29). This model, investigating risk factors in a cohort of 1384 MGUS, identified three risk factors, M-protein >15g/L, abnormal FLCr, and non-IgG isotype (50, 112). The latter, non-IgG isotype, has not been confirmed as an independent risk factor in other studies (68, 69, 115-117), but rather IgA isotype. Thus, the non-IgG isotype could be regarded as an unreliable risk factor. In addition to established risk factors, several other risk factors have been investigated. The majority of these reflect the tumor burden or the end-organ damage caused by PC infiltration in BM (65, 66, 68, 117-119). Immunoparesis has been suggested in multiple studies as a potential risk factor (65, 66, 68, 117). The consistency of increased risk in multiple studies would suggest its potential use in predictive models.

With the current IMWG risk prediction score, patients classified as low risk still have a 5% risk of progression at 20 years (29). With this relatively low risk of progression, one might advocate that low-risk patients could be omitted from follow up. On the other hand, as both M-protein and FLC are comparatively non-invasive tests, monitoring these biomarkers would be feasible in most MGUS patients. An evolving MGUS, any annual increase over a period of three years, has been recognized as a risk factor for malignant transformation (116). Similarly, changes from low-risk to high-risk MGUS prior to MM progression have been observed in MGUS patients when assessed with the IMWG risk prediction model (117). This observation indicates that FLC, in addition to M-protein, is an essential biomarker for monitoring. However, cut-off in temporal changes in biomarkers associated with MGUS progression and how to interpret the risk of progression when monitoring MGUS patients with FLC has not been investigated to date.

Table 3 Risk factors, assessed in peripheral blood, for progression in monoclonal gammopathy of undetermined significance

	Blade et al (115)	Cesana et al (65)	Kyle et al (112) / Rajkumar et al (50)/ Katzmann et al(120)	Rosinol et al (116)	Tureson et al (68)	Sandecka et al (69)	Landgren et al (117)
MGUS patients	120	1104	1384	359	728	4887	685
Progress to MM	13 (10%)	43 (4%)	115 (6%)	32 (9%)	53 (7%)	162 (9%)	187 (27%)
Median age	61	63*	72	66	74	63	69
Median follow-up, months	56	65	185/185/NR	93	120	48	**
IMWG risk factors							
M-protein >15g/L	No	NR	Yes	Yes	Yes	Yes	Yes
Abnormal FLCr	NR	NR	Yes	NR	Yes	Yes	Yes
Non-IgG subtype	Only IgA	Yes	Yes	Only IgA	No	No	Only IgA
Additional risk factors							
Age	No	NR	No	No	NR	Yes	NR
Immunoparesis	NR	Yes	No	NR	Yes	Yes	Yes
Hemoglobin<120g/L	NR	NR	NR	NR	Yes	Yes	NR
iFLC	NR	NR	NR/Yes/Yes	NR	NR	NR	NR

MGUS= monoclonal gammopathy of undetermined significance, MM=multiple myeloma, IMWG=international myeloma working group, MP=monoclonal protein in serum, FLCr=serum free light chain ratio, iFLC=involved serum free light chain, NR= not reported/**not evaluated**, Yes= a significant risk factor, No= not a significant risk factor, *including SMM patients, ** patients included 1993-2011

1.4.4 Smoldering Multiple Myeloma

Like MGUS, SMM is a premalignant stage to symptomatic MM, albeit with a higher risk of progression, 10% annual risk of progression in the first five years after diagnosis (95). As in MGUS, there is no end-organ damage in SMM; however, M-protein levels (≥30g/L) and/or the BMPCs (10-60%) are consistent with MM findings. The diagnosis criteria for SMM have changed during the years (25, 121) due to the identification of ultra-high-risk factors. These ultra-high-risk factors, ≥BMPC >60%, iFLCr ≥100 and 1 or more focal lesion on MRI, were initially considered risk factors for progression to MM but have since been redefined as MDE (25, 122-125).

Evidence shows that treatment of high-risk SMM prolongs the time to progression (TTP) and even overall survival (OS) (126-128). However, as the cancer treatment investigated can have serious side-effects, including an increased risk of secondary malignancies (129), and not all SMM will develop MM, it is essential to identify those patients where the benefit will overweigh the risk of side-effects.

Risk prediction in SMM

Risk factors for SMM progression to MM have been extensively studied. Similar to MGUS, the M-protein size is a significant risk factor for progression also in SMM (52, 70, 71, 95, 130-134). The first Mayo clinic prediction score defined a group of SMM with M-protein>30g/L and BMPC>10%, where the 5-year progression was 68% in patients with both risk factors present at diagnosis (95). The risk prediction model was further refined with the incorporation of the iFLCr (52). A predictive value has also been associated with immunoparesis (70, 71), albumin (131), and FLCr/iFLCr (52, 71, 131, 132). Several of these studies included SMM patients according to the earlier diagnosis criteria (121) before MDE was defined. Thus, these studies could have included symptomatic MM in their cohorts. More recently, including SMM patients based on the 2014 criteria, defined BMPC (>20%), M-protein (>20g/L), or iFLCr (>20) as predictors of progression to MM(132). This observation has since been validated in a large multicenter study and incorporated into the SMM IMWG risk stratification (54). Although the assays used for FLC determination in the multicenter study are not specified, one can assume that it would have included patients assessed with Freelite and N-Latex as centers in Europe were included in the study. As the iFLC and iFLCr are highly dependent on the assay utilized (61-64), it would be important to validate the risk prediction model in an N-Latex FLC cohort.

The evolving SMM, a successive increase of M-protein during follow-up, was initially described as a risk factor for progression to MM (130). Suggested dynamic risk factors for progression of SMM to MM have been an absolute and relative increase in M-protein (133-136), the relative increase of iFLCr (136), and iFLC (135), as well as an absolute decrease in Hb (133, 135). The definition of both absolute and relative increase in M-protein and the timeframe over which changes had to occur differed between these studies, making comparisons difficult. The relative increase could potentially overestimate the risk of progression in patients with a minor M-protein. The studies either tried to adjust for this with either a more remarkable relative change in low-level M-protein or an absolute minimum

change. Even though there are differences between these studies, they all show dynamic changes of the tumor surrogate markers such as M-protein and FLC could further differentiate patients at risk of progression.

1.4.5 Multiple Myeloma

The 5-year survival rate in MM is rapidly increasing with the advance of novel therapies, from approximately 40% in 2002 to 54% in 2010-2016 (137). However, MM is still considered essentially incurable. Thus, a majority of patients will relapse and develop resistance to existing therapies.

At baseline, the iFLC ratio has emerged as a prognostic marker for MM in several reports, where high baseline iFLC is associated with worse overall and event-free survival (138-140). However, other studies have not successfully demonstrated a correlation with survival (141, 142). In one of the later studies, early FLC response (at two months) was superior to early M-protein response in predicting overall response (142). Earlier response by iFLC as a superior factor in predicting overall response has been supported in several studies (139, 142, 143). Both relative and absolute changes are well defined for M-protein and uM-protein, while assessment by iFLC is currently only recommended in patients without measurable M-protein/ uM-protein. Also, there is a lack of cut-offs for iFLC for response evaluation in MM when the M-protein is evaluable in sPEP or uPEP. With a sensitivity close to 100%, when iFLC, sPEP, and IFE is combined, it has been suggested that monitoring should be performed with FLC instead of uM-protein (58).

In MM, biomarkers can be expected to fluctuate with the response to treatment and the disease's progression. Thus, evaluation of dynamic changes in the follow-up of MM is crucial to determine response to treatment as well as to identify when biochemical progression occurs (30).

Risk prediction in MM

The prediction models in MM focus on defining the risk at diagnosis rather than evaluating changes over time. Several prognostic variables in newly diagnosed MM have been identified, such as the subtype of M-protein, C-reactive protein, albumin, β2-microglobulin (β2M), FLC, and CAs. The international staging system (ISS) is relatively easy to evaluate due to the incorporation of only serum markers, β2M, and albumin, reflecting both tumor burden and bone marrow microenvironment (144). CAs have been extensively investigated as risk factors in MM, and several CAs have been assessed together with ISS (145-148). The Revised International Staging System (R-ISS) builds on the ISS while also including CAs associated with high-risk MM and lactate dehydrogenase (Table 4) (149).

Table 4. Prognostication by ISS and R-ISS

	Stage	Criteria
ISS	I	β2M<3.5mg/L and serum albumin ≥35g/L
	II	β2M<3.5mg/L and serum albumin <35g/L or β2M 3.5-5.5mg/L regardless of serum albumin level
	III	β2M>5.5mg/L
R-ISS	I	ISS I and no high-risk CAs and normal LDH
	II	Neither R-ISS I or III
	III	ISS III and high-risk CAs or elevated LDH

ISS= international staging system, R-ISS=revised international staging system, β2M=beta-2-microglobulin, LDH= lactate dehydrogenase, high risk CA= del(17p) and/or t(4;14) and/or t(14;16)

1.5 RESEARCH GAP

With a heterogeneous risk of progression in premalignant stages of PCD and heterogeneous outcomes to treatment in active MM, the need to identify high-risk individuals is vital.

Dynamic changes in biomarkers have not been extensively studied in MGUS and is of interest to enable earlier detection of progression. While dynamic changes have been explored in SMM, the results have been non-conclusive. Preventative measures to limit the progression to MM are being investigated in SMM and potentially be considered for high-risk MGUS in the future. However, this intervention can have serious side-effects. Thus, identifying patients that might benefit the most, those at the highest risk of progression, from intervention is crucial.

Currently, FLC should be evaluated at diagnosis to assess PCD and for risk prediction. As these recommendations have been based on studies performed with one of several available assays, there is a need to investigate risk factors when using other available assays.

Thus, this Ph.D. project focuses on serum biomarkers' for the monitoring of premalignant PCD and treatment demanding MM.

2 RESEARCH AIMS

The aim of this thesis was to adapt laboratory diagnostics for prognostication of plasma cell dyscrasias. The specific objectives of the studies were:

Study I

- To evaluate the diagnostic value of dynamic changes in protein assays as markers for progression in MGUS

Study II

- To evaluate temporal changes in biomarkers as risk factors for progression from SMM

Study III

- To compare the difference in time to response and progression in MM assessed by protein electrophoresis and sFLC

Study IV

- To identify risk factors for increased mortality in non-progressing MGUS

3 MATERIALS AND METHODS

3.1 SETTING AND OVERVIEW

The publicly funded Swedish healthcare system entails every citizen to equal care. The Stockholm-Gotland region is one of the six regions of the healthcare system. In 2009-2020, this region's population increased from 2.1 to 2.4 million inhabitants (from 22 to 23% of the Swedish population) (150). The Stockholm-Gotland region is the largest region in Sweden and the study base for **studies I-IV**.

Studies I-IV are retrospective registry-based cohort studies (Table 5). The main database constructed to perform these studies included retrospective data from laboratory, electronic medical journals, national death registries, and biobanks.

Table 5. Overview of studies I-IV

	Study I	Study II	Study III	Study IV
Study design	Cohort	Cohort	Cohort	Cohort
Data collection	Retrospective	Retrospective	Retrospective	Retrospective
Study	Region-wide, Stockholm; Sweden	Region-wide, Stockholm; Sweden	Region-wide, Stockholm; Sweden	Region-wide, Stockholm; Sweden
Study population	Patients >18years with MGUS	Patients >18 years with SMM	Patients >18 years with treatment demanding MM	Patients >18 years with MGUS and no progression to MM
Time period	2009-2017	2009-2020	2009-2017	2009-2020
No of patients	987	126	450	1103
Outcome	Progression to MM	Progression to MM	Response and progression	Death Prevalence of co-morbidities

3.2 STUDY POPULATION AND STUDY DESIGN

The study design defines a study's ability to answer the research question. Observational studies such as retrospective cohort studies, due to their inherent design, assess correlation but not causality. Thus, these studies can tell us which factors that are associated with the event of interest but not claim that a factor cause-specific events. The individuals included in a cohort study are followed over time from the exposure until the outcome, death, or follow-up. The study population is defined with inclusion and exclusion criteria.

The study populations included in **studies I-IV** were identified from a database of laboratory parameters. All adult patients subjected to s/uPEP and FLC assessment between 2009 to 2017 at Karolinska University Laboratory (KUL) were identified (n=4756). We selected individuals from the respective database depending on each study's inclusion and exclusion criteria.

Study I investigated the association of M-protein/uM-protein and iFLC elevation, in individuals with MGUS, with the risk of progression to symptomatic MM. A total of 987 individuals with MGUS were included. The inclusion criteria were MGUS diagnosis with matched samples of iFLC and M-protein or uM-protein at MGUS diagnosis. Exclusion criteria were M-protein >30g/L, IgM heavy chain, progression to a hematological disorder other than MM, an MGUS or MM diagnosis prior to the first FLC sample, and lack of samples at least six months prior to the date of MM diagnosis in the case of progressions. We also excluded individuals with CRAB or MDE. The primary outcome was progression to MM, defined as the onset of symptomatic MM. The temporal changes of M-protein and iFLC were investigated in a subgroup of patients (n=516) with a minimum of 2 serial samples taken three months or more apart during the study period. Patients were followed until progression to symptomatic MM, death, or end of follow-up.

In **study II**, we included 126 patients with SMM to investigate both static risk factors at the diagnosis of SMM and dynamic risk factors during the follow-up, associated with a risk of progression to symptomatic MM. Inclusion criteria were an SMM diagnosis by the 2014 IMWG criteria. Patients were excluded if they had two or fewer matching iFLC and M-protein/uM-protein samples at least six before the onset of symptomatic MM. Patients were followed until progression to symptomatic MM, death, or end of follow-up, August 2020.

In **study III**, we identified patients with MM and sequentially samples of FLC and M-protein/uM-protein to assess differences in time to response (TTR) and TTP when measured by iFLC, M-protein, or uM-protein. We excluded patients without laboratory assessment of both iFLC and M-protein or uM-protein at MM diagnosis and those lacking additional measurement within 100 days of MM diagnosis. We also excluded patients who did not respond to first-line treatment. A total of 450 patients were included in the study population and followed for up to three response and progression cycles.

Study IV was performed to assess the co-morbidity and cause of death patterns in individuals with MGUS. We identified individuals in the database without a treatment demanding PCD or other hematological malignancy. These individuals' electronic medical journals were assessed from January 2020 to February 2020 to verify an MGUS diagnosis at any time until the end of follow-up and extract ICD codes for diagnoses other than PCDs and hematological

malignancies. Causes of death were acquired from the national board of health and welfare death registry. We included 1103 MGUS patients diagnosed between 1982 and 2017. Patients were followed until the date of death, lost-to-follow-up, or end of follow-up, February 2020.

3.3 MATERIALS

Sweden is well known for the long tradition of systematically collecting data on citizens and individuals with permanent residency. An individual residing in Sweden is assigned a personal identification number (PIN) consisting of birth date, a control digit, and a three-digit unique code that includes a sex-specific digit (YYMMDD-XXXX). Since the introduction in 1947, the PIN is extensively used in nationwide registers and medical journals. This PIN allows linking between registers and enables extensive cohort studies with long and complete follow-ups (151).

Case identification

The KUL Information System (LIS) at Clinical Chemistry (Flexlab/LifeCare, Tieto, Helsinki, Finland) was used to identify cases. A data retrieval algorithm was defined to identify cases for inclusion. The algorithm's order was first to identify age >18 years, followed by an FLC assessment and lastly, an analysis of M-protein or uM-protein within seven days of the FLC assessment. Data available within ± seven days of a matched FLC and M-protein/uM-protein measurement were extracted when all the algorithms' conditions were met. In cases where multiple measurements of a biomarker were available, only the measurement closest in time to the FLC measurement was retrieved. Laboratory data from September 1st, 2009, until April 1st, 2017, was extracted in the database's first closure. In total, 4756 individual cases fulfilled all three criteria at one or more timepoints. The Karolinska University Laboratory IT department pseudonymized all data during the extraction process from Flexlab.

Database construction

Laboratory data from clinical chemistry

Laboratory parameters included, when available, in the extraction from FlexLab were; complete blood cell count including erythrocyte indices, creatinine, iohexol, cystatin c, eGFR, calcium, beta-2-microglobulin, albumin in serum and urine, orosomucoid, CRP, antitrypsin, FLC κ, FLC λ, FLCr, total immunoglobulins (IgG, IgA, and IgM), urine HC, total IgG in urine, total κ and λ in urine, size and isotype of M-protein and uM-protein and whether IFE/uIFE had been performed. Parameters calculated in the database were eGFR, iFLC, and iFLCr. EGFR was calculated using the LMRev formula(152). IFLC is calculated as involved FLC-uninvolved FLC and iFLCr as involved FLC/uninvolved FLC.

In total 30,052-time points with approximately 3 million laboratory values were included in the database.

At KUL, a trained clinical chemist assesses the M-protein in sPEP/uPEP and IFE together with total immunoglobulin levels. The clinical chemist writes a free text statement containing information on the presence of M-protein, size and isotype of the M-protein, and whether IFE has been performed. The free text results were manually coded into numerical values. In

brief, 39842 sPEP/uPEP and IFE were coded as follows. The presence of an M-protein was coded as 1 and no M-protein as 0. The heavy chain was coded; IgG=1, IgA=2, no heavy chain in sIFE/uIFE=3, IgM and other=4, two or more M-proteins=6, and oligoclonality=6. The light chain was coded as κ=1 and λ =2. After the first round of manual coding, a random 5% of samples were selected and controlled by another individual.

Bone marrow assessments (study II and III)

The cases identified in the laboratory database extraction were linked to a clinical database containing results from FISH performed on BM samples. In brief, PBMC is separated with density gradient centrifugation (LymphoprepTM, Axis-Shield, Oslo, Norway). The samples then undergo purification with CD138 magnetic beads selection (Miltenyi Biotec, Bergisch Gladbach, Germany) before being added to hybridization slides (2–4 x 104 cells/spot) and air-dried overnight. Probes targeting 1q21/8p21, 6q21/15q22, 17p13.1/19q13, 9p21/9q21, 13q14/qter, and for translocations t(4;14) (p16.3;q32.3), t(11;14)(q13;q32.3), t(14;16)(q32.3;q23) (Kreatech, Amsterdam, the Netherlands) are used for detection of chromosomal aberrations. Two hundred nuclei are evaluated for each probe set. Additions and deletions are determined using a 10% cut-off for positive observations except for del(17p), where the cut-off is 60%.

Medical records

The electronic medical records code each healthcare visit according to the Swedish Revisions of the International Classification of Disease (ICD) system (ICD-10: 1997 onwards). These records were used in **studies I-IV** and reviewed for diagnoses and dates of MGUS, SMM, and MM, other plasma cell disorders, and hematological disorders. In **study IV**, ICD diagnoses relating to chronic and acute disorders were identified. ICD-codes and dates associated with the codes for cardiac, renal, liver, pulmonary, other cancers, infectious, inflammatory and immune disorders were included in the database. The electronic medical records were queried twice to acquire diagnoses and diagnosis dates, in 2017-2018 for **studies I and III**, and again in 2020 for **studies II and IV**.

Cause of death register.

This register is held by the national board of health and welfare and contains data on the ICD codes for underlying and leading causes of death that were used in **study IV**.

Progression to MM

The date of progression from MGUS or SMM was defined as the first date when symptomatic MM was assigned by a specialist in conjunction with a hematology clinic visit.

Response and progression after symptomatic MM

The IMWG criteria for response and biochemical progression were used to assess response and relapse (Table 6) (30).

Table 6. IMWG definitions of biochemical changes for response and progression in multiple myeloma

	M-protein	**uM-protein**	**iFLC**
Stable disease	Does not meet the criteria for complete response, very good partial response, partial response, minimal response, or progressive disease		
Minimal response	49-25% reduction	50-89% reduction	--
Partial response	≥50% reduction	≥90% reduction or <200mg/24h	≥50% reduction in iFLC
Very good partial response	≥90% reduction or not detectable electrophoresis	<100mg/24h	-
Complete response	Negative immunofixation	Negative immunofixation	-
Stringent complete response	Complete response		AND normal FLC ratio
Biochemical progression	≥25% increase from lowest		
AND absolute increase	≥5g/L or ≥10g/L if lowest M-protein was >30g/L	≥200mg/24h	≥100mg/L in iFLC

IMWG=international myeloma working group, M-protein=monoclonal protein in serum/plasma, uM-protein=monoclonal protein in urine, iFLC=involved serum free light chain

Glasgow Prognostic Score

In **study IV**, we graded the inflammation by Glasgow prognostics score (GPS), table 7 (153).

Table 7. The Glasgow prognostic score

	Points
CRP ≤ 10mg/L and albumin ≥35 g/L	0
CRP > 10mg/L and albumin ≥35 g/L	1
CRP ≤ 10mg/L and albumin <35 g/L	1
CRP >10mg/L and albumin <35 g/L	2

Routine chemistry assays (Study I-IV)

Routine laboratory parameters were measured with accredited routine assays at KUL. FLC measurements were conducted with latex-enhanced immunonephelometric assay (Siemens Healthcare GmbH, Erlangen, Germany). Protein electrophoresis and immunofixation were performed with agarose gels on the Hydrasys/Hydrasys 2 platform (Sebia, Lisses, France). Total immunoglobulin (IgG, IgA, and IgM) concentrations were analyzed using immunoturbidimetric assay (Roche Diagnostics GmbH, Mannheim, Germany).

3.4 STATISTICAL ANALYSIS

The statistical analysis was performed as described in detail in the separate papers and are explained briefly here. In general, a two-sided p-value <0.05 was considered statistically significant.

Descriptive analyses (study I-IV)

Categorical variables are presented as counts with percentages, and continuous variables are presented as median and interquartile ranges (IQR). Pearson's chi-square test was used to analyze the differences between groups for categorical variables. For continuous variables, the independent student T-test was performed in the case of normally distributed variables. Kruskal-Wallis and Mann-Whitney U-test were performed for non-normally distributed variables depending on the number of groups.

Receiver operating characteristics and area under the curve (study I-II)

Receiver operating characteristics (ROC)

ROC analyses are used to assess the diagnostic accuracy, sensitivity and specificity (Table 8), of a test. A binary outcome and ordinal or continuous predictor variables are required to perform a ROC analysis. The sensitivity for each level of the predictor variable is plotted on the y-axis, and the 1-specificity is plotted on the x-axis. A variable with a ROC curve plotted close to y=1 and x=0 will have a higher probability of distinguishing between diseased and non-diseased.

Table 8. Assessment of diagnostic test

		TEST		
		Diseased	Non-diseased	
TRUTH	Diseased	True positive (A)	False negatives (B)	Sensitivity = A/(A+B)
	Non-diseased	False positives (C)	True negatives (D)	Specificity = D/(C+D)
		PPV =A/(A+C)	NPV =B/(B+D)	

PPV=positive predictive value, NPV= negative predictive value

The area under the ROC curve (AUC)

The AUC represents the overall discriminatory ability of the predictor variable. The higher the AUC, the better the model distinguishes between patients with the disease and no disease. The AUC can be used to assess/quantification the diagnostic accuracy of a test. In a perfect test with 100% sensitivity and 100% specificity, the AUC would be 1.0. In contrast, a test that cannot discriminate between diseased and non-disease would have an AUC of 0.5. The AUC can be interpreted as follows: if a test has AUC 0.8, the test value of a random diseased individual will be higher than that of a random non-diseased individual 80% of the time.

Youden's J statistics.

The Youden J index can determine the optimal cut-offs for the predictive variables evaluated with ROC AUC. A high value of a Youden index indicates a combination of both high sensitivity and specificity. Youden J index is calculated as follows: J=sensitivity(%)+specificity(%)-100.

ROC and AUC analysis were used in **study I** to determine the discriminatory ability, sensitivity, and specificity of the values assessed for relative and absolute increases. In **study II**, ROC and AUC analysis was performed to assess potential predictive variables, and the Youden index was calculated to identify the optimal cut-offs for each variable.

Time-to-event analysis

A critical outcome variable evaluated in all the studies was the time from the diagnosis of a PCD to a critical event. In survival analysis, the time-to-event is the primary interest. The time is calculated from a defined point in time, often the study's inclusion or the time of exposure to a critical event. An essential feature of the time-to-event analysis is the presence of censoring. When an individual's survival time is unknown, censoring occurs. For all four studies, patients were censored if they were (i) alive at last follow-up, (ii) death from a cause other than MM, (iii) lost to follow-up. The date used at the last follow-up date in censored cases was the medical journal's last visit date.

Cox proportional hazard regression (studies I-IV)

Cox regression analysis is a method for assessing the risk associated between variables and the survival rate. The model assumes that the risk of an event, often progression or death, is a function of the independent variables. Prospective risk factors and possible confounders can be assessed in univariate and multivariate analyses. When assessing groups with different exposure, the risk of an event (hazard) associated with the exposure variable can be estimated.

Cox regression was used to analyze potential predictors of progression to MM (**studies I-II**) and risk of death (**study IV**). The prospective risk factors were first assessed in univariate analyses. Significant risk factors in the univariate analyses were entered in the multivariate analyses.

In **study I**, serum and urine M-protein and FLC's continuous values were assessed as log-transformed due to the skewed distribution. Univariate cox regression was used to assess the

predictive capability of increases of either M-protein or uM-protein or iFLC. In the predictive analysis, three different prognostic variables' indicators within a specific period, the absolute change, absolute threshold, and relative change, were evaluated. These indicators are a dummy variable, where the change in a specific period is tested; For an absolute change, we assessed the difference between iFLC, M-protein, or uM-protein at time, t_i, versus the value of iFLC, M-protein, or uM-protein at the time of MGUS diagnosis, t_0. Then, for the relative change threshold, we look at the percent increase from t_0 to t_i. For absolute threshold, we assessed if the value of iFLC, M-protein, or uM-protein at t_i was above the investigated cut-off regardless of the value at t_0.

Each prognostic variable was then tested via a Cox regression for each three months interval. The cox regression results were visualized by plotting the hazard rate with confidence intervals in each of those regressions.

Kaplan-Meier survival curve and log-rank test

KM plots are a statistical method that visualizes the time to an event obtained from survival tables. The cumulative probabilities of an event can be compared between groups with different exposure with the log-rank test.

In **studies I and II**, we used KM plots to visualize the cumulative risk of progression between the risk groups based on risk factors identified in multivariate cox regression. As there were more than two groups to compare, we used log-rank pairwise over strata to compare each group's difference. KM was also used in **study IV** to compare the difference in survival between patients with different heavy chain groups and by inflammatory status.

In **study III**, Kaplan-Meier analysis was used to compare differences in TTR and TTP for iFLC and M-protein or uM-protein. We calculated the time to the event according to Table 9. In brief, the date when a partial response or better was first obtained in each treatment line was used to define a response. The date of biochemical relapse was defined as the timepoint when an increase compared to the lowest value observed in a response cycle.

Table 9. Calculation of time to event in **study III**

		Time from	**Time to**
First cycle	TTR	Diagnose date	The first date of the partial response of better
	TTP	Date of the best response	Date of biochemical progression
Second cycle	TTR	Date of the highest value of the first progression	The first date of the partial response of better
	TTP	Date of the best response	Date of biochemical progression
Third cycle	TTR	Date of the highest value of the second progression	The first date of the partial response of better
	TTP	Date of the best response	Date of biochemical progression

TTP = time to response, TTP = time to progression

3.5 ETHICAL AND LEGAL ASPECTS

All studies were conducted following the declaration of Helsinki and approved by the Swedish Ethical Review Authority. EPM: 2017/349-31 and 2019-06564 and EPM 2014/526-31/3 and 2015/973-32 respectively.

The biological samples of patients with symptomatic MM used for FISH assessment in **studies II-III** were registered as a biobank in the Stockholm Medical Biobank. These patients provide a signed informed consent before sampling for the biobank.

The databases in **studies I and III** analyzed by the Netherland company Ingress-Health were pseudonymized before the data sharing. The data transfer was detailed in a Medical Transfer Agreement (MTA) and a Personal Data Processors Agreement (PDPA) between Karolinska University Hospital and Ingress-Health.

There are different aspects of ethical considerations in research projects involving human subjects, from the general human right to moral and ethical principles. While several guidelines, recommendations, and consensus documents exist, they are all to some extend based on the fundamental ethical principle applicable to all humans: the principle of respect for autonomy and integrity, the principle of doing no harm, and the principle of justice, which is discussed below in brief.

For **studies II and III**, the study subjects' written informed consent was given at the hematology clinical before the patients were sampled for routine assessment. If they accepted to participate in the study, an extra tube of bone marrow was drawn. To draw an extra sample in a patient already supposed to be sampled is a minimal health risk. According to the local guidelines as well as the ethical permit, patients would only be sampled if a sample were to be drawn for routine care. The sample was supposed to be drawn last after routine samples had been obtained, and in the case of difficulties sampling, only routing samples were collected.

For both studies, the patients may indeed have felt exposed to the health care professionals' goodwill, perhaps wondering whether refusing to participate and possibly giving an extra sample would menially influence their care quality. However, it is also common that patients invited to participate in studies have a sense of philanthropy and content at the prospect of improving the care of future patients. It is possible that patients were worried about receiving informed consent regarding evaluating risk factors in the bone marrow.

In **studies I-IV**, the retrospective data collected were pseudonymized with a unique study code by the Karolinska University Laboratories IT department during the data extraction. Only pseudonymized data was handled during the statistical processing of the studies. The individual study subject was not informed or asked for participation. According to art 89 in the GDPR, there is an exception for handling registry-based and historical data in a research project of public interest, meaning that all participants do not have to be informed, if not possible. In this project, it would not be possible to inform all subjects due to a large number of study subjects included, and due to historical data handling, several of the study subjects could be anticipated to be deceased. The study was approved to be performed without the informed consent of subjects included. The lack of informed consent is a dilemma as the

study subjects' integrity could be affected by the database's inclusion, regardless of the pseudonymity.

Management of sensitive personal information is an important ethical issue, and it is essential to make sure that data security is not breached. The sensitive key code linking personal data with the study code has been saved on special encrypted USB-memories.

A delicate question in biomarker studies is how to deal with assay results, namely pathologic results. A common notion is that it is wrong to withhold assay results from patients that could potentially impact their future health or health management. For the FISH analysis database, the results had been communicated to the physician. Regarding the studies of dynamic changes, as these results were already reported in a clinical setting, no further communication with the ordering physician was deemed necessary.

One of the more serious ethical conundrums is whether it is sound to further the research in additional PCD markers, given that increased sensitivity of assays of PCDs is improved, leading to the possible increase of asymptomatic cases. Nevertheless, it still holds that clinical risk stratification is challenging and that better biomarker than the currently available markers and the improved application of available biomarkers could positively affect patients' management with uncertain risk profiles.

4 RESULTS

4.1 STUDY I

To assess dynamic changes in biomarkers and the associated risk of progression to MM, we included 987 individuals with MGU in this retrospective cohort study. A total of 83 patients progressed to symptomatic MM during the follow-up. The median M-protein levels were significantly higher in patients who later developed MM. We could also see higher median urine M-protein and iFLC in patients that progressed to MM (Table 10).

Table 10. Patient Characteristics*

	Non-progressors (N=904)	Progressors (N=83)	P-value
Gender, male no. (%)	494 (55)	38 (46)	0.21
Age, median (range)	69 (26-96)	66 (35-86)	0.51
Heavy chain type, no (%)			
IgG	572 (72)	55 (69)	0.54
Non-IgG	227 (28)	25 (31)	
	Median (IQR)		
Hemoglobin, g/dL	129 (118-140)	128 (115-137)	0.36
Creatinine, µmol/L	83 (68-110)	78 (66-103)	0.56
eGFR, mL/min/1,73 m²	65 (47-77)	66 (52-78)	0.66
Calcium, mmol/L	2.3 (2.2-2.4)	2.3 (2.2-2.4)	0.96
M-protein, g/dL	6 (1-11)	16 (7-25)	**<0.001**
uM-protein, mg/L	7 (4-20)	8 (0-31)	**<0.001**
iFLC, mg/L	10 (0-30)	43 (10-219)	**<0.001**

*Selected characteristics adapted from paper I. P values obtained with
M-protein = monoclonal protein in serum/plasma, uM-protein= monoclonal protein in urine, iFLC=involved serum free light chain, eGFR= estimated glomerular filtration rate

Potential predictive risk factor for progression

We observed that age, M-protein, abnormal FLCr, and iFLC were associated with progression to MM in the univariate Cox regression. The HR of progression to MM was 2.10 (95%CI 1.31-2.40) for patients older than 65 years at MGUS diagnosis. The HR was observed with iFLC above 100mg/L (HR 4.22, 95% CI 2.66-6.70) compared to iFLC ≤100mg/L. Patients with a non-IgG heavy chain showed no increased risk of progression than those with an IgG-heavy chain.

As both iFLC and FLC ratios are derived from the same laboratory parameters, we combined these two variables. Patients were grouped as iFLC ≤100mg/L and normal FLCr, iFLC ≤100mg/L and abnormal FLCr or iFLC>100mg/L regardless of FLCr. In the multivariate model, iFLC above 100mg/L was an independent and strong risk factor associated with multiple myeloma progression. In patients with iFLC ≤100mg/L, we did not observe any significant difference between those with normal and abnormal FLC ratio (Table 11)

Table 11. Hazards ratios of risk of progression among 987 MGUS patients		
	HR (95% CI)	P-value
M-protein		
≤15g/L	1.00	
>15g/L	3.24 (1.96-5.35)	**<0.001**
iFLC/FLCr		
iFLC ≤100mg/L and normal FLCr	1.00	
iFLC ≤100mg/L and abnormal FLCr	1.24 (0.68-2.22)	0.46
iFLC >100mg/L	2.93 (1.57-5.47)	**0.001**
Age		
18-65	1.00	**0.003**
>65	2.21 (1.30-3.74)	
MGUS=monoclonal gammopathy of undetermined significance, HR=hazard ratio, CI=confidence interval, M-protein=monoclonal protein in serum/plasma, iFLC=involved serum free light chain, FLCr=serum free light chain ratio P-values obtained with multivariate Cox regression		

We included these three variables, M-protein >15g/L, iFLC >100mg/L, and age >65 years, to create a clinical risk prediction model. 301 (30%) of the patients had no identified risk factor, 585 (59%) any one of the risk factors, and 101 (10%) two or three risk factors at the time of diagnosing. The cumulative probability of progression at five-years was significantly higher in patients with all three risk factors (31%) than those with no risk factor (2%), Figure 7.

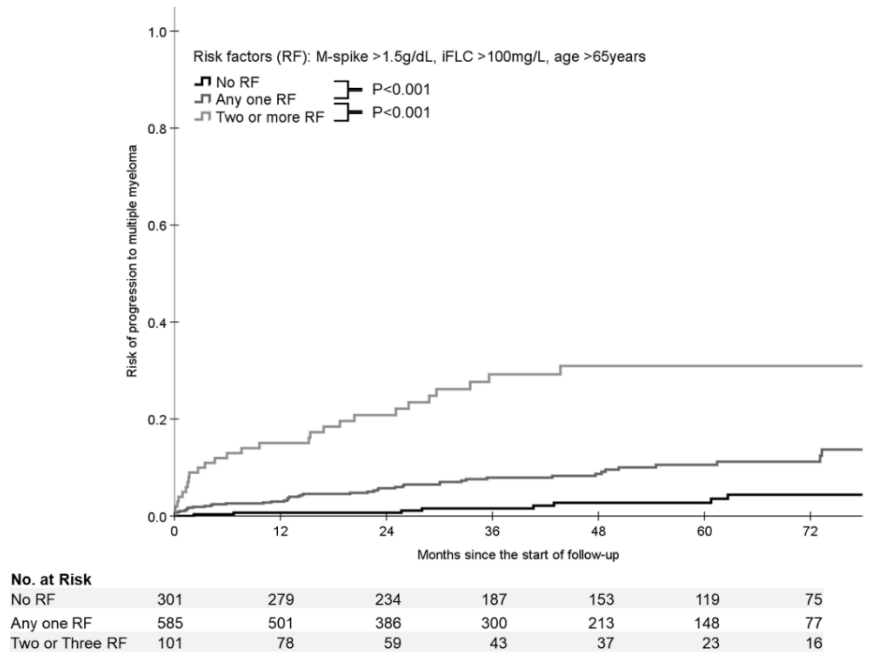


Figure 7. Cumulative probability of progression from monoclonal gammopathy of undetermined significance to multiple myeloma grouped by the three risk factors M-protein>15g/L, iFLC>100mg/L, and age >65 years

Biomarker dynamics and the risk of progression

We found that dynamic increases in iFLC >100mg/L were significantly associated with increased risk of progression, both from the baseline value (Figure 8A) and regardless of baseline value (Figure 8B).

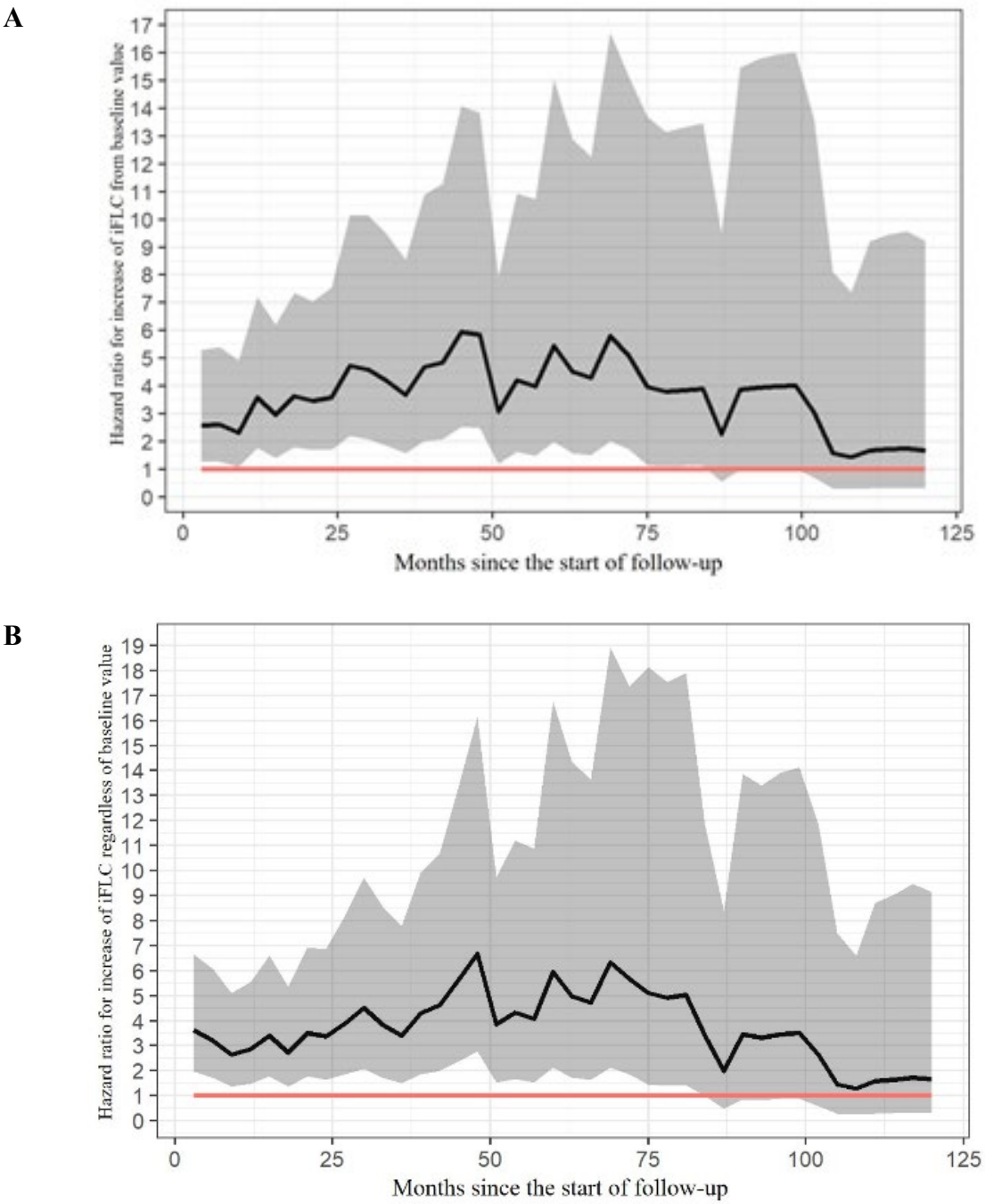


Figure 8. The risk of progression from monoclonal gammopathy of undermined significance to multiple myeloma assessed by dynamic increases of involved serum free light chains. The black line shows the univariate Cox regression hazard ratios at three months intervals. The red line shows when the hazard ratio is equal to one. **A.** Absolute increase of 100 mg/L of plasma iFLC from the baseline value. **B.** Increase in serum iFLC >100 mg/L at any time during follow-up regardless of the baseline value.

4.2 STUDY II

We identified 126 patients diagnosed with SMM and a minimum of three serial samples prior to MM diagnosis or the last follow-up date to investigate the association of temporal changes in biomarkers and the risk of progression to symptomatic MM. Compared to non-progressing patients, the patients progressing to symptomatic MM (n=44, 35%) had higher M-protein levels at the time of SMM diagnosis.

The risk of progression at SMM diagnosis

We assessed how previously published risk prediction scores were applied to our cohort by performing uni- and multivariate analysis. As the levels of iFLCr of 20 and above were not significant in univariate cox regression, we assessed the optimal cut-off for iFLCr in our cohort by ROC analysis. iFLCr at diagnosis's predicted progression to MM with a ROC AUC of 0.63 (95% CI 0.51-0.74) and the cut-off of 8.2mg/L had a sensitivity of 55% and specificity of 69%. However, only BMPC >20% and M-protein >20g/L were independent risk factors in the multivariate, including iFLC with 8mg/L as a cut-off.

Temporal changes in biomarkers during SMM follow-up

We then evaluated the delta changes over time in plasma M-protein, iFLC, iFLCr, and Hb (Figure 9). Decreases in Hb within the first year after SMM had no discriminatory ability in our cohort, AUC 0.55 (95% CI 0.44-0.65) for the relative and absolute increase in Hb. Therefore, we did not attempt to identify Hb cut-offs and did not include the marker in further assessments.

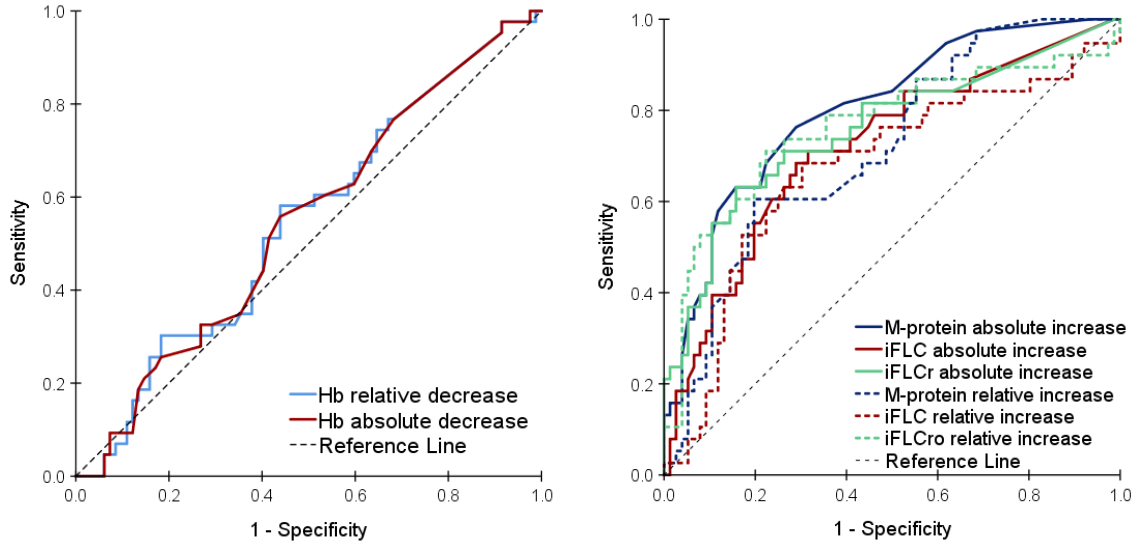


Figure 9. Receiver operator characteristics curve of delta changes in **A**. decreases in Hb within 12 months of smoldering multiple myeloma diagnosis. **B**. absolute and relative increases in M-protein, iFLC, and iFLCr from smoldering multiple myeloma diagnosis until six months before symptomatic multiple myeloma or last follow-up. M-protein=monoclonal protein in serum/plasma, iFLC=involved serum free light chain, iFLCr=involved serum free light chain ratio

The predictive accuracy for progression to MM was good for an absolute increase of M-protein and fair for relative M-protein increase. We could observe that the ROC AUC was fair for both relative and absolute increases of iFLC and iFLCr (Table 12).

Table 12 Receiver operator characteristic area under the curve assessing the predictive accuracy of absolute and relative change for multiple myeloma progression.

		AUC (95%CI)	Cut-off	Sensitivity	Specificity
Absolute	M-protein increase	0.80 (0.72-0.89)	4.5	76%	71%
	iFLC increase	0.72 (0.62-0.82)	21	71%	68%
	iFLCr increase	0.76 (0.66-0.86)	4.5	63%	84%
	Hb decrease	0.55 (0.44-0.65)	-	-	-
Relative	M-protein increase	0.71 (0.62-0.81)	13%	61%	80%
	iFLC increase	0.69 (0.58-0.80)	14%	68%	70%
	iFLCr increase	0.76 (0.66-0.87)	14%	71%	78%
	Hb decrease	0.55 (0.44-0.65)	-	-	-

Optimal cut-off assessed with Youden J index together with corresponding sensitivity and specificity AUC=area under the curve, M-protein=monoclonal protein in serum/plasma, iFLC =involved serum free light chain, iFLCr=involved serum free light chain ratio

We then evaluated the potentially predictive risk factors with Cox regression and found that absolute increase of M-protein >5g/L and iFLCr >4.5 were independently associated with increased risk of progression, HR 2.40 (95%CI 1.16-497) and HR 2.57 (95%CI 1.28-5.19), respectively. We could show that patients with either one or both risk factors increased during the follow-up had a worse TTP than those without either risk factor (32 months and not reached, p<0.001) (Figure 10).

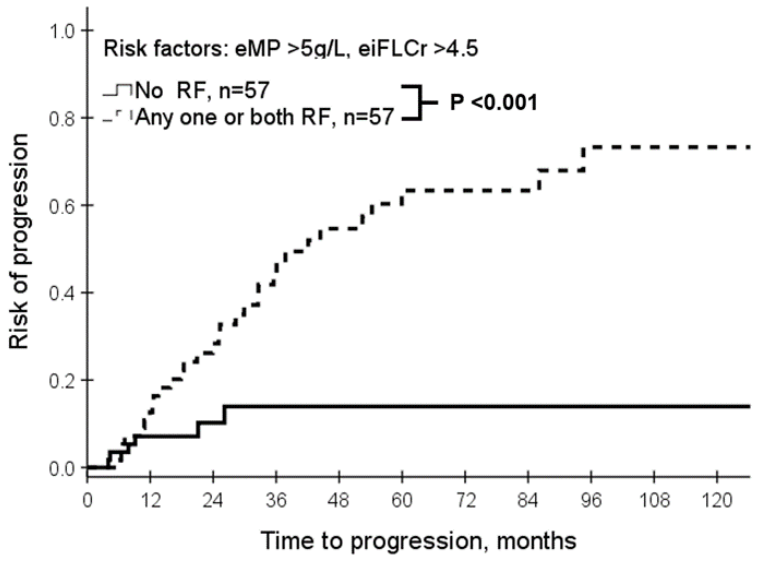


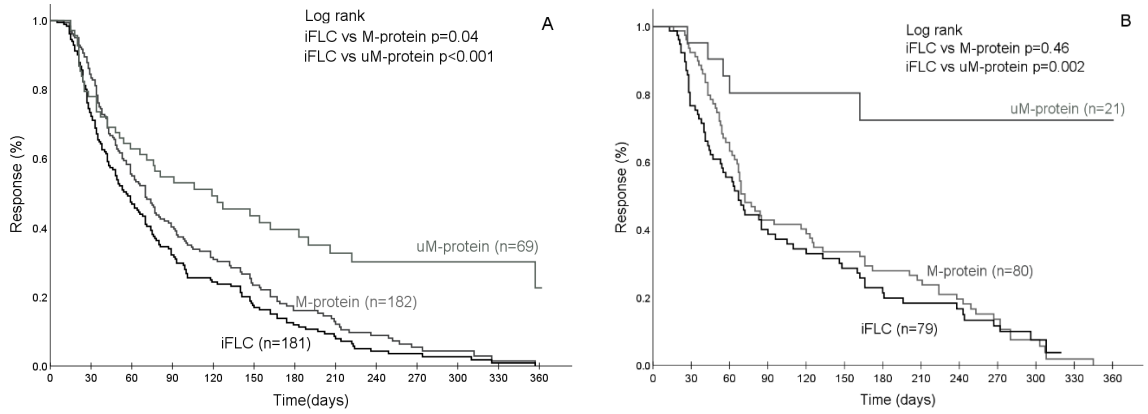
Figure 10. Time to progression in SMM patients stratified by evolving absolute increases. eMP = absolute increase in monoclonal protein in serum/plasma, eiFLCr = absolute increase of involved serum free light chain ratio

4.3 STUDY III

We included 450 symptomatic MM patients with sequential retrospective samples to investigate temporal differences between M-protein, uM-protein, and iFLC when assessing response and biochemical progression. Of these, 205 (45%) had a measurable disease in both M-protein and iFLC (>10g/l M-protein and >100mg/L iFLC with abnormal ratio) at the time of their symptomatic MM diagnose. Measurable disease by M-protein only was observed in 107 (24%) patients, and 107 (24%) had a measurable disease only by iFLC and uM-protein. The remaining patients had an M-protein of ≤10g/l and uM-protein ≤ 200mg/24h and iFLC ≤100mg/L.

The temporal difference in response detection

Overall, we observed responses significantly earlier with iFLC than M-protein in 1st line treatment; the median TTR was 2.2 months for iFLC and 5.6 months for M-protein. We then grouped the patients by measurable disease at diagnosis. The response could be detected earlier by iFLC in the patients with disease in M-protein and iFLC (Figure 11A) and those with a measurable disease in iFLC (median TTR 1.4 months) and uM-protein (median TTR 1.9 months), p=0,003. We did not observe any difference in patients with a plasma M-protein >10g/L and iFLC ≤100mg/L (Figure 11B)



	Median months	95% CI	Median months	95% CI
iFLC	1.8	1.4.-2.2	2.2	1.7-2.7
M-protein	2.3	1.9-2..7	2.3	1.9-2.9
uM-protein	3.9	1.7-6.1	NR	NR

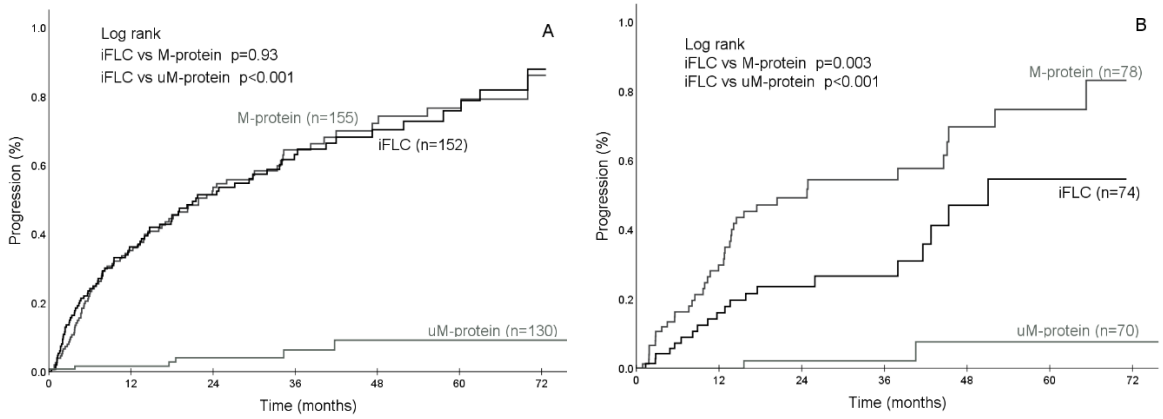
Figure 9. Detection of response in first line with involved serum free light chain (iFLC), M-protein, and uM-protein **A**. Patients with measurable disease in M-protein and iFLC (>10g/l M-protein and >100mg/L iFLC with abnormal ratio)**B**. Patients with measurable disease in M-protein only (>10g/l M-protein and ≤100mg/L iFLC) NR=nor reached

Immunoglobulin subtypes and the temporal response detection

To evaluate if the heavy chain type could affect response detection, we did a subgroup analysis of patients with IgG (n=273, 61%) and IgA (n=89, 20%) heavy chains. We did not observe any difference in response detection in the 1st line for patients with IgA heavy chain, 2.0 months (95% CI: 1.4-2.6) for iFLC and 2.4 months (95% CI: 1.6-3.2) for M-protein, p=0.14. In patients with an IgG SMM, a response was observed earlier when assessed by iFLC, 1.7 months (95% CI: 1.4-2.1) compared to M-protein, 2.3 months (95% CI: 2.0-2.6), p=0.007.

Detection of progression

Next, we evaluated the time to progression in the whole cohort and by groups of measurable disease. We did not observe a biochemical progression earlier by either biomarker in the complete cohort. In the subgroup analyses by measurable diseases group, progression was observed earlier when assessed with M-protein compared to iFLC in patients with and M-protein >10g/L and iFLC ≤100mg/L at the MM diagnosis (Figure 12B), while no difference was observed in those patients that had a measurable disease in both iFLC and M-protein (Figure 12A).



	Median months	**95% CI**	**Median months**	**95% CI**
iFLC	21.8	15.3-28.5	51.1	-
M-protein	21.5	7.5-24.6	24.8	3.3-46.3
uM-protein	NR	NR	NR	NR

Figure 12. Detection of the first progression with involved serum free light chain (iFLC), M-protein, and uM-protein **A**. Patients with a measurable disease in M-protein and iFLC (>10g/l M-protein and >100mg/L iFLC with abnormal ratio). B. Patients with a measurable disease in M-protein only (>10g/l M-protein and ≤100mg/L iFLC), NR=nor reached

4.4 STUDY IV

In this retrospective study, we included 1103 individuals with MGUS that had not progressed to MM or another treatment demeaning hematological malignancy to describe the pattern of co-morbidities and cause of death. Of these individuals, 55% were male, and 45% were female. The median age in the whole cohort was 69 years (IQR 60-77).

We observed that most patients had a known co-morbidity (n=819, 82%) at the time of MGUS diagnosis or developing during follow-up. The most prevalent co-morbidity was cardiac diseases, where heart failure accounted for 187 of the cardiac disease cases. The frequency of heart failure was 30% and 26% in patients with IgM and LC-MGUS compared to 20% and 18% in IgA and IgG MGUS.

Causes of death by the heavy chain subtype

One hundred and eighty-one deaths were observed during the follow-up. We found that patients with LC-MGUS, while only making up 8% of the cohort, accounted for 34% of all deaths observed. Therefore, we grouped patients by heavy chain subtypes. For patients with IgA, IgM, or LC-MGUS, a cardiac event was the most common cause of death, while cancer was the most common cause in IgG MGUS (Table 13)**.**

Table 13. Causes of death among MGUS patients grouped by the heavy chain subtype of the monoclonal protein

	Dead	***Cardiac***	***Cancer***	***Renal***	***Infection***	***Other***
IgG, n=716 (65%)	101 (14)	20 (20)	22 (22)	14 (14)	14 (14)	31 (31)
IgA, n=178 (16%)	30 (17)	10 (33)	8 (27)	4 (13)	2 (7)	6 (20)
IgM and other, n=116 (11%)	19 (16)	6 (32)	3 (16)	1 (5)	4 (21)	5 (26)
LC-MGUS, n=93 (8%)	32 (34)	9 (28)	8 (25)	5 (16)	5 (16)	5 (16)

MGUS=monoclonal gammopathy of undetermined significance, LC-MGUS=light chain MGUS

Risk factors associated with overall survival in MGUS

We investigated the association between covariates and overall survival by cox regression models. Of several variables that were found to be associated with survival in the univariate analysis, male gender, age≥65, plasma albumin<35, eGFR 60-30 and <30, as well as LC-MGUS, were independent risk factors in multivariate analysis. As plasma albumin also is an inflammatory marker, we evaluated if inflammation influenced the overall survival. GPS-score were used to group patients, and we could observe that patients with GPS 1+2 were older, with lower median hemoglobin and median eGFR compared to patients with GPS 0. The multivariate analysis including GPS-score instead of albumin also showed that LC-MGUS (HR 2.66, 95% CI 1.56-4.51) together with eGFR 60-30 (HR 2.11, 95% CI 1.24-3.34) and <30 (HR 2.90, 95% CI 1.74-4.86), age≥65 (HR 3.94, 95% CI 2.07-7.51) and male gender (HR 1.65, 95% CI 1.11-2.46) were independently associated with overall survival.

5 DISCUSSION

5.1 METHODOLOGICAL CONCERNS

Epidemiological studies can describe the incidence and prevalence of disease and estimate the effect of variables and risks. Population-based cohort studies are common in epidemiology and appropriate to study associations of exposure on rare outcomes, such as the progression from MGUS to MM, which are studied in this thesis. Given that the internal validity is high, the cohort studies can provide good scientific evidence. The Swedish population-based registers provide a unique source of information for such studies.

Validity

Studies investigating the associations of variables and outcomes are affected by two types of validity, internal and external. Internal validity reflects the extent to which the study populations observed results reflect the truth without influence from other variables. External validity means to what extent the results can be generalized to another population than the study population. The internal validity is affected by systematic errors, such as bias and confounders. External validation of a predictive model is essential to confirm generalizability. The studies' external validity in this thesis is strengthened by large region-based cohorts and the use of real-life data to identify exposure and outcomes. However, as N-Latex FLC was used, this may influence the generalizability of **studies I-III** results. Only MGUS patients without progression to a hematological malignancy were included in **study IV**, which influences the possibility to generalize. As the risk prediction models in this thesis have not been externally validated, the result should be considered hypothesis-generating.

Internal validity

Bias

Statistical bias occurs when a systematic error is introduced, making the study population unrepresentative of the general population. The types of errors introduced by bias can cause the removal of genuine associations and evoke associations that are not there in reality. The effect of bias cannot entirely be removed or adjusted for in the statistical analysis, making it essential to avoid bias already at the design phase. When designing a study, it is thus necessary to consider several types of bias.

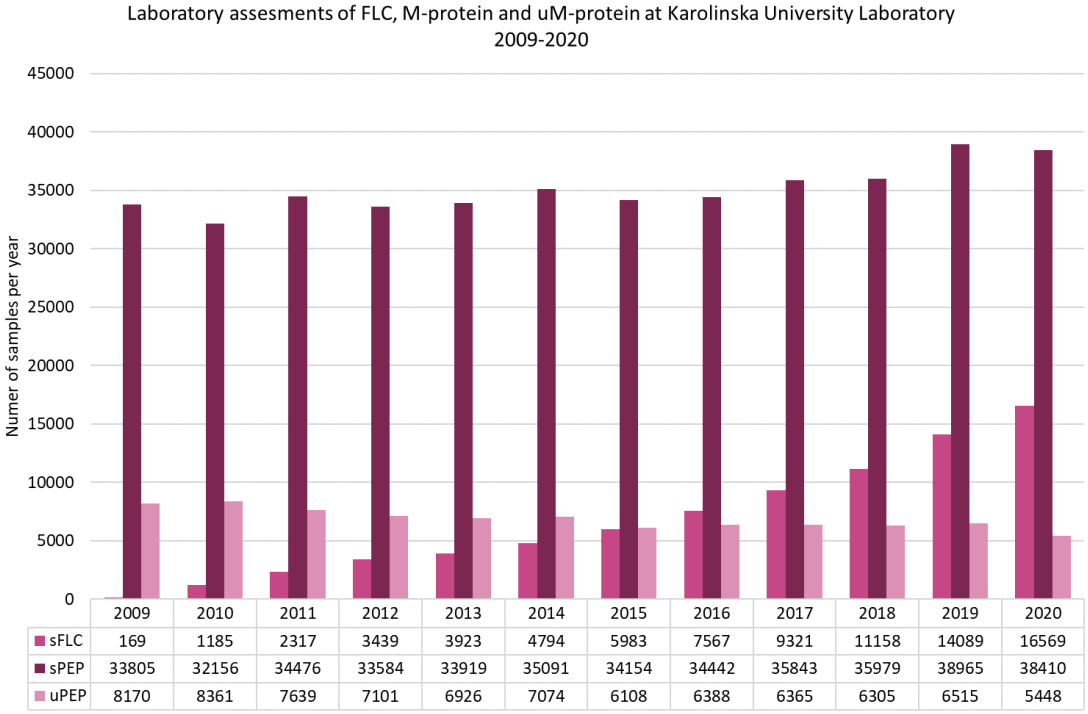
Selection bias

Selection bias occurs when the study population is selected so that the sample population is not representative of the population in terms of exposure or outcome. Non-participation or non-sampling is a risk in epidemiological studies, which can introduce a selection bias if those participating or samples are not representative of the general population.

The Swedish health care system ensures that the population has access to medical services. Thus, it is not anticipated that the inhabitants avoid seeking medical care due to financial concerns and that registries reflect the population. In the Stockholm region, four out of five of the hematology units and a majority of general practitioners refer patients for samplings to

the Karolinska University Laboratory. Thus, selection bias is significantly reduced when selecting the study population based on retrospective laboratory samplings.

The main concern for all four studies is that only individuals with FLC assessment at KUL were included. Therefore, not all episodes of the disease-related conditions have been included in the database as individuals will also have been assessed with M-protein or uM-protein only (Figure 13). Additionally, the assessment of FLC was introduced at KUL in 2009, which affects **Studies I-IV** as the time interval in selecting the study population was restricted. A difference in time to the adaption of new biomarkers will affect which patients are included in the laboratory database. Indeed, FLC was not included in the standardized recommendations for general practitioners until 2018. In **studies I and IV**, where we were investigating patients with MGUS, it could be plausible that since individuals with MGUS with a higher risk of progression are referred to hematological clinics for assessment and thus tend to be assessed for FLC more frequently. However, we could observe that the majority of patients included were low-risk MGUS.



	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
sFLC	169	1185	2317	3439	3923	4794	5983	7567	9321	11158	14089	16569
sPEP	33805	32156	34476	33584	33919	35091	34154	34442	35843	35979	38965	38410
uPEP	8170	8361	7639	7101	6926	7074	6108	6388	6365	6305	6515	5448

Figure 13. Numbers av analyzed samples for the assays serum free light chain (FLC) and protein electrophoresis in serum/plasma (sPEP) and urine (uPEP) per each year from 2009 until 2020 at Karolinska University Laboratory

In **studies I, II, and III**, the exclusion of patients without sequential samples could lead to a selection of more heavily monitored patients that could represent MGUS and SMM at high risk of progression to MM in **studies I and II** and possibly MM patients where relapse was suspected in **study III**.

Observational bias

Observational bias is an error where the information regarding exposure or outcome is misclassified. The observational bias can be differential or nondifferential. Differential occurs when the misclassification or measurement error is different between the groups investigated, leading to either under or overestimating the association. When the misclassification is equal in exposed and unexposed groups, the observational bias is nondifferential, leading to a diluted association. The original database included only individuals with matched samples with FLC and M-protein/uM-protein, reducing the risk of exposure misclassification.

In **studies I and III**, we used the ICD-codes registered in electronic medical journals assigned from the hematological clinic or general practitioners in conjunction with a visit to decrease the risk of misclassification. In **study III**, additional data was collected from two MM databases containing data on treatment and cytogenetics. These two additional databases contained information on symptomatic MM, which was used to verify the previously entered data and further decrease misclassification risk. For **study II and IV**, an update of the diagnoses in the database was performed in 2020. In the 2020 update, ICD-codes were included regardless of which clinic that assigned them.

Confounding

Confounders are variables that are associated with both the exposure and the outcome but without casual relation. The bias introduced by confounding factors will depend on the strength of the correlation between the confounding factor, the dependent factor, and the independent variables. This correlation can cause under and overestimation of an independent variable's effect and mask an actual effect in regression models. Statistical models should be adjusted for confounders to avoid bias in the estimates of the association. Residual confounding or omitted variable bias refers to the remaining confounding that has not been controlled in the study analyses. Due to the retrospective nature of **study I-IV**, there might be residual confounding present as only available factor measurements could be included in the database. When available, confounding was assessed in multivariable regression analyses.

Random error

Chance findings, either seeing a difference when there is none (type I error) or not seeing an actual difference that is there (type II error), can never be entirely eliminated. Reducing the outcome parameters and increasing sample size reduces the risk of chance findings. Typically, p-values, assessing the probability that a similar find could be seen by chance, and confidence intervals (CI), showing the range in which, the true mean of the population can be found, is used. The significance level was predefined to 0.05, which corresponds to a 95% CI for all the studies in this thesis. The risk of type I error can be increased when overfitting a model. Using no more than one covariate per 10-20 observations of the outcome is commonly sufficient to avoid overfitting. We assessed the potential covariates in **studies I, II, and IV** by univariate analysis before introducing them in a multivariate model to reduce the risk of overfitting.

5.2 GENERAL DISCUSSION

The improving diagnostics of PCD enable a better follow-up of patients and risk stratification. Predictive models assessing the risk of progression to symptomatic MM have suggested combining dynamic changes of serum tumor surrogate markers with more invasive bone marrow assessment for SMM patients. In MGUS, it was recently shown that a trend for increasing M-protein could be observed prior to progression to symptomatic MM.

The studies included in this thesis are strengthened by the substantial region-based material and the vast number of available sequential observations. Validation of diagnosis and disease-related variables against the electronic medical journals and complementary databases on treatment and cytogenetics enabled a real-life assessment. The thesis focused on assessing static and temporal risk factors associated with progression to symptomatic MM (**studies I-II**), dynamic changes during follow-up of symptomatic MM (**study III**), or risk factors related to increased risk of death in patients with MGUS (**study IV**). In **study I**, we could demonstrate that temporal changes of iFLC were a vital biomarker to assess in patients with MGUS. The results from this study give insight into how the monitoring of MGUS patients could be improved. In **study II**, the temporal changes relating to progression in SMM patients were investigated. We observed increases of both M-protein and iFLCr over time were strong and independent factors that predicted an increased risk of progression compared to the prediction with static biomarker levels. Further, we did not find an association between decreasing hemoglobin and progression to symptomatic MM. Additionally, we identified patient and disease-specific predictors that already at diagnosis were associated with progression. The results from **studies I and II** highlight the importance of including FLC assessments in monitoring the pre-malignant PCDs.

As there is a difference in half-time between intact Igs and FLC, we designed **study III** to investigate if differences in time to response and progression could be seen in MM patients. In the complete cohort, we observed that the median time to partial response or better was shorter when detecting response by iFLC compared to M-protein. This find could also be seen in patients in groups with measurable diseases. In contrast, M-protein proved to detect progression faster in certain groups of patients with low levels of iFLC at symptomatic MM diagnose. Finally, we focused on excess mortality in patients with MGUS in **study IV**, where we could observe that the grade of inflammation and the subtype of MGUS was associated with the overall survival.

5.3 MAIN FINDINGS

5.3.1 Temporal risk prediction in MGUS and SMM

The natural history of the pre-malignant PCDs, MGUS and SMM, is well defined, with increasing evidence of evolving changes that impact progression and further divides the entities into evolving and non-evolving subtypes. Our objective in **studies I and II** was to identify temporal risk factors associated with progression to symptomatic MM.

The main finding from **study I** was the increased risk of progression seen in MGUS patients where iFLC increased by 100mg/l or more during the follow-up. This risk factor was associated with a significant and high HR until 6.25 years of follow-up. Moreover, we

demonstrated that these dynamic increases in iFLC were a superior risk factor compared to that of M-protein elevations, where the latter only were significantly associated with risk of progression at a few time points. Although the increase in iFLC was associated with a high HR over time, it must be noted that the ROC AUC was 0.68, thus showing only a poor/fair discriminatory ability to identify the patients at high risk of progression. A fair number of patients would still have to be monitored to ensure that all progressors were included.

In contrast to our observations in **study I**, it has been suggested that M-protein and not iFLC levels show a trend for increasing over time (117). However, potential cut-offs were not investigated. Different FLC assays were utilized in these two studies, which could account for the discrepancies/differences. While it is well known that the Freelite assay and the N-latex assay will detect different levels of both κ and λ, it is currently not known how levels would differ during a dynamic follow-up. However, as abnormal FLCr is observed more frequently in MM than MGUS(28), it is reasonable to assume an increase in iFLC, reflecting monoclonal production, would be seen during follow-up. We, therefore, believe that the iFLC increase is an essential predictor of MM progression.

Study II added support to the significant evidence that in recent years has suggested incorporating temporal evaluation of biomarkers in SMM follow-up. Our study showed that absolute increases of M-protein and iFLCr were significant predictors of progression. Predictive models incorporating the dynamic changes in M-protein have indicated that a relative increase over time in M-protein is associated with a higher risk of progression(133-136). The cut-offs for the relative increase and the time frames over which increase should occur have differed between these studies. Only two of the predictive models included an absolute minimum increase together with the relative increase(133, 136). As a relative increase alone could include patients with a meager absolute increase, particularly in patients with M-protein <30g/L, an absolute minimum increase is essential. Indeed, when we assessed relative and absolute increased by ROC analysis, we could observe a higher AUC for an absolute increase of M-protein compared to a relative increase of the same. Interestingly, when we assessed the optimal cut-off in our cohort for M-protein in **study II**, we found a level of 4.5g/L. This level is similar to the absolute minimum increase, 5g/L, previously reported by Ravi et al. and Atrash et al. as a risk factor(133, 136). We thus believe that this is a significant risk factor in prediction progression to MM.

A relative increase in iFLC of 169% or more within the first year of SMM diagnosis has been reported as an independent risk factor for progression(135). We could not confirm this finding in **study II**. Also, an absolute increase of iFLC showed a better discriminatory ability than a relative increase in our cohort. Our results showed that an absolute increase of 20mg/L was significant in univariate but not an independent factor. In contrast, we observed that the absolute increase of eiFLCr ≥4.5 could better discriminate progression from non-progressors and was an independent factor. Two critical differences in **study II**, besides incorporating absolute increases rather than relative, from previous dynamic prediction models are N-Latex FLC assay and incorporating only dynamic markers. The use of a different assay compared to the earlier prediction models can affect the results' generalizability. However, until an international standard is available for FLC, it is crucial to investigate the biomarker's utility in different cohorts.

Another important observation in **study II** was that decrease of hemoglobin during the first year of diagnosis was not associated with increased risk or progression. Declines in hemoglobin have been assessed in previous studies with discrepant results(133, 135, 136). As anemia development is one of the CRAB symptoms, it is not unthinkable that hemoglobin reduction would correlate with progression. However, anemia is not uncommon in an elderly population due to other diseases and thus is an unspecific marker. In general, patients were younger in the studies where the dynamic decline in hemoglobin was a significant risk factor, median age 60 and 64 years(133, 135), compared to median age 68 in the study where no significance for hemoglobin was observed(136). Similarly, to Atrash and all, the patients included in our **study II** were older, with a median age of 67 years. This age difference could be one explanation for the discrepancies in the results. Thus, the underlying cause of the reduction in hemoglobin needs to be evaluated from case to case rather than used a general risk factor.

The results show that dynamic increases of tumor surrogates such as M-protein, iFLC, and iFLCr after MGUS and SMM diagnosis are important predictors for symptomatic MM progression. These results should be externally validated but could potentially have clinical importance in these patients' follow-ups. Based on **studies I and II** results, we recommend that FLC be evaluated together with M-protein to monitor MGUS and SMM patients.

5.3.2 Predictors of progression at diagnosis in MGUS and SMM

To evaluate existing predictive models before suggesting a new one is recommended. Several published risk scores to evaluate the risk of progression from MGUS and SMM to symptomatic MM have been proposed. In s**tudies I and II,** we evaluated the impact of suggested risk factors present at diagnosis of MGUS (**study I**) and SMM (**study II**) and potential new risk factors.

In **study II**, we evaluated several previously published risk scores. We could confirm M-protein>20g/L and BMPC>20% as independent risk factors at SMM diagnosis in our cohort. An interesting observation was that we could not validate iFLCr as an independent risk factor when assessing risk at diagnosis, while a delta change of iFLCr during follow-up were significant. iFLCr has been included in many of the published predictive models, albeit with different cut-offs and primary at the time of diagnosis(52, 71, 131, 132). It is well known that the assessment of FLC is assay dependent, and this could be one possible reason that iFLCr was not a risk factor in our cohort. Even when an optimal cut-off for iFLCr at diagnosis was determined by ROC analysis, the variable was not independently associated with progression.

The IMWG risk stratification incorporated three risk factors to assess the risk of progression in MGUS, M-protein >15g/L, abnormal FLCr, and non-IgG isotype(29). In **study I**, we could not observe that non-IgG isotype was associated with an increased risk of progression. The exclusion of IgM MGUS from our cohort could influence the result. However, this finding supports recent studies where the risk of progression associated with a non-IgG subtype has not been confirmed(66, 68, 69). Therefore, we suggest that a non-IgG subtype should not be considered a risk factor for progression.

Abnormal FLCr is a prevalent risk factor, present in 33-47% of the MGUS cohorts reported(50, 68, 69). Indeed, we observed that 40% of patients in **study I** had abnormal FLCr

at MGUS diagnosis. Even at a low tumor burden, FLC can be produced and give rise to an abnormal FLCr. Incorporating a risk factor observed at low tumor burden, which can classify one-third to half of the population as at risk, would potentially give a large proportion of the population to need continued monitoring. In contrast, iFLC, a marker that reflects the tumor burden, could better identify a high risk of progression depending on the cut-off used. Thus, we combined iFLC and FLCr into three groups and could observe that an abnormal FLCr when the iFLC levels were low, equal to or below 100mg/L, was not a significant risk factor for progression to MM. In contrast, iFLC >100mg/L, regardless of FLCr, was an independent and strong risk factor for progression to MM. As the assay used in **study I** to assess FLC differs from that used in many previous studies, the results would need validation in an external cohort, preferably one where other assays are used for FLC assessment.

Older age has been suggested as a risk factor of progression in a large Czech MGUS cohort, where the HR was increased for patients with age ≥60 years(69). Similarly, in **study I**, we could identify age >65 as a significant risk factor of progression. The observation in our cohort supports the findings from the previous study. In contrast, age as a risk factor was not observed in the Mayo Clinic cohort's extensive studies(50, 112, 120). The Mayo clinic studies included MGUS patients diagnosed in the years 1960-1994, while the Czech study and **study I** included patients diagnosed in the 2000s and forward. Additionally, a large proportion of patients in the Mayo clinic cohort were 70 years or older at MGUS diagnosis, 59%, compared to 44.9% in **study I** and 30.3% being ≥69 years in the Czech cohort. It could be possible that age as a risk factor was not observed in the Mayo clinic cohort due to the differences in the study populations.

One concern about **studies I and II** is the lack of BM examination in both cohorts. The lack of BM assessment could have led to misclassification of SMM patients as MGUS in **study I**. Additionally, the insufficient data on BM hindered the assessment of risk factors proposed, such as aberrant plasma cells and aneuploidy in MGUS. In **study II**, assessing dynamic changes of clonal PC in the BM could have provided insight into the correlation of these with the serum markers. However, studies I and II aimed to identify risk factors that were less invasive than BM assessment. Therefore, we believe that **studies I and II** give further insight into risk stratification in MGUS and SMM.

5.3.3 Dynamics in response and progression assessment

Study III aimed to compare the time to response and progression assessed by M-protein and iFLC in patients with MM. Patients with symptomatic MM undergoing treatment need to be monitored to evaluate the response to treatment and detect biochemical progression. **Study III** is the first investigation, to our knowledge, comparing dynamics of M-protein and iFLC in response and progression for symptomatic MM. A large number of biomarker observations is a strength of the study that enabled stratification of the patients based on M-protein and iFLC.

The IMWG has included both relative and absolute changes in their definition of response and progression. Using the IMWG criteria, we could classify response and progression according to the relative and absolute differences in M-protein/uM-protein and iFLC in all patients over time. We observed that detection of response overall occurred earlier with iFLC

than M-protien. However, multiple factors could impact the response detection, such as type the subtype of Ig and M-protein and iFLC levels at the time of MM diagnosis. Recycling of IgG leads to a longer half-life than for both κ and λ FLC. Therefore, we hypothesized that detection of response could be observed earlier with iFLC than M-protein in IgG MM. In our study, the median TTR was 1.7 months with iFLC and 2.3 months with M-protein. Interestingly, while IgA also has a longer half-life than FLC κ and λ, we did not observe any difference between median TTR between M-protein and iFLC in patients with IgA. The low number of patients with IgA MM, n=89, could be one reason for the lack of difference. It would be of interest to further investigate patients with IgA MM by the levels of M-protein and iFLC at MM diagnosis to look at the possible difference.

By grouping patients according to the levels of M-protein/uM-protein and iFLC at MM diagnosis(30), we could investigate how response detection by the different biomarkers differs depending on the biomarker levels at MM diagnosis. The results from **study III** indicate the assessment with iFLC could enable an earlier response detection in symptomatic MM patients except for patients where iFLC were not measurable. These results support that assessment of MM should be performed with iFLC rather than uM-protein(58). It might even be plausible to suggest that select patients could be monitored exclusively with iFLC and only verify a response with M-protein once it has been observed in iFLC. We did not assess any correlation of difference in response times with the treatment that patients received. Therefore, it would be highly interesting for further studies to investigate whether a difference between the response detection by different assays is associated with treatment.

In contrast to our observations on response detection with iFLC, we could not observe that iFLC detected a biochemical progression earlier than M-protein. While iFLC appeared to detect progression earlier than uM-protein, uM-protein was assessed more infrequently in our cohort. This difference in uM-protein and iFLC assessment makes it difficult to speculate on either assay's superiority in progression detection. Previous studies had suggested that FLC assessment could identify biochemical progression earlier with FLC. This observation could only be partially validated in **study III**. Patients with MM with a progression 18months or later would be identified earlier with iFLC than M-protein. However, as this is a select subgroup, **study III** results cannot support omitting M-protein evaluation in MM monitoring.

In summary, iFLC is a non-inferior biomarker when monitoring MM patients' response and progression regardless of Ig subtype. However, in patients with low levels of iFLC at diagnosis, it should be combined with M-protein assessment when evaluating biochemical progression. In line with the previous suggestion, we strongly recommend that monitoring of FLC is included in future guidelines for symptomatic MM follow-up.

5.3.4 The excess mortality in MGUS

In **study IV**, we aimed to explore the factors associated with excess mortality in MGUS. We observed that individuals with LC-MGUS had a substantially increased risk of death than MGUS patients with other isotypes (HR 2.66, 95%CI 1.56-4.51). To our knowledge, **study IV** is the first observation where individuals with LC-MGUS have inferior survival compared to patients with intact M-protein MGUS. However, the relatively short follow-up could impact the results as only 181 deaths were observed during the follow-up. As we only had a

relatively small fraction of LC-MGUS, 9%, in the cohort, one could speculate that these could also impact the results.

Regarding the reduced overall survival observed in patients with LC-MGUS, we can only assess a correlation and not causality in **study IV**. In the general population, non-clonal FLCs have been shown to predict a lower overall survival, independently of age, gender, and renal function. It could be speculated that it is not the monoclonal FLC in LC-MGUS that is the cause of increased excess mortality, but rather an FLC overload regardless of clonality.

One reason for the increased risk of death in individuals with LC-MGUS could, of course, be clinically unnoticed or, for some reason, unregistered progression to a lymphoproliferative disorder. Particularly, progression to AL amyloidosis can be challenging to diagnose, and the disorder can go undetected. The observation that the patients with LC-MGUS tended to more death by cardiac failure could indicate a missed AL amyloidosis diagnosis. The lack of information on clinical assessment and electrocardiography makes it challenging to assess the risk of misdiagnosis in the cohort. However, as shown, elevated polyclonal FLC levels can be a prognostic marker in chronic heart failure(154) and correlate with left ventricular function in patients with ST-elevation myocardial infarction(155). A strong connection between inflammation, seen as increased FLC, and atherosclerosis could be one underlying explanation and a possible field for future investigation

Hypoalbuminemia was a factor associated with increased risk of death in our cohort, similar to an earlier study (114). Although albumin is a negative acute phase reactant and indicates inflammation, there are several other causes for hypoalbuminemia, such as chronic kidney disease with increased loss or liver failure with reduced synthesis, to mention a few. To further assess the role of inflammation we used the GPS score, which combines albumin and CRP to grade inflammation. Interestingly, more patients with inflammation (GPS 1-2) had iFLC levels above 100mg/L than those without inflammation (GPS 0). This could suggest that the inflammation could have triggered malignant transformation. One alternative explanation is that the biological activity of iFLC can cause the inflammation. To further this observation of the potential association of inflammation and survival in MGUS it would be of value to grade inflammation by another acute-phase reactant than albumin. However, we can only see association and not causality in this type of study and further research are warranted to clarify the underlying mechanism of this observation.

6 CONCLUSIONS AND POINT OF PERSPECTIVE

This thesis has enabled detailed investigations in the dynamic changes of biomarkers for benign and symptomatic PCDS and supports the increasing evidence that FLC assessment should be assessed when monitoring PCDs.

In study I, we observed that the risk of progression in MGUS patients was associated with several variables both at diagnosis and during the monitoring of patients. Notably, a dynamic increase was a clinically significant risk factor for progression to MM. Predicting progression in MGUS patients could help guide clinical decisions.

In study II, we observed that FLCr and MP's absolute increases were significant risk factors for SMM progression. With early intervention trials in SMM showing promising results, identifying high-risk SMM by evolving FLCr and MP may support their inclusion in the trials.

Results from study III showed that assessment with iFLC during monitoring could detect a response earlier. Earlier detection of response could have a clinical impact in enabling changes in treatment.

In study IV, patients with LC-MGUS appeared to have a dismal outcome compared to patients with intact Ig MGUS. We also observed an association of inflammation and survival in MGUS patients. These results warrant further studies into the genesis of cardiac disorders in LC-MGUS.

Point of perspective

This thesis has not investigated the correlation of dynamic increases during MGUS and SMM with outcomes after progression to MM. Thus, to further investigate the clinical relevance of dynamic biomarkers, potential differences in OS, and progression-free survival after progression to MM between individuals with evolving and non-evolving MGUS and SMM are of interest to evaluate. Moreover, comparisons of dynamic risk factors by different assays to characterize these biomarkers' changes are central to incorporating these evaluations in clinical practice. Additionally, investigations into an FLC standard could enable harmonization that could affect the current risk models.

Furthermore, to understand the connection of heart disease in LC-MGUS, we suggest that the condition's biochemical, genetic, and clinical characterization should be a future focus. If future studies can bring an understanding to the pathogenesis and isolate risk factors for mortality and morbidity in patients with LC-MGUS, prediction models could be developed and possibly start early intervention.

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