Inflammatory biomarkers with focus on Calprotectin (S100A8/S100A9) and S100A12 (EN-RAGE)

Method development and application in acute radiation proctitis and rheumatoid arthritis patients

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Scientific environment

The present work was carried out at Institute of Medicine, Section of Oncology, University of Bergen. Parts of the study were done at Department of Immunology and Transfusion Medicine, Ullevaal University Hospital, Oslo; Department of Immunology and Transfusion Medicine, Haukeland University Hospital (HUH), Bergen, and Department of rheumatology, HUH. The cooperation from National Institute for Medical Research, Physical Biochemistry Division, London, was necessary for the research on S100A12. Centre for Clinical Research, HUH, participated in the statistical work.

Acknowledgements

During my residency in Porsgrunn in 1990, Professor Truls Erik Bjerklund Johansen recruited me to scientific work and encouraged me to pursue a PhD thesis, with the tentative title: "Normal tissue side effects of radiation in gynaecological cancer patients". During my years at The Norwegian Radium Hospital I realized that many patients treated with radiation therapy for cervical cancers were cured, but suffered from late side-effects. Professor Martin Hauer-Jensen invited me to examine a patient material on late radiation enteropathy. He suggested that I contacted Olav Dahl, Magne K. Fagerhol and Nils Hovdenak. This turned out to be the "winning team" for my PhD-thesis. My working place the last 6 years has been with Professor Olav Dahl at Department of Oncology, Haukeland University Hospital (HUH), as my principal supervisor. The planning of studies, applying for grants, and preparing the first paper of this thesis were done while working at the Hyperthermia project. Olav's touching and convincing statements when we were applying for grants, his open attitude towards my ideas for the research, and his rapid reading of manuscript drafts are some of his good qualities that I have appreciated during these years. He early stated: "You are not picking the easy route for your research". He was right, as we set out to intervene to prevent radiation proctitis by giving seal oil before and during radiation therapy. However, we wanted to find objective measurements to evaluate any effects, and therefore the biomarkers became so central in this work.

Professor Magne K. Fagerhol at Ullevaal University Hospital, Oslo, has included me in his work, teaching me that a day not perfectly suited for research work does almost not exist. His enthusiasm for immunology, and in particular "his" proteins, calprotectin and S100A12 the recent years, is indeed contagious. In addition to sharing his knowledge with me, patiently teaching and repeating what he just told me, he also has involved me in work that will include new challenges for us the years to come. As my co-supervisor, he was always prepared to help me out during the various challenges that we met preparing this thesis.

Abbreviations

AA	Arachidonic acid
AGE	Advanced glycation end products
Anti-CCP	Anti-cyclic citrullinated peptide
Ca^{2+}, Zn^{2+} and Cu^{2+}	Ions of calcium, zinc and copper
CRP	C-reactive protein
CV disease	Cardiovascular disease
Da	Dalton
DAS28	Disease Activity Score 28
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
ESR	Erythrocyte sedimentation rate
ExRA	Extra-articular manifestations of rheumatoid arthritis
FAs	Fatty acids
HAQ	Health Assessment Questionnaire
IBD	Inflammatory bowel disease
ICAM-1	Intercellular Adhesion Molecule-1
Ig	Immunoglobulin

IL	Interleukin
LTB	Leukotriene B
MS	Mass Spectrometry
PDB	Protein data bank
PGE	Prostaglandin E
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RF	Rheumatoid factor
ROS	Reactive oxygen species
RT	Radiotherapy
SPDV	Swiss-Prot Deep Viewer
TBARS	Thiobarbituric acid reactive substances
TNF	Tumour Necrosis Factor
VCAM-1	Vascular Cell Adhesion Molecule-1

Abstract

Assessments of diseases are usually performed by a combination of clinical symptoms, signs and objective measurements. Biochemical markers of inflammation are important in evaluation of treatment of acute and chronic diseases. In this thesis we have focused on two inflammatory diseases: acute radiation proctitis and rheumatoid arthritis. We examined established and new biomarkers, with focus on two closely related proteins, calprotectin (S100A8/S100A9) and S100A12 (EN-RAGE). These and other biomarkers were compared to clinical symptoms and signs with the aim of defining their usefulness in monitoring the course of the diseases.

Characterizing the changes induced by radiation therapy to the pelvic region showed incongruent time profiles in symptoms, histological scoring of inflammation and biochemical measurements. Compared to the examination before start of radiation therapy, biopsies from rectal mucosa showed a maximal inflammation at the first examination during radiation therapy, while symptoms were at the most intense towards the end of treatment. Vitamin E concentrations in blood were significantly lower during treatment but returned to normal within a month. Calprotectin and lactoferrin concentrations in stool samples increased during therapy and were the most promising biomarkers for radiation proctitis in our study.

Development of an ELISA immunoassay for the quantification of S100A12 in blood showed that calcium concentration influenced the estimated S100A12 concentrations. Various experiments led to the conclusions that the antibodies used in this assay could only recognize S100A12 when calcium were above a threshold and that S100A12 were found in many molecular sized complexes (oligomers) in blood. S100A12 was found to bind to heparin and this will be further examined in the future.

Calprotectin and CRP in blood correlated with disease activity of rheumatoid arthritis. S100A12 was associated with a more severe disease course in terms of extra-articular disease manifestations and the presence of cardiovascular disease.

S100A12 correlated with rheumatoid factor and anti-CCP and may thus be of prognostic value.

In summary, radiation proctitis occurs in biopsies before symptoms are evident. This is important knowledge for studies with interventions to prevent development of late radiation enteropathy. Stool markers are at present the best suited objective means of assessing interventions. The quantification of S100A12 in blood samples are recommended to be done in serum samples, since both anticoagulants EDTA and heparin may influence conformational changes of S100A12 protein structure and hence the measured concentrations. The usefulness of S100A12 as a biomarker in rheumatoid arthritis and other inflammatory diseases warrants further examinations.

List of publications

I   Larsen A, Hovdenak N, Karlsdottir A, Wentzel-Larsen T, Dahl O, Fagerhol MK. Faecal calprotectin and lactoferrin as markers of acute radiation proctitis: a pilot study of eight stool markers. Scand J Gastroenterol 2004 Nov;39(11):1113-8

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II   Larsen A, Bronstein IB, Dahl O, Wentzel-Larsen T, Kristoffersen EK, Fagerhol MK. Quantification of S100A12 (EN-RAGE) in blood varies with sampling method, calcium and heparin. Accepted Scand J Immunol 2006

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III   Larsen A, Bjørge B, Klementsen B, Helgeland L, Wentzel-Larsen T, Fagerhol MK, Hovdenak N, Dahl O. Time patterns of changes in biomarkers, symptoms and histopathology during pelvic radiotherapy. Accepted Acta Oncologica 2006

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IV   Madland TM*, Larsen A*, Kristoffersen EK, Fagerhol MK, Brun JG. S100- proteins in rheumatoid arthritis: Calprotectin and S100A12 are associated with disease activity, extra-articular manifestations and concomitant cardiovascular disease. Submitted to Scand J Rheum Dec 2006

*The first two authors contributed equally to this work

Contents

1. General introduction

1.1 Biomarkers as indicators of disease activity

The term biomarker will be used in this study to distinguish the measurement of an endogenous substance from clinical parameters related to disease processes. Biomarkers can be divided in groups related to common properties, like antibodies, cytokines, adhesion molecules, acute phase proteins (e.g. C-reactive protein, CRP) or leukocyte proteins (e.g. calprotectin and S100A12). Another classification is to use the biochemical nature of the molecules to define proteins, lipids or carbohydrates and compounds derived from these as the category, e.g. eicosanoids as lipid mediators in inflammatory processes.

Some biomarkers can reflect disease activity by increased or decreased concentration. Other biomarkers are useful in differential diagnostics but not to monitor disease, e.g. rheumatoid factor (RF) in rheumatoid arthritis (RA). Biomarkers like RF and calprotectin may yield prognostic information in the individual patient, namely increased risk of future disease manifestations.

If available, an ideal biomarker will be preferred to scoring systems of disease activity since they are objective means of evaluating pathology and treatment efficacy.

The ideal biomarker should also be easily applicable for routine clinical purposes, stable in blood or stool samples, fluctuate with disease severity and normalize following successful therapy or recovery.

1.2 Leukocyte proteins in inflammation

The rapidly responding innate immune system consists of: 1) physical and chemical barriers, e.g. epithelial surfaces; 2) phagocytes and natural killer cells; 3) blood proteins and 4) cytokines (1). The phagocytic cells are mainly the macrophages and polymorphonuclear neutrophil granulocytes (neutrophils), the latter being the most abundant type of circulating leukocytes and the major cell type mediating acute inflammatory responses to bacterial infections (1). The increase in plasma concentrations of several proteins, mostly synthesized in the liver as a systemic inflammatory response, is up-regulated by cytokines like tumour necrosis factor-α (TNF-α) and IL-6 (interleukin-6) (1). Cytokines are proteins secreted by the cells of innate and adaptive immunity as a response to microbes and other antigens. The same protein may be synthesized by many cell types, including endothelial cells and other tissue cells, and activate different effector cells to eliminate microbes and other antigens (1). This activation of leukocytes initiates up-regulation and secretion of various molecules. The up-regulation implies activation of genes, increased concentration of mRNA and protein synthesis. Inflammation is characterized by recruitment and activation of the leukocytes at sites of infection or tissue damage and extravasation of several plasma proteins (1). Circulating proteins include members of the complement system, coagulation factors, CRP and mannose-binding lectin. These are particularly important in the innate immune system (1). Examples of laboratory investigations related to inflammation are erythrocyte sedimentation rate (ESR), CRP, leukocyte count, platelets, fibrinogen, serum amyloid A, ferritin, α_1-antitrypsin, complement factors, albumin and transferrin. The last two are found in decreased and the others in increased concentrations during inflammation (2).

The proteins transferrin and lactoferrin are synthesized in leucocytes. They are carrier proteins of iron and prevent iron from catalyzing redox reactions during transport in the circulation (3). The most abundant cytosolic protein in phagocytic cells, calprotectin, has become a clinically useful biomarker in inflammatory bowel disease (IBD) when measured in stool samples (4-6). This is due to high concentration and

stability of calprotectin in stools and correlation of disease activity and leukocyte excretion in stools (7). Although not specific for IBD, fecal calprotectin measurements are useful in screening for organic bowel diseases, and monitoring disease activity of known IBD, thereby reducing the need for endoscopic examinations. Lactoferrin in stool samples is another leukocyte protein examined as a potential biomarker of intestinal mucosal inflammation (8-10). Granulocyte transmigration into the bowel lumen during radiation therapy was assessed by measurement of transferrin in stools of rats and found to be predictive of later radiation enteropathy (11). Both α_1-antitrypsin and lactoferrin in stools have been evaluated as markers of disease activity of IBD (8;9).

1.2.1 S100 proteins calprotectin (S100A8/A9) and S100A12

Calprotectin was first called L1, Leukocyte protein candidate 1, as the first protein that might reflect granulocyte turnover in vivo (12). The name calprotectin was suggested when the protein was found to have antimicrobial properties (13). Calprotectin has a molecular mass of about 36 kilo Dalton (kDa) and consists of subunit polypeptide chains, L1 $_{Heavy}$ and L1 $_{Light}$ chains with molecular masses of about 13 and 8 kDa, respectively (14;15). The functional protein is referred to as calprotectin, since the subunits have not been reported to occur as free, individual proteins in vivo (16). The subunits L1 $_{Heavy}$ and L1 $_{Light}$ chains have identical amino acid sequences of MRP-14 and MRP-8 (17). Other groups have named subunits calgranulin A and B (18). Additional synonyms are listed by Ravasi et al (19). Calprotectin constitutes about 60% of the proteins in cytosol of neutrophils (20).

Calprotectin is mainly found in cells of the monocyte-derived cell lineage, in peripheral neutrophils and monocytes (12;21) and macrophages in formalin-fixed and paraffin-embedded routine material (22;23). Other tissues and squamous type epithelia also express calprotectin (22;24).

The term "S100 protein" was originally used to describe proteins that were "soluble in a 100% saturated solution with ammonium sulphate" (25). This family of proteins is one of the classes of Ca^{2+}-binding proteins that regulate the levels of cytosolic calcium concentration by being buffers, or acting as second messengers by altering their activities depending on the calcium concentration (26-28). They are members of the EF-hand superfamily of Ca^{2+}-binding proteins, of which more than 600 have been identified from the human genome (27). Twenty-one of at least 25 members of S100 proteins have their genes in a cluster located on human chromosome 1q21, and a nomenclature according to this position has been suggested (19;29). S100 proteins are proposed to have both intracellular and extracellular functions, supposedly as trigger or activator proteins; by contrast, other Ca^{2+}-binding proteins are considered to act mainly as buffers (26;28). The majority of S100 protein interactions are calcium-dependent (27).

The three-dimensional, conformational state of S100 proteins are modulated by the calcium concentration, and S100 proteins form both homo- and hetero-dimers and larger complexes (26;27;30). Ca^{2+}-binding is responsible for changes in the global shape and charge distribution of S100A4, S100A6 and calprotectin (31-33).
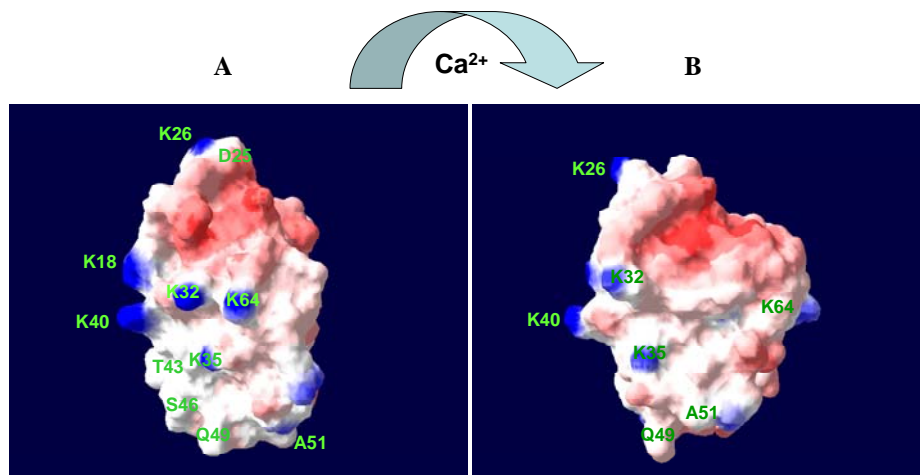
Figure 1. Comparison of S100A6 structures in the apo (A) and calcium bound (B) states

Molecular surface representation of the asymmetric unit of S100A6 (in the apo form , Protein Data Bank (PDB) http://www.rcsb.org/pdb/Welcome.do entry 1K9P and in calcium bound form PDB entry 1K96) coloured by electrostatic potential with the two forms of protein in the same orientation. Increasing saturation of negative and positive potential is represented by red and blue respectively. Location of different residues shows the conformational changes in the presence of calcium.

The images are oriented with calcium binding loop on the top. The figure was created using Swiss-Prot Deep Viewer software (SPDV). The electrostatic potential was calculated by SPDV.

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Calprotectin is suggested to be a hetero-oligomer, consisting of one or more trimers (16;20;34). In the absence of calcium, recombinant S100A8 and S100A9 are thought to form stable heterodimers, while the oligomerization in the presence of calcium is discussed in the literature (35). Heterotetramer (S100A8/S100A9)$_2$ was recently suggested as the functional state necessary for the formation of microtubules in yeast cells (36). Five different combinations of complexes were suggested to occur in extracts from carotid arteries with atherosclerosis (37).
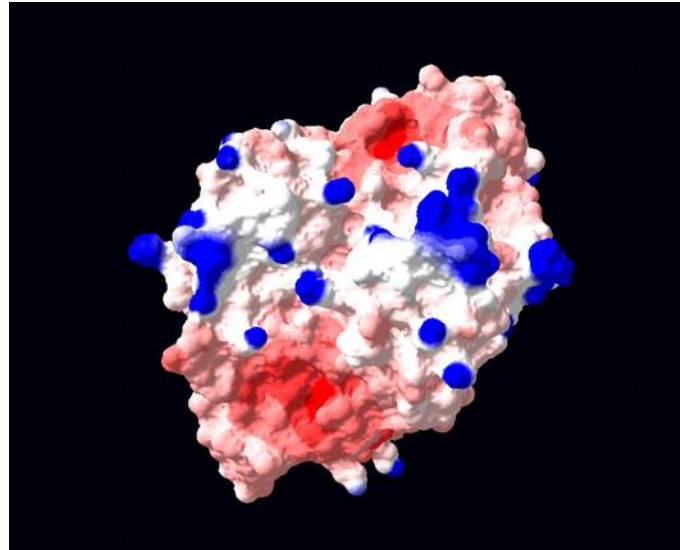
Figure 2. Calprotectin

Molecular surface representation of calprotectin coloured by electrostatic potential. Increasing saturation of negative and positive potential is represented by red and blue respectively. Location of different residues shows the conformational changes in the presence of calcium.

The figure was created using SPDV. The electrostatic potential was calculated by SPDV.

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Non-covalently associated complexes of S100 proteins are considered a prerequisite for their biological functions in vivo. One example of this was studied by two independent groups, agreeing that complex formation of S100A8 and S100A9 was necessary for the binding of polyunsaturated fatty acids (PUFAs), e.g. arachidonic acid (AA) (38;39). If calcium concentrations were below a critical level the binding capacity was lost. One of the research groups induced complex binding of S100A8 and S100A9 by zinc or copper instead of calcium, but these complexes had conformational differences from the calcium-induced protein complex and could not bind AA (40). A recently described clinical syndrome with recurrent infections,

hepatosplenomegaly, anemia and evidence of systemic inflammation was characterized by hyperzincaemia and hypercalprotectinaemia (41). Calprotectin was found to inhibit matrix metalloproteinases by sequestration of zinc (13;42).The antimicrobial activity of calprotectin was abolished by addition of zinc (43). S100A8 and S100A9 alone had no antimicrobial activity (43). This may suggest that a high affinity zinc binding site is generated by complex formation between the subunits. The affinity for zinc is thought to be high enough for calprotectin to reduce the zinc concentration sufficiently to inhibit microbial growth.

Calprotectin and a more recently described member of the S100 family, S100A12, have many functions related to inflammation (26;44;45). S100A12 was first called p6 (46) due to an estimated molecular weight of 6.5 kDa. It constitute 5-8 % of cytosolic proteins of neutrophils (in human, porcine or rabbit) (46-48). S100A12 was found to be a ligand for the receptor for advanced glycation end products (RAGE) (49), and therefore called EN-RAGE (extracellular newly identified RAGE binding protein, EN-). Different research groups have assigned various names for this protein: calgranulin C, calcium binding protein in amniotic fluid-1 (CAAF-1), MRP-6, CGRP (19;50;51).

The concentrations of Ca^{2+}, Zn^{2+} and Cu^{2+} were shown to influence the three-dimensional structure (conformational state) of S100A12 (52-54). Calcium and probably other ions, determine whether S100A12 is found as monomers, dimers or oligomers. In signal transduction of inflammation, the concentration of the Ca^{2+} ions in the milieu around S100A12 is proposed to be the determinant of whether S100A12 participate in a signal cascade or not. The hexamer is suggested to be the form that interacts with RAGE (30). Two different conformational states of the hexamer are shown in fig. 3
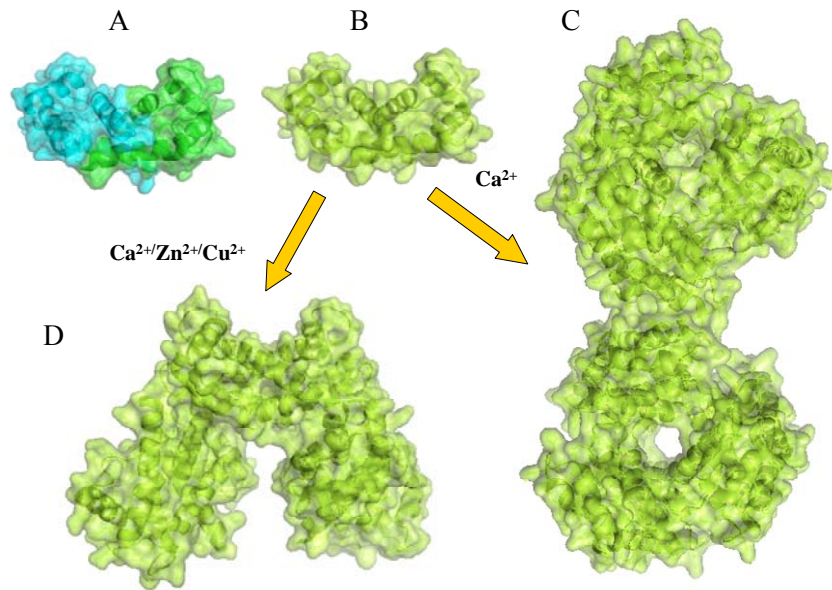
Figure 3. Structural organisation of S100A12 oligomer

The diagrams A and B illustrates how the two subunits of S100A12 (PDB entry 1E8A) (coloured by blue and green to differentiate the subunits in A) makes extensive surface contacts. Two monomers are firmly holding each other. The diagrams C (PDB entry 1GQM) and D (PDB entry 1ODB) illustrates how the dimer associate into two types of oligomers. Interactions between subunits in the hexamer (C) are mediated by the addition of six calcium atoms. Images are shown in a semitransparent surface representation. The route of assembly is purely illustrative and represent assembly of the oligomeric particles in crystal. The figure was made using the program PyMol.

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1.2.2 Endothel, RAGE and atherosclerosis

Calprotectin and S100A12 has mainly been found in monocytes and neutrophils (19-22;45;46), important effector cells of immune responses. Recruitment of leukocytes from the circulation is initiated by the binding of cytokines (and other molecules with chemotactic properties) to endothelial cell heparan sulfate glycosaminoglycans,

which activate the rolling leukocytes and convert the integrins to a high-affinity state (1). This is followed by firm adhesion of leukocytes, which then migrate through the endothelium. Both calprotectin and S100A12 have chemotactic properties (45;55-57). Calprotectin and S100A9 were found to bind to endothelial cells via the heparan sulfate proteoglycans (58), and S100A9 had a high affinity for heparin. Immunohistochemical studies found S100A8 and/or S100A9 in macrophages, foam cells, extracellular matrix and in early and advanced calcified deposits of atherosclerotic vessels (37). These research groups suggest that the presence of S100A8 and S100A9 may reduce oxidative damage (37;58). On the other hand, calprotectin in high concentrations (like those seen in plasma during bacterial infections) induced apoptosis in vitro in all cell types tested so far (59).

In contrast to the suggested protective anti-oxidant function of calprotectin (37;58), endothelial cells showed increased release of IL-6, soluble intercellular adhesion molecule-1 (ICAM-1) and soluble vascular cell adhesion molecule-1 (VCAM-1) after stimulation with calprotectin, but not if incubated with either S100A8 or S100A9 alone (60). However, this effect was only found after preincubation with advanced glycation end products (AGE). AGE are glycosylated lipids and proteins that are found in increased concentrations with increasing age and in patients with diabetes mellitus, atherosclerosis and other chronic diseases (61;62). The authors hypothesized from their observations that S100A8/S100A9 binds to various cell receptors depending on the surrounding milieu, leading to differentiated downstream signalling (60). Calprotectin concentrations increased almost 100-fold when measured in plasma before and within minutes after finishing marathon-running (63). Neutrophil count increased only 3.4-fold in the same individuals' samples. The amount of calprotectin per neutrophil were in the same range after 30-km running as in healthy controls, and calprotectin in plasma increased 20-fold (63). Even though some of this increase might be caused by crushing of leukocytes, the finding supports two in vitro experiments describing secretion of calprotectin from human neutrophils (64;65). The significance of the increased calprotectin concentration is unknown. The publications discussed above suggest antioxidant functions as well as proinflammatory effects on

endothelial cells under some conditions. Hypoxia and tissue damage caused by the running can be regarded as proinflammatory events.

S100A12 was found in macrophages in autopsy specimens from coronary arteries in a study of sudden cardiac death (66). The non-diabetic subjects had less macrophage infiltrate in their arteries than the diabetic subjects. In sections from diabetes mellitus type 2 more healed infarcts and cardiomegaly was found, while type 1 diabetic subjects had low rates of healed infarcts and acute thrombosis. The autoimmunity aspect of type 1 diabetes was speculated to be involved in the observed differences. The research group that first described the protein as a ligand for RAGE (49) found that S100A12: 1) increased the expression of ICAM-1 and VCAM-1 adhesion molecules on human endothelial cells; 2) increased the generation of TNF-α, IL-1β and IL-2 from leukocytes; and 3) acted chemotactic for macrophages. The interaction of S100A12 with RAGE was also shown by Moroz et al (30). At present, the prevailing perception is that RAGE acts as a receptor recognizing patterns of three-dimensional structures, such as β-sheets and fibrils, rather than specific amino acid sequences (i.e. primary structure) (61;62). This explains that RAGE can be activated by many ligands. The ligand-receptor interaction induce a sustained post-receptor signal which include activation of p21^{ras}, MAP kinases and the NF-κB pathway (61;62). Blocking of RAGE by the soluble fragment of the receptor (sRAGE) can attenuate inflammatory responses in many animal models (49;61), but it does not completely abolish these, probably because the ligands of RAGE can interact with other cell surface molecules or receptors (58;61;62). Application of sRAGE in the animal models showed reduced late complications in diabetes, prevention of micro- and macrovascular diseases and improved the outcome of experimental colitis (49;61).

1.2.3 Measurements of calprotectin and S100A12

The clinical application of calprotectin and S100A12 as biomarkers of inflammatory diseases and infections have been suggested in many disease states (44;59;67). Up-regulation of genes indicates a first step towards synthesis of a functional protein. Gene expression of blood mononuclear leukocytes showed up-regulation of mRNA for S100A8 and S100A12 proteins (and other substances) in rheumatoid arthritis' patients (68;69). Patients with Kawasaki vasculitis had up-regulated gene expression for S100A8, S100A9 and S100A12 during the acute phase of the disease (70;71). In the study by Abe et al. (70), plasma concentrations of calprotectin were estimated by two different enzyme-linked immunosorbent assay (ELISA) kits. One of the kits measured S100A8/S100A9 heterocomplex without cross-reaction to monomers of S100A8 or S100A9 (70;72), and the other kit detected S100A9 without cross-reaction to S100A8/S100A9 heterocomplex. The publication of the study was followed by a correspondence regarding the quantification of S100A8, S100A9 and complexes of these proteins (72). The research group with Foell, Roth and colleagues discussed the possibility of pitfalls in detection of S100 proteins in patient samples related to the complex formation in vivo and in vitro (72). Some of the questions raised where: 1) Do the protein standards in vitro form the same heterocomplexes as the relevant heterocomplex in vivo when recombinant S100A8 and S100A9 are used? 2) Have the various antibodies used been characterized with respect to specificity and affinity to their epitopes? 3) Can the quantification of S100- proteins in one ELISA kit be compared to quantification in another ELISA kit in the absence of a "gold standard" to which both kits have been calibrated?

Similar questions apply to published results of S100A12 quantification in two different ELISAs developed by independent research groups (73;74). S100A12 was found in increased amount quantified by ELISA in serum from Kawasaki vasculitis' patients (75;76), and in rheumatoid, juvenile and psoriatic arthritis' patients (73;77). Another research group found higher concentrations in EDTA plasma in patients with diabetes mellitus type 2 than in healthy controls (74). However, none of these two

groups examined the possible influences on the estimated concentration from collection tube anticoagulants (EDTA plasma), time delays in clinical routine, laboratory handling or other aspects that might be important for reliable results. With respect to calprotectin, EDTA plasma was recommended due to stability before separation of plasma or serum from the blood sample (78). Serum concentrations were about twice as high as plasma. The in vitro increase of calprotectin in conjunction with coagulation was regarded as interfering with the interpretation of the circulating calprotectin concentration (78). As mentioned above, commercially available ELISA kits for S100A8, S100A9, the complex of these or the native protein calprotectin, are based on various standards and antibodies, which may affect research results and interpretations (72). The quantification of calprotectin developed in Oslo, is based on calprotectin purified from human granulocytes as standard, and polyclonal antibodies raised in rabbits against this standard (4;12;15;78-80). The published ELISAs for S100A12 were developed as rabbit polyclonal antibodies to S100A12 purified from human granulocytes (73) or with two mouse monoclonal antibodies against recombinant S100A12 (74). The concentrations published for S100A12 from these two research groups have not been calibrated against each other or an international standard. Furthermore, it is possible that conformational changes of S100A12 in vitro might apply to the protein in blood, in a manner that affects the antigenic epitopes and thus the interaction with certain antibodies used in ELISAs. With a homology of amino acid sequences in S100A8, S100A9 and S100A12 of about 40-70% (55), a rabbit polyclonal antibody raised against S100A8 was found to cross-react with S100A9 and S100A12 (p6) (46).

Quantification of S100 proteins in ELISAs depend on specific antibodies to interact with epitopes that might be changed during sampling or analysis, thus the method is dependent on conditions that preserves the three-dimensional conformation of the protein for a reliable result. Mass spectrometric (MS) examination of proteins is a technique that could allow a large number of samples of blood or other body fluids to be analyzed for many proteins in a short time (81;82). The editorial of Clinical Chemistry described MS-based diagnostic as "the upcoming revolution in disease

detection" (82). The quantification of biomarker proteins using MS might be a useful diagnostic tool in the future. For the time being, MS techniques are used to discover proteins that could serve as biomarkers, e.g. in RA (83;84). S100A8, S100A9 and S100A12 were found as candidate biomarkers in synovial fluid and serum from RA patients (83;84). Serum concentrations of S100A8, S100A9 and S100A12 were found to be 6-fold, 14-fold and 111-fold higher, respectively, in patients with erosive RA than in healthy controls (84). MS quantification of pooled serum samples from 15 healthy controls showed a concentration of S100A12 of 5.3 ng/ml (μg/L) while the 15 patients with erosive RA had from 38 to 80 ng/ml in three different experiments with the pools of sera. The same MS quantification was used to estimate CRP, and compared this to quantification with immunoassay. The results showed significant discrepancies in concentrations measured by the two methods with the immunoassay most often estimating higher concentrations than MS. However, the ratios between healthy controls and RA patients were comparable for MS and immunoassay quantification of CRP (84).

1.3 Lipids as biochemimical indicators and therapeutic tools

The activation of leukocytes during an inflammatory response leads to de novo synthesis and release of cytokines, lipid mediators, enzymes and biogenic amines (e.g. histamines) (1). Lipid-derived mediators have a variety of effects on leukocytes, blood vessels and other tissues. Two important groups of such mediators are prostaglandins (PGs) and leukotrienes, collectively called eicosanoids (1;85). They are derived from the fatty acids (FAs) via the cyclooxygenase or lipoxygenase pathways, most often from the 20-carbon long polyunsaturated FA (PUFA) arachidonic acid (AA) since this is the most abundant FA in inflammatory cells (85-

87). The omega (denoted ω or n) nomenclature indicates on which carbon the first double binding in unsaturated FAs occurs, counting from the methyl end of the FA. In general, n-6 FAs like AA are abundant in the Western diet, whereas n-3 FAs are scarce, but found in seal, oily fish and concentrated oils from such sources (87). The overall effect on inflammation of eicosanoids depends on which cells are involved, the nature of the stimulus, concentration of the different eicosanoids generated and the sensitivity of the target cells and tissues. In such a complex interplay, PGE_2 may have either pro- or anti-inflammatory effects (85;87). There are indications, but not clear evidence, that the eicosanoid mediators derived from eicosapentaenoic acid (EPA) and other n-3 PUFAs have less potent pro-inflammatory effects, or even possess anti-inflammatory effects compared to eicosanoids derived from AA (87). Increased dietary intake of EPA and other n-3 PUFAs affected the FA composition of human inflammatory cells in a dose-dependent fashion with alterations in concentrations of eicosanoids (reviewed in (87)). Measurements of eicosanoids may be useful as biomarkers indicating the inflammatory state of the organ/system in question (87-89). The double bonds in PUFAs are susceptible to lipid oxidation, and reactive oxygen species (ROS) may initiate a chain reaction of lipid peroxidation (85;87;90). Antioxidants vitamins A, C and E, zinc and other substances can interrupt these reactions during initiation or propagation stages (90). The lipid antioxidant vitamin E is particularly important in the protection of PUFAs. Lipid peroxidation is often assesed by measuring thiobarbituric acid reactive substances (TBARS) (90).

In two clinical studies where IBD patients were given seal oil enterally by a nasoduodenal feeding tube for 10 days, IBD disease activity, joint pain index, serum cholesterol level and the rectal mucosa biopsies' n-6/n-3 ratio were significantly decreased (86;91). Compared to rectal biopsies from a control group without inflammatory reactions in the mucosa, the amount of PUFAs (given as the ratio of n-6/n-3) indicated a shift towards a normal ratio after treatment with seal oil (from mean ratio 6.6 to 2.2) (86;91). Only weak evidence supported the hypothesis that n-3

PUFAs supplementation from fish oil preparations could be beneficial in IBD in 13 clinical studies reviewed by Calder (87).

Radiotherapy (RT) induced proctitis may have some similarities to IBD microscopically, endoscopically and clinically (92;93), and such common features led to the hypothesis that knowledge from IBD studies might be applicable in RT induced proctitis. Biopsies from rectal mucosa in patients about to receive RT for prostate cancer were analyzed for FAs (published in a thesis for master degree in nutrition (94)). Ten patients were examined within days before start of RT, during and after RT (a total of five examinations). The results indicated a shift in the composition of FAs as radiation dose increased, with significantly more n-6 PUFAs and less n-3 PUFAs in the biopsies taken nine weeks after RT. Three and nine weeks after completed RT a significant increase of eicosatrien acid (mead acid, 20:3 n-9) was found, indicating a deficiency of essential FAs (94).

Dietary interventions aimed at reducing the risk of developing cancer in a healthy person is a different situation from two closely related topics: protection of normal tissue that is about to be exposed to RT versus protection of the malignant cells that preferentially should be susceptible to RT. Finally, treatment of late RT effects in a cured cancer patient is yet a separate topic, as there presumably should be no need to consider tumour cells.

In the above referred study (94), the FAs in normal rectal mucosa were studied before, during and after the oxidative stress generated by the RT. The response to RT of normal tissue may be different from that of malignant cells. In vitro studies of effects on cancer cells showed that fewer colorectal adenocarcinoma cells survived if they were incubated with n-3 PUFAs prior to the single radiotherapy exposure (95). There were indications that n-3 PUFAs induced apoptosis in these experiments also without the radiation. If the cells were incubated with vitamin E and n-3 PUFAs before radiation, the increased cell death due to n-3 PUFAs was abolished (95). The authors therefore suggested that lipid peroxidation was involved in the antitumor properties of PUFAs. Comparable results and interpretation were found using a rat

model with induced mammary cancer (96). The rats were divided into three groups before a single radiation exposure: a control group, a group that were fed a diet with high n-3 PUFAs content and a group fed with high n-3 PUFAs content plus vitamin E. Tumor sizes were more reduced in the group fed with high n-3 PUFAs (60% reduction in tumor size) than in the control group (31%), with the combined n-3 PUFAs and vitamin E closer to the control group (36% reduction in tumor size) (96). A third research group found similar results with pancreatic cancer cell lines and a leukaemic cell line (97). They examined not only effects of radiation, PUFAs and vitamin E, but also how the timing of radiation and incubation with PUFAs influenced cell survival (97). In the publications from Benais-Pont and colleagues (95) cells were incubated with PUFAs before radiation, while Hawkins and colleagues found that more pancreatic cells died if incubated with PUFAs after the radiation rather than before or simultaneously with the radiation (97). In 1966 a paper described the enhanced effect of radiation by adding a compound that increased the amount of ROS in the cancer cell culture (98). At the 40-years anniversary of this journal, the editorial board chose this and six papers from the first issues to be reprinted and commented (99-101). The principle of enhancing anti-tumor effect by generating ROS is re-emerging in cancer research today (100;102). The complex protein cellular signalling systems recognise radiation-induced oxidative damage to DNA and plasma membrane lipids (102). This leads in turn to stimulation of intracellular signalling pathways, which modulate the activity of genes controlling apoptosis, cell-cycle arrest or repair of damaged molecules including DNA (102). Apoptosis can be initiated if the cellular membrane has been damaged by radiation and a second messenger for this signalling pathway is ceramide (102-104). Ceramide can be synthesized *de novo* or generated by enzymatic hydrolysis of sphingomyelinase from sphingomyelin, which is the major membrane sphingolipid (102;104). Radiation activates sphingomyelinase (102;103), and modulation of ceramide signalling might be useful for enhancement of RT effects.

Studies on the effects of a FA analogue (tetradecylthioacetic acid, TTA) in cell cultures showed that it induced apoptosis in malignant cells (glioma and leukaemia cells) as well as in normal T-cells (105;106).

As to date, supplementation of PUFAs in conjunction with oncological treatment seems rather to enhance cell killing than protect tumor cells (95;97;107). However, the above referred experiments with cells or animals still differ greatly from RT of patients using fractionated radiation. Concerning the normal tissues, Wen and colleagues studied oral mucosa reactions of radiation in mice and tumor effect in nude mice (107). They showed protection of mucosal radiation effects from injections with n-3 PUFAs. Inhibition of tumor growth was seen with n-3 PUFAs injections as the sole treatment, but maximal effect observed when PUFAs and radiation were combined (107).

It might not be advisable to administer vitamin E in conjunction with oncological treatment until the effects on tumor growth have been thoroughly studied. However, as a potent chain-breaker of unwanted lipid peroxidation, the use of vitamin E might be appropriate after completion of oncological treatment. Dietary interventions after RT was reported in 17 studies (108). The authors concluded that evidence was limited, but antioxidant supplementation might be useful (108).

1.4 Acute radiation proctitis

Radiotherapy (RT) is the main oncological treatment modality for many cancer patients (109-111). Estimates show that 20% to 50% of patients with pelvic cancers undergo RT (110;112) and up to 50% of patients with abdominal or pelvic cancers may have RT as part of their treatment (111). This gives estimates of 11 000-12 000 new patients treated each year in United Kingdom (110). Taking in account that half of survivors might suffer some degree of bowel dysfunction after abdominopelvic RT, more than 1 million patients in the USA may suffer from RT sequelae (111). In

Norway, 3818 new cases of prostate cancer were diagnosed in 2004 (113), 1137 rectal cancers and around 1000 other abdominopelvic cancers. Conservative estimates of patients treated with abdominopelvic RT would be about 2000 each year. Major technological improvements in radiation treatment planning and delivery have allowed increased doses given to the tumour while reducing the dose to normal tissues in neighbouring locations (fig. 4). However, generally the tumour dose is increased to obtain better tumour control while the normal tissues are irradiated at the same dose level, albeit in smaller volumes (114). Studies have shown that increased dose per fraction, higher total RT dose and reduced overall treatment time increased the severity and frequency of late radiation enteropathy in a rat model (115;116). However, much research remains to elucidate why some patients develop late radiation enteropathy. The prevalence of moderate to severe late bowel effects (three months after completion of RT) varies from 5% to above 30% (113;117-121). Clinical studies have shown that the risk of developing late radiation enteropathy increases if gastrointestinal symptoms of acute radiation enteropathy were present during RT (118;120-125). The processes start during the course of RT, and analyzing for changes induced by RT during the acute phase is a step toward understanding the chronic, progressive enteropathy that might ensue.

4A)

4B)
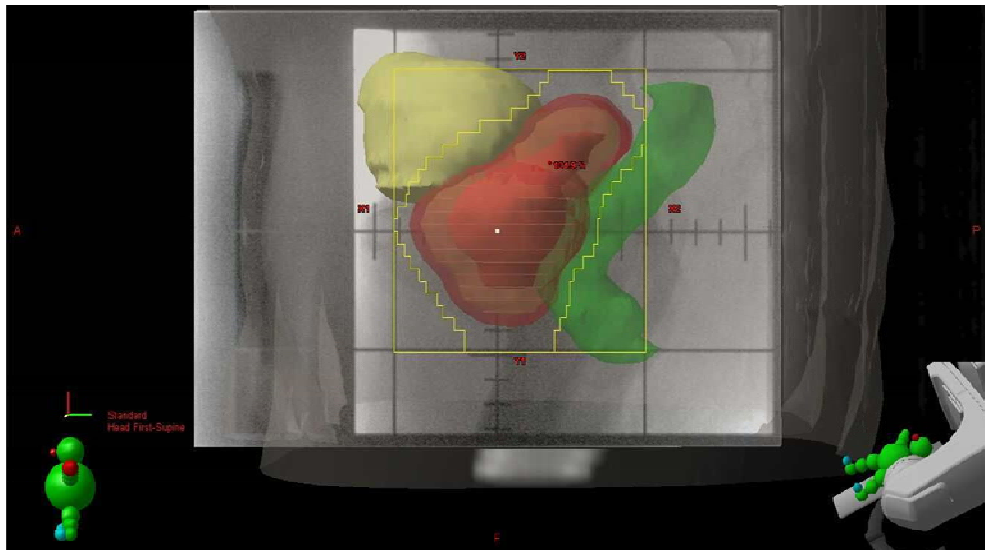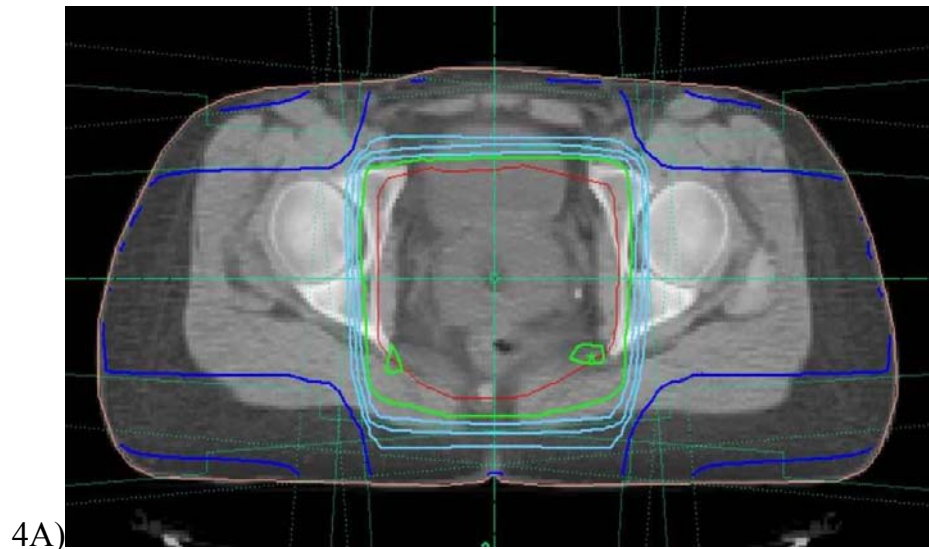
Figure 4. Conformal radiotherapy of pelvic region

A) Computer tomography planned four-field RT. Transversal section in height with hips, supine position. Red line in centre indicate clinical target volume (cervical cancer), to receive 100% of RT dose, dark blue lined delineate expected 30% of RT dose in these areas.

B) Three-dimensional illustration of prostate with seminal vesicles, clinical target volume by treatment of prostate cancer (red organ), urinary bladder (yellow organ) and rectum (green organ) visualised from the left side view of the patient (Helax-model patient in lower left corner). Yellow lines: multileaf collimator delineating the RT field borders. In the lower right corner: Helax-model patient lying on treatment equipment.

RT effects are caused by reactive oxygen species (ROS) generated in tissues (109). These short-lived substances start chain reactions leading to damage on DNA and other molecules like plasma membrane lipids (102;103). Cells are more susceptible at some stages during the cell cyclus (G2 and mitosis most sensitive (109)). If the damage on the cell and in particular on DNA is irreversible, the cell stops dividing, and eventually dies. Most cancer cells divide relatively frequent and are hence more often vulnerable to RT. Epithelial cells of intestines have a high proliferation rate and are at risk of RT damage. However, death of stem cells in the intestinal crypts is probably not the main event leading to radiation-induced late effects rather it is a consequence of apoptosis of endothelial cells of small vessels (126;127).

The biological changes during RT have been studied in cells and animal models, and more recently in humans. Prediction of normal tissue tolerance to RT and interventions to alter the sensitivity individually are topics of major concern in radiobiology research (109). A model for the clinical normal tissue radiosensitivity was proposed in a review (128) taking numerous gene responses into consideration, assuming suboptimal function of gene products. This model implies that e.g. alterations in DNA repair genes, genes coding for scavengers of ROS, overexpression of the gene for tumour growth factor-β1 and other responses to radiation damage may combine into the observed individual tolerance for normal tissue complications (128). Large-scale and well-planned clinical studies to examine the possibility of predicting individual RT complication risk were recommended.

The physiological environment in the tissues can be influenced e.g. by cessation of smoking during RT. Studies in animal models have shown that carbon monoxide inhalation (similar to tobacco smoking) protects tumour cells (129), thus a higher radiation dose is required to obtain tumour control. The use of objective measurements of smoking cessation has been recommended in clinical trials evaluating the effect of smoking on survival after RT (130). Dietary interventions during RT have been examined in 18 studies reviewed by McGough (108), recommending further research with low-fat diets, probiotic supplementation and

elemental diets. Transient decrease in nutritional intake and slowly developing cobalamin deficiency were recently described in rectal cancer patients during RT (131;132). Smoking was the strongest predictor of late radiation complications in nearly 3500 cervical cancer patients (117), and differences in diets between the ethnic groups was suggested as an explanation to differences in late radiation effects. In addition to smoking habits and ethnical relations, body composition were important, in that thin women had an increased risk of intestinal complications while obese women were more likely to have serious bladder complications (117).

Time course of gastrointestinal symptoms was not congruent to the histopathological grading of inflammation in rectal mucosa (92;93;120). Biopsies are considered "gold standard" in defining inflammation. The discrepancy in time course with maximal inflammation at first examination after start of RT while symptoms reach their maximum towards the end of RT, could have important implications for future prophylactic interventions to mitigate acute and late radiation enteropathy. The possibility to obtain rectal mucosa biopsies in prostate cancer patients, receiving rather homogeneous radiation doses, makes this a feasible clinical model to study physiological changes induced by RT. The complexities of the tissue damage and repair processes that take place during the weeks of fractionated RT have been divided in four categories to better understand the key processes:1) Epithelial injury; 2) Inflammation; 3) Endothelial cell changes and activation of the coagulation system; and 4) Proliferation and repair (133).

Gastrointestinal symptoms like diarrhoea, blood on stools and abdominal pains are shared by radiation enteropathy and IBD. In the case of radiation enteropathy, the initiating event of the ensuing inflammation is the repeated generation of ROS. A natural defence mechanism of the innate immune system is also the generation of ROS by leukocytes (1). However, in IBD the ROS produced by leucocytes infiltrating in the mucosa destroy tissues and decrease the amount of important antioxidants (134). Oral ferrous iron therapy increased oxidative stress in patients with IBD assessed by plasma reduced cysteine and reduced glutathione (135). Lipid

peroxidation, measured by malondialdehyde in plasma, increased in IBD-patients after oral and intravenous iron therapy (136;137). Antioxidant vitamin supplementation in Crohn's disease reduced oxidative stress measured in plasma and breath tests (138). Whether such factors relevant in IBD could also be applicable in RT remains to be studied. Obviously, the deleterious effect on cancer cells must not be compromised by interventions aimed at reducing normal tissue late radiation effects. There are controversies regarding the use of antioxidant supplements while the tumour is still untreated (139).

1.5 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation of the synovium of joints and destruction of the cartilage and bone. The diagnosis is mainly clinical, based on criteria from the American College of Rheumatology, revised in 1987 (85). Four of seven criteria must be demonstrated in the patient, and have a duration of more than six weeks. One criterion is radiographic changes and the only laboratory criterion included is the finding of serum rheumatoid factor (RF). Interpreted together with the clinical picture, RF may support a diagnosis of RA. However, finding of RF in serum does not exclude other related diseases, and RF is found in 5% of the normal population (140). Approximately 10% of RA patients will remain RF negative (140). In a study examining gene expression in peripheral blood mononuclear cells from RA patients and healthy controls, the RF positive and RF negative patients could not be discriminated, while RA patients and healthy controls could (69). RF is hence used in the differential diagnosis of RA but also as a prognostic biomarker. During the first half of the twentieth century RF was discovered by Erik Waaler (1940, Oslo) and later Harry M. Rose and Charles A. Ragan (1948, New York) (85;141). Waaler recollects how his observations of "the

factor, which was a globulin, reacted as an antibody with another globulin. This other globulin was an antibody, which behaved as if it were an antigen" (141). At the time his finding seemed "remarkable, even improbable" (141). The laboratory techniques applied have changed since then, but RF remains important in rheumatology (85;140;142). High levels of serum RF are associated with a worse prognosis in RA (140). Waaler or Latex tests mainly identify RF of IgM class of immunoglobulins (Ig). Using ELISAs for detection of IgM, IgG, IgE or IgA type RFs, discriminative functions of the subtypes of RFs indicate that IgG RF is a risk factor for development of vasculitis, while IgA RF may correlate with vasculitis, bone erosions and a more severe disease course (140). Lately another biomarker, anti-cyclic citrullinated peptide antibody (anti-CCP antibody) has been found useful in combination with RF both in differential diagnosis and prognosis of RA (143;144). Both sensitivity and specificity were improved by combining the two biomarkers. The first description of autoantibodies against citrullinated peptides came in 1964 (144). Although easily applicable in routine use by commercial ELISA kits, its role in rheumatology is not yet clearly established.

The chronic, systemic inflammation in RA is thought to participate in the development of atherosclerosis of RA patients, contributing to their higher morbidity and mortality (145-148). The reduced long term survival was related to cardiovascular (CV) disease in females and malignant diseases in men in a Norwegian study of 147 RA patients (147). After correlating for established risk factors for CV disease, RA disease duration, higher deformed joint count and increased ESR and CRP remained associated with an increased likelihood of carotid plaque in 631 RA patients (145). Another study found that disease duration predicted serious atherosclerotic plaques while methotrexate appeared to be protective on atherosclerosis development (149). In RA patients the chronic inflammation may contribute independently or synergistically with atherosclerotic risk factors (smoking, hypertension, diabetes and hypercholesterolemia) (148). The premature atherosclerosis in RA patients has been referred to as "accelerated atherosclerosis" and was suggested as an extra-articular feature of RA (ExRA) (148). Although

atherosclerosis is considered an inflammatory disease (150;151), the histopathological and pathogenetic mechanisms in the heterogenous group of clinical syndromes collectively called systemic vasculitides are a different disease entity, thought to be mediated by circulating immune complexes (85).

The acute phase reaction (measured by ESR and CRP) is commonly used to monitor RA disease activity and response to therapy, but intercurrent diseases may complicate the interpretation of elevated ESR and CRP. Furthermore, RA patients often have CRP in the range of 30-40 mg/L when disease activity is moderate (152). "High-sensitivity" CRP assays detecting concentrations below 1 mg/L have been found to predict CV disease risk in apparently healthy individuals (150;153). In RA patients this is unlikely to be helpful as a screening for CV disease.

Calprotectin was found in high concentrations in synovial fluid in RA patients (17;154). Calprotectin levels in blood samples correlated with RA disease activity but their prognostic value remains to be defined (155-157). They may be useful with regard to radiological changes of joint destruction (158). Peripheral blood cell gene expression profiles showed S100A8, S100A9 and S100A12 to have higher expression in RA patients than healthy controls (69). Another study of peripheral blood cell gene expression profiles found that out of 4 500 genes expressed, 81 genes were expressed differently in RA patients than healthy controls (68). In a logistic regression analysis of each gene's ability to classify samples (patients versus controls), S100A12 were among the four best (68). S100A12 was found in synovial fluid from RA patients (45;73) and serum concentrations of S100A12 correlated with disease activity in RA (67;73).

2. Aims of the study

The overall objective of the present study was to evaluate biomarkers calprotectin and S100A12 in two diseases characterized by inflammatory reactions.

The specific aims of the study were:

Search for candidate biomarkers that mirror disease activity in acute radiation proctitis in samples from blood, stools and/or mucosal biopsies (*Paper I and III*).

Describe changes occurring during the course of acute radiation proctitis in clinical parameters, histopathological graded inflammation in rectal mucosa biopsies and candidate biomarkers. Evaluate their relationship with each other (*Paper III*).

Confirm the preliminary results regarding RT induced relative deficiency of essential n-3 FAs and plan and execute an interventional study where seal oil should be given to prostate cancer patients scheduled for RT *(Paper III).*

Develop a reliable method to quantify a new biomarker, S100A12 (*Paper II*).

Evaluate serum calprotectin and S100A12 as biomarkers in rheumatoid arthritis, specifically how each correlate with other biomarkers, disease activity, extra-articular manifestations and concomitant cardiovascular disease (*Paper IV*).

3. Materials and Methods

3.1 Patients and study design

Paper I and *III* included patients consecutively presenting at the outpatient clinic of Department of Oncology, Haukeland University Hospital scheduled for conformal radiotherapy (RT) for localized prostate cancer. Exclusion criterion was gastrointestinal disease. These longitudinal studies examined patients within the week before start of RT, two and six weeks into the treatment and (*Paper III* only) four weeks after completion of the RT. *Paper II* included healthy controls, which in this context meant absence of chronic diseases that had been shown or hypothesized to be related to increased blood concentrations of calprotectin or S100A12. The healthy controls were staff at Haukeland University Hospital. In addition 150 blood donors were examined in order to establish a reference range. *Paper IV* was a cross-sectional study of 129 rheumatoid arthritis patients, consecutively included when admitted to the out-patient clinic of Department of Rheumatology.

3.2 Laboratory methods

Paper II describes the development of an ELISA for S100A12. This was a central part of the PhD work, and necessary for the results in *Paper III* and *IV*.

The S100A12 assay was developed in cooperation with Igor B. Bronstein, National Institute of Medical Research, London, and Magne K. Fagerhol, Department of Immunology and Transfusion Medicine, Ullevaal University Hospital. Recombinant S100A12 was provided by Igor B. Bronstein, and the rest of the method developed at Ullevaal University Hospital. Polyclonal antibodies were obtained by immunization of rabbits with the recombinant S100A12. Immunoaffinity purified anti-S100A12 was prepared by use of a column where S100A12 had been covalently linked to agarose beads. These antibodies were conjugated with alkaline phosphatase (ALP).

The IgG-fraction of rabbit anti-S100A12 serum was used for coating of microwell plates (MaxiSorp, Nunc, Denmark). Dilutions of the recombinant S100A12 were used as standards. For the assay, standards and samples were diluted in tris-buffered saline with 10 g/L bovine serum albumin and 0.1 g/L sodium azide as an antimicrobial. The first incubation was for two hours and the second, with ALP-conjugate, for one hour. Between incubations wells were washed four times. Optical densities were read at 405 nm after reaction with substrate for about 30 minutes.

Calprotectin was measured by an ELISA (79), both at Department of Immunology and Transfusion Medicine, Ullevaal University Hospital, and Department of Immunology and Transfusion Medicine, Haukeland University Hospital.

S100A12 and calprotectin ELISAs were used to analyze blood samples in *Papers II-IV* as well as stool samples in *Paper I* and *III* (only calprotectin in *Paper I*).

Stool samples in *Papers I* and *III* were additionally analyzed with purchased ELISA-kits for lactoferrin (IBD-CHEK[TM] and IBD-SCAN[TM] from TechLab, Blacksburg, VA), TNF-α (BIOTRAK[TM] from Amersham Pharmacia Biotech Limited, Buckinghamshire, England), leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2) and tromboxane B_2 (R&D Systems Europe, Oxon, England). Transferrin was determined by an ELISA very similar to that for calprotectin using reagents prepared at Ullevaal University Hospital. α1-antitrypsin was measured by nephelometry using an automated instrument from Behring, Germany.

Routine laboratory methods at Haukeland University Hospital were used for the following components in blood: haemoglobin, leukocyte count, platelets, C-reactive protein, erythrocyte sedimentation rate (ESR), albumin, creatinin, liver enzymes. Rheumatoid factor (RF) was determined by Waaler's test. Anti-CCP was determined in an ELISA assay (QUANTA LiteTM CCP IgG ELISA, INOVA Diagnostics Inc, San Diego, CA, USA).

National Institute of Nutrition and Seafood Research, Bergen, analyzed biopsies and blood samples for 37 different fatty acids (FAs), and blood samples were analyzed

for LTB$_4$, vitamin E and TBARS. FA composition was determined by a gas liquid chromatography (GLC) (159;160). LTB$_4$ was analyzed by ELISA-kits from R&D Systems, London, United Kingdom. Vitamin E content (tocopherol isomers $\alpha,\beta,\gamma,\delta$) was analyzed with high performance liquid chromatography (HPLC) (161). TBARS was measured spectrophotometrically (162).

3.3 Symptoms, diet and histopathology in radiation proctitis

In *Paper III* symptoms of gastrointestinal toxicity induced by the radiotherapy was recorded with a previously used questionnaire (93) as well as the relevant questions from a validated questionnaire, QUWF94 (163). Scores were graded from zero, meaning no problem with the item in question, and numbers 1-10, with 10 representing the most severe of the problem.

Paper III recorded dietary habits with respect to nutriments rich in potentially beneficial fatty acids (fish, nuts and supplements with n-3 FAs), antioxidants (fruit, vegetables and micronutriment supplements) and cultured dairy products. The questions were "How many days last week did you eat….".

Paper III assessed the histopathological inflammation in biopsies from rectal mucosa as the radiation dose increased. Nine characteristic features were graded 0-4, with 0 as normal and 4 for maximal pathology.

3.4 Rheumatoid arthritis' definitions of disease

The RA diagnosis was made according to the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (164). The investigator's and the patient's global assessments of the disease activity and pain last week were recorded on a visual analogue scale (VAS). A 28-joint count for

tender and swollen joints was performed (165). The composite disease activity score DAS28 was calculated (166). The Health Assessment Questionnaire (HAQ) (167) was used as functional disability score. Cardiovascular (CV) disease was considered present if the patient had been diagnosed with angina pectoris, myocardial infarction or congestive heart failure by a cardiologist. Secondary Sjögren's syndrome was considered present if two of three were present: Xerostomia, keratoconjunctivitis sicca (reduced tear secretion as measured by Schirmer's test < 5 mm/5 minutes or a positive Rose Bengal test) or serological evidence as positive SS-A, SS-B, RF or elevated IgG. Other ExRA were clinically judged based on presence of subcutaneous nodules, scleritis or episcleritis, peripheral neuropathy not explained by other conditions or pulmonary fibrosis. Felty's syndrome was defined as splenomegaly and persistent neutropenia ($<1.5x10^9$).

3.5 Statistics

In the longitudinal studies (*Paper I and III*) changes in each parameter (symptom scores, biopsy grading) and laboratory measurements were compared to baseline, i.e. before start of radiotherapy. The samples sizes of 15 and 20 patients were not sufficient to infer how the data were distributed. Non-parametric tests were therefore applied, and exact paired permutation tests chosen, since this makes use of the magnitude of the measurements, and not only rank order, which is the case with exact Wilcoxon paired samples test. Spearman rank order correlations were used to compare different parameters at each examination time.

In *Paper II*, the sample size of 150 blood donors allowed assumptions of the distribution of the concentrations of calprotectin and S100A12 in blood samples. After log-transformation, the distributions of both proteins were close to normally distributed, compared to the skewed distribution, most pronounced for S100A12, before transformation. Hence, we used linear mixed effects models, Pearson

correlations or paired-samples t-tests, as appropriate, for log-transformed values. Reference intervals for S100A12 and calprotectin for serum, EDTA plasma and heparin plasma were calculated separately for each gender as mean ± 2 standard deviations on a log scale and transformed back. For S100A12 in serum the influence of age and gender was examined using a quadratic regression with possibly different relationships for women and men.

The cross-sectional study and sample size of 129 patients in *Paper IV* allowed regressions between parameters to be explored. Univariate regression between variables was analysed. Spearman rank order correlation test was used to compare clinical and laboratory variables. The Mann-Whitney test was used to compare different subgroups of the patients. Independent associations for extra articular rheumatoid arthritis manifestations and concomitant cardiovascular disease were examined by multiple logistic regression analyses with a forward stepwise design, and odds ratio (OR) and 95% confidences interval (CI) given for any significant associations.

Two-tailed p-values <0.05 were considered statistically significant.

4. Summary of results

4.1 Paper I

Faecal calprotectin and lactoferrin as markers of acute radiation proctitis: a pilot study of eight stool markers

The faecal excretion of eight markers of gut inflammation was examined in stool samples from 15 patients with prostate cancer scheduled for radiation therapy. Of the five proteins only calprotectin and lactoferrin concentrations increased significantly during radiation treatment (p=0.0005 and p=0.019). α1-antitrypsin could not be detected in any sample and transferrin in nine of 45 samples. No changes during treatment were found for TNF-α nor the three eicosanoids PGE_2, LTB_4 and thromboxane B_2.

4.2 Paper II

Quantification of S100A12 (EN-RAGE) in blood varies with sampling method, calcium and heparin

Studies of S100A12 in serum, EDTA and heparin plasmata showed that the protein was highly influenced by the anticoagulants in vacutainers, calcium concentration in buffers and handling of samples. The choice of vacutainer used for drawing blood,

the time span before separation of plasma or serum and repeated freeze-thaw cycles were all shown to influence the estimated concentration of S100A12 in the ELISA. Conformational changes in S100A12 structure and the ability to form dimers and oligomers were suggested as the molecular basis of our findings. The hypothesis that S100A12 binds to heparin in a fashion that affects the influence of calcium concentration was generated on basis of the results. The conformational changes of S100A12 in EDTA plasma influenced the ability of our polyclonal antibodies to recognize S100A12. Individual S100A12 levels did not change substantially during a five week sampling period. Serum was proposed to be the best medium for S100A12 measurements in patients. Based upon testing of 150 blood donors we suggested reference intervals of S100A12 in serum to be 49-1340 µg/L for women and 27-1750 µg/L for men. The estimated mean concentrations were 234 µg/L in serum samples (range 12-15791 µg/L), 114 µg/L (range 3-17282 µg/L) in re-calcified EDTA plasma and 48 µg/L (range 2-14843 µg/L) in heparin plasma.

4.3 Paper III

Time patterns of changes in biomarkers, symptoms and histopathology during pelvic radiotherapy

The increase in histopathological inflammation in rectal mucosa biopsies from 20 prostate cancer patients reached a maximum 2 weeks after start of radiotherapy (RT). Symptoms of gastrointestinal toxicity increased with higher radiation dose and had not returned to pre-treatment level 4 weeks after RT. Lactoferrin concentrations in stools increased significantly in week 6. Significant decreases of vitamin E, leukocyte

count, haemoglobin and some groups of fatty acids in blood samples were discovered, while a few fatty acids increased significantly during the study period.

4.4 Paper IV

S100-proteins in rheumatoid arthritis: Calprotectin and S100A12 are associated with disease activity, extra-articular manifestations and concomitant cardiovascular disease

Serum concentrations of calprotectin and S100A12 were found to correlate significantly ($p<0.01$) with the seven different clinical outcome measurements of RA disease activity (DAS28, HAQ etc.) in the 129 patients. Of the clinical indices, DAS28 was found to have the most substantial correlation (rho$\geq$0.51) to calprotectin, S100A12, ESR and CRP (all $p<0.01$). Patients with ExRA, positive RF or anti-CCP had significantly higher S100A12 levels ($p\leq0.001$) than patients without these characteristics. Calprotectin showed a weaker relationship to the same characteristics ($p=0.06$ for the presence of ExRA, $p=0.01$ and $p=0.03$ for positive RF and anti-CCP, respectively). Logistic regression analyses on ESR, CRP, calprotectin, S100A12, anti-CCP and RF titre showed that RF was associated with ExRA (OR 1.19 for each titre step, CI 1.03-1.37) and S100A12 was associated with a diagnosis of cardiovascular disease (OR 1.055, CI 1.011-1.101). Concentrations of calprotectin and S100A12 did not correlate with age, disease duration, gender or current treatment with disease-modifying antirheumatic drugs or peroral steroids.

5.   General discussion

5.1 Calprotectin and S100A12

Calprotectin and S100A12 as biomarkers in stools and blood during RT

Calprotectin has been found useful as a biomarker in stool and blood samples in many diseases, particularly those characterized by inflammation (44;59). *Paper I* indicated that fecal calprotectin could be a clinical, objective marker to monitor gastrointestinal toxicity during RT. Even though the patients and RT were comparable in *Papers I* and *III*, as were the stools collection and analysis, we could not confirm the increase in fecal calprotectin as the radiation doses were increasing. In IBD, both the extent and intensity of inflammation in the bowel are of greater magnitude than those after abdominal or pelvic RT. During relapses of IBD fecal calprotectin generally increase 10- to 20-fold (5;80;168). Patients with prostate cancer receive a RT field with a relatively small volume of intestines included, while some other patient groups (gynaecological cancers, bladder cancers, testicular cancers, colorectal cancers) usually need larger RT fields to cover tumour and lymph node areas. They are consequently more prone to suffer from gastrointestinal toxicity during RT. In *Paper III* we examined S100A12 in stool samples. Concentrations were low, and the indications suggesting S100A12 to be considerably less stable than calprotectin, lead us to recommend using calprotectin rather than encourage further studies on the quantification of S100A12 in stools. Neither calprotectin nor S100A12 in blood or stools showed major changes during RT.

Protein conformational state and consequences for the quantification of S100A12 in clinical use

In *Paper II* the newly developed ELISA for quantification of S100A12 showed that the concentration of S100A12 increased in EDTA plasma if collection tubes were left before separation of plasma; by contrast, serum from blood in gel tubes had stable concentrations independent of when separation took place, examined up to 48 hours after blood was drawn. Repeated freeze/thaw cycles did not influence S100A12 concentrations in most of the sera, but for a few individuals' samples, both freeze/thaw cycles and storage in 4°C increased the measured concentrations of S100A12. Leaving sera at room temperature increased the concentrations; similarly addition of calcium to the assay buffer gave significantly higher readings. These observations were interpreted in the light of a limitation of the ELISA technique combined with knowledge from crystallization experiments of S100A12 (54) and our own gel permeation chromatography findings. We suggest that the increased concentrations observed in serum samples could be explained by formation of larger complexes of S100A12, and that such complexes falsely are quantified as if more S100A12 are present in the serum sample. We also concluded that the antibody or antibodies present in our ELISA depends on the formation of dimers or larger complexes of S100A12 to be present in the sample, and that epitopes on the conformational state found in EDTA plasma hardly can be detected by the developed ELISA.

Paper II included comparisons between calprotectin and S100A12 regarding stability during blood sampling and analysis. Some of the new data regarding calprotectin was not published and is described here. Calprotectin concentrations were unchanged when serum from blood stored in gel coated tubes (p=0.64) or plasma from heparinized blood (p=0.11) were harvested after 24 hours delay, while the concentration in EDTA blood increased significantly (mean 1.4 and 2.3 mg/L at 1 and 24 hours, respectively, p=0.018). In clinical studies, 2 hours has been the time

span accepted before separation of plasma. Calprotectin estimates in serum and plasma were unaffected by Ca^{2+}- concentration in dilution buffers from 0 to final concentrations of about 6 and 21 mM (all p≥0.35). Calprotectin concentrations in serum were not significantly changed by ten freeze/ thaw cycles to -20ºC (means 1.4 and 1.5, range 1.2-1.8 mg/L, p=0.35) or to -70ºC (means 2.3 and 2.2, range 1.6-3.0 mg/L, p=0.40). Calprotectin concentrations in EDTA plasma decreased when stored at room-temperature for three weeks (means 2.1 and 1.5, range 0.9-2.8 mg/L, p=0.033). Eleven healthy volunteers were examined seven times during five consecutive weeks, samples taken after breakfast and before noon. There was no significant change by time in EDTA plasma (p=0.69) during the time period of the experiment (fig. 5). The standard deviation for within-individual variation was 0.33 (95% CI 0.29 – 0.37). Calprotectin serum and plasma concentrations were elevated in one individual probably related to bronchitis. Three other serum samples were elevated for unknown reasons. All S100A12 concentrations were within the suggested reference intervals (S100A12 data in *Paper II*).
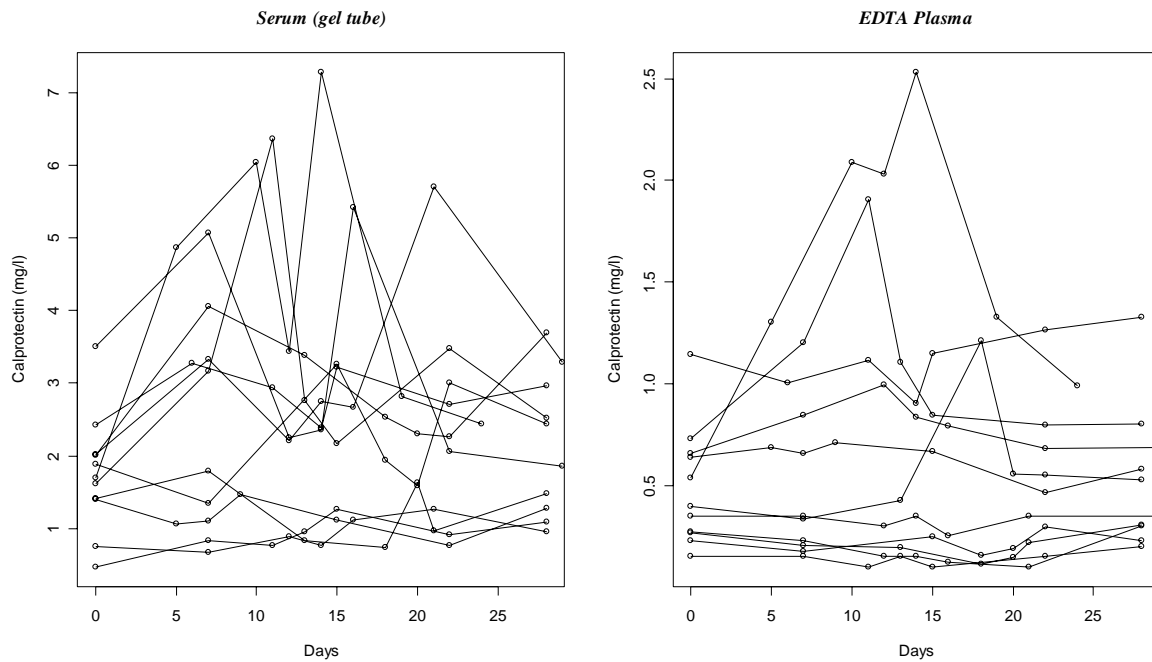
Figure 5. Concentrations of calprotectin during five weeks

Variation in eleven healthy volunteers' samples (seven examinations in five weeks). Lines represent separate volunteers.

Figure made by Tore Wentzel-Larsen in R software. Printed with kind permission.

Due to our findings in *Paper II* we have indications that our polyclonal rabbit antibodies were either mainly from one clone, possibly also from other clones which produce antibodies against epitopes only found when dimers or oligomers of S100A12 are present. The consequence of this assumption is that we consider the conformational changes of S100A12 in EDTA plasma to be of such a nature that our antibody can not estimate the concentration of S100A12 in plasma samples. However, when the plasma samples were diluted in a buffer containing calcium, and the molarity exceeded the 4 millimols bound by EDTA in the collection tube, S100A12 could be quantified also in EDTA plasma samples. This raised a new question: at which concentration of calcium can we expect S100A12 to be found in the concentration present in the blood of the patient? Both serum and EDTA plasma samples had seemingly higher estimated concentrations of S100A12 if more calcium (above physiological concentrations, i.e. 2 mM in blood circulation) were added in

dilution buffers before quantification. For the estimation of reference intervals, 6 mM calcium was arbitrarily chosen based on the 4 mM calcium bound by EDTA and the 2 mM in circulation. The assay developed in *Paper II* is hence not satisfactorily for future quantification of EDTA samples; this would require antibodies directed at epitopes found also on S100A12 in EDTA samples, i.e. independent of calcium concentration in the sample.

EDTA samples from long distance runners showed a 20-fold increase in calprotectin concentration (63). Five healthy persons (age 32-55 years) were examined before and after an endurance cross-country run of 30 km. The examination times were: 1- 12 hours before start, immediately (within 10 minutes) after the race and 6 h, 18 h, 48 h and 72 h after the race. EDTA blood was drawn and transported on ice to laboratory for centrifugation within two hours. The plasmas were frozen at -50ºC. When the assay for quantification of S100A12 was developed, the runners gave their consent to examination of their samples. We choose to estimate S100A12 even though some uncertainties exists regarding the concentration due to EDTA and the subsequent addition of calcium in dilution buffers, and the samples also inadvertently had been thawed and stored in room temperature for three weeks before freezing and storage at -20ºC before the S100A12 analysis. For these reasons, the results are unpublished, but may give indications for future research on S100A12. As shown in fig. 6, S100A12 in re-calcified EDTA-plasma increased 77-fold from before (mean 358 µg/L) until immediately after (mean 27 642 µg/L) (p<0.001) a 30 km cross country running competition. Based upon the rate of decrease towards the normal after the run, an estimated half time in the circulation was 3.1 hours (CI 2.1-5.5), compared to about five hours for calprotectin (63) . This half-life estimate was calculated based on a linear mixed effect model, as ln(0.5) divided by the estimated slope in the cross-country run data. Neutrophil counts increased 3.2-fold from $4.0 \cdot 10^9$ to $12.8 \cdot 10^9$ cells/l during the run and had normalized 18 hours after the finish (63). We speculate that the much higher increase in S100A12 concentration compared to the increase in neutrophil count could indicate active release of S100A12, possibly also leakage if neutrophils are crushed in the soles of the feet. However, considering the higher

content of calprotectin compared to S100A12 in each neutrophil cell, active release of S100A12 seems more likely to explain the increased S100A12 concentration. The physiological reason for this may be related to endothelial damage during exhaustive physical exercise and involvement of neutrophils in the removal of microthrombi, re-establishement of a smooth endothelial surface, i.e. inflammation and tissue repair.
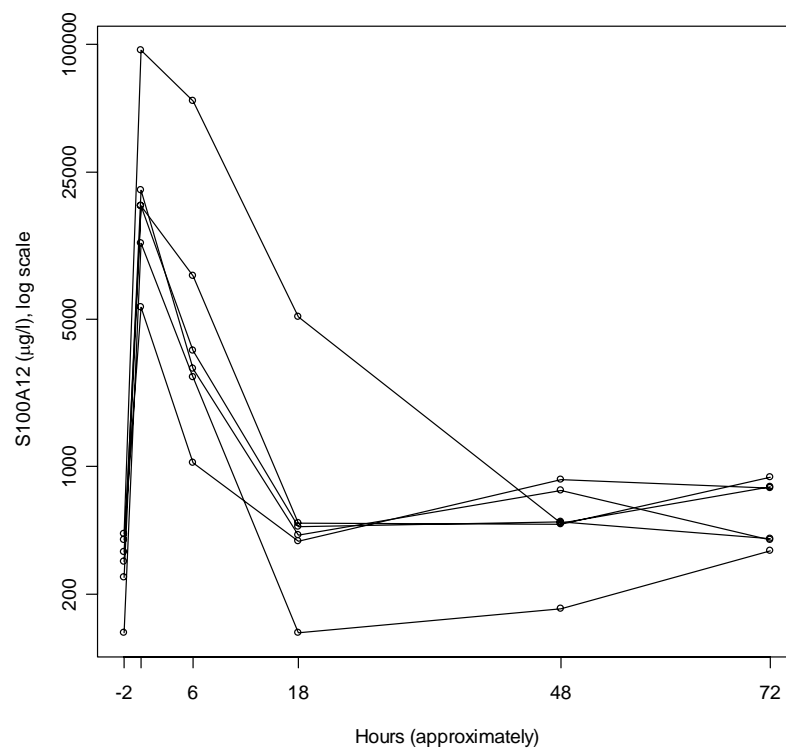

Figure 6. S100A12 in EDTA plasma before and after 30 km cross-country running

(before the start of the run is marked by -2)

Figure made by Tore Wentzel-Larsen in R software. Printed with kind permission.

Binding between heparin and calprotectin and S100A12

Heparin and heparan sulphate are linear carbohydrates belonging to the group of glycosaminoglycans (GAG). One of the functions of heparan sulphate is mediated from the endothelial cell, where it is bound and participate in the recruitment of leukocytes to the site of inflammation in tissues (1;169). Two studies have described S100A9 and the S100A8/A9 complex binding to endothelial cells by binding to heparan sulphate structures of endothelial cell surface glycosaminoglycans (37;58). Both these research groups have proposed a role for these proteins in reducing oxidative damage to the endothelial cells. Heparin binds to many proteins, and most likely also to S100A12, due to its homology with calprotectin (51;55). When analyzing serum from gel coated tubes after addition of calcium, S100A12 concentrations were significantly higher than without adding calcium (*Paper II*). However, in heparin plasma addition of calcium did not change S100A12 estimates. This may suggest that the binding of heparin interferes with the calcium induced conformational changes of S100A12. In order to examine the influence of heparin as an anticoagulant in collection tubes, serum samples were added either heparin-coated sepharose beads (HI Trap Heparin, Pharmacia AB, Uppsala, Sweden) or native sepharose beads and incubated overnight on a rotator. Supernatants were collected and assayed for S100A12 and calprotectin. Some of the supernatants were subsequently exposed to new heparin-coated beads for a second overnight incubation and assayed for calprotectin. The experiment (partly unpublished data) showed that incubation of serum with heparin-coated sepharose beads reduced the S100A12 concentration significantly from 579 µg/L (incubated with beads without heparin) to 15µg/L (p<0.001). The calprotectin concentration did not change. However, after a second incubation with new heparin-coated beads, calprotectin concentration were reduced (mean 2.1 mg/L after first incubation, 0.4 mg/L after second incubation, p<0.001). We concluded that S100A12 binds to heparin, and this may support our hypothesis on the lack of conformational changes when calcium was added to heparin plasma. This indicates that S100A12 has a higher affinity to heparin when compared to calprotectin under the conditions in serum.

Reference ranges for calprotectin and S100A12 was suggested, based upon quantification of the two proteins in three different types of collection tubes from each of 150 blood donors. Our recommendation in *Paper II* is that serum samples should be used for S100A12. Considering the stability of calprotectin in serum samples, the reference intervals for calprotectin in serum might also be useful, even though calprotectin has previously been recommended to be analysed in EDTA plasma (78), based on the in vitro increase during coagulation.

In *Paper IV* a selection of commonly used biomarkers for inflammation and rheumatoid arthritis were compared to each other and to indices of disease activity. The newly developed ELISA for S100A12 was applied, and serum samples were used for the estimation of S100A12 and calprotectin. Previously calprotectin has been measured in EDTA plasma (154-157). *Paper IV* showed that calprotectin in serum correlated with disease activity, and hence may be used as a biomarker of RA disease activity in EDTA plasma or serum. Correlations were comparable to those of CRP. The relationship of calprotectin to RF and ExRA were slightly less significant than that of S100A12, and may be indicative of the utility of calprotectin as a marker of disease activity rather than a predictive biomarker. This is in line with previous results, showing that calprotectin in plasma did not predict joint destruction in subsequent years (157). DAS28 was the clinical scoring system that correlated best to the laboratory measurements in *Paper IV*. This may in part rely on the fact that ESR is one of the variables entered into the calculation of this composite disease activity score (http://www.umcn.nl/userfiles/other/DAS28_ne.xls).

The diagnosis of CV disease in this study was only based on information in the patients' records, and the patients were not subjected to any screening or objective examination with regard to CV disease. Since CV diseases are considered to develop over a long time span, most likely more patients would have been diagnosed with CV

disease if subjected to a systematic examination. Even with these reservations, logistic regression analysis showed an association of concentration of S100A12 and CV disease, while this was not found for ESR, CRP, calprotectin, anti-CCP and RF titre. RF positive patients have a more severe disease course, and S100A12 might have a role as a predictive biomarker in RA. Previous publications on S100A12, RA and disease activity included few patients compared to *Paper IV* (73), but we confirmed the correlation of S100A12 to disease activity. However, correlations were better for calprotectin and CRP than ESR and S100A12 (*Paper IV*).

5.2 Other biomarkers and correlation to disease

In *Paper I* our results indicated usefulness of lactoferrin and calprotectin measured in stool samples, and these two were therefore also examined in *Paper III*. Only lactoferrin were found to increase with increasing radiation dose in *Paper III*. The six remaining stool markers examined in *Paper I*, and the new candidate marker S100A12, were not found to increase during RT (*Paper III*).

By defining biomarkers as clinical applicable measurements, the analysis of FAs in rectal mucosa biopsies is a too invasive and troublesome examination to qualify as a suitable biomarker. Compared to published ratios of n-6/n-3 PUFAs (86;91), the patients examined in *Paper III* had slightly higher ratio (median 3.6) throughout the whole study period than the healthy control group (mean 2.2) and the IBD patients after seal oil treatment (mean 2.2). The ratio decreased from 6.6 to 2.2 in the IBD patients (86;91) after treatment. We did not find any significant change induced by the RT (*Paper III*), and endoscopic, histological and anamnestic information did not indicate that the patients suffered from any inflammatory reactions before RT. Although many of the study conditions (selection of patients, RT, biopsy handling and analysis) were similar in *Paper III* and the study by Norlie, (94), we could not confirm the results suggesting a deficiency of essential FAs developing during RT.

The planned interventional study was therefore postponed, until the scientific basis is of such a nature that it is timely to do this. Hypotheses from studies of IBD may not be representative of the inflammatory processes in acute proctitis during RT. The antioxidant vitamin E was decreased as radiation dose increased in *Paper III*. This could indicate an increased consumption of antioxidants during RT. Administration of antioxidants during RT is controversial (139), but our observations might indicate that further mapping of nutritional and oxidative status could be warranted during the course of RT.

In the case of interventional studies, it would be preferable to have available biomarkers to evaluate the effects in a treated versus a control group of patients. At present we have measurement of lactoferrin, and possibly also calprotectin, in stools as the most promising of the nine markers examined in this study.

We found no correlation of clinical scoring systems, histopathological scoring and laboratory markers (*Paper III*). Systems of grading disease activity have been developed in many diseases and we applied two questionnaires for acute radiation proctitis in prostate cancer patients (93;163). Time profiles of gastrointestinal toxicity and histopathological scoring of inflammatory changes showed discrepancy in that the biopsies were already at a maximum of inflammation at the first examination after start of RT, while symptoms increased during the whole RT period (*Paper III*). The implications of this finding, if confirmed, may be of importance when planning when to start interventions to prevent acute radiation proctitis.

5.3 Future research

Our study has shown that calprotectin and S100A12 are promising biomarkers in patients with rheumatoid arthritis. As biomarkers of the radiation proctitis during RT

stool samples seems most appropriate, with lactoferrin and calprotectin as possible biomarkers. Stool samples are non-invasive, acceptable for the patients and possible to use in clinical studies. The patient group of prostate cancer patients selected for our study had a modest bowel inflammation during RT compared to patients with IBD. Future studies should examine fecal lactoferrin and calprotectin in other patient groups with the aim of monitoring acute radiation enteritis.

The reported increase in high-sensitivity CRP during RT (170) and other biomarkers in blood indicating oxidative stress, e.g. plasma malondialdehyde, should be evaluated in future studies.

Methods for the quantification of S100A12 need to be addressed with the findings of our study in mind. We recommend that S100A12 is assayed in serum from blood collected in gel coated tubes; samples should be analyzed either before freezing, or shortly after thawing (within a day). Further studies should examine the reliability of assay results in ELISA after serum storage in 4˚C more than two days, or repeated freeze/thaw cycles. The hypothesized conformational changes and oligomerization of S100A12 is a potentially caveat for ELISA. Our and other research groups have found correlation of S100A12 and disease activity in RA patients. Immunoassays and MS should be examined as future quantification methods. With reliable methods available, other inflammatory or infectious diseases may benefit from measurements of S100A12 in blood.

Laboratory studies of S100A12 should test whether the oligomerization state of S100A12 in blood samples are comparable to the hypothesized hexamer in vitro (54). The preliminary data of S100A12 binding to heparin should be characterized. The clinical role of S100A12 in inflammation should be examined in various patient groups, and the association to CV disease and increase after physical exercise are findings relevant for such studies.

Dietary needs during RT were addressed by Guren et al (131;132). They found decreased cobalamin during and one year after RT for rectal cancer. Future studies should aim at identifying specific nutritional needs used as a tool to alter the course of normal tissue radiation effects both during and in the long time follow up of patients. The discovery of predictive profiles identifying patients at risk of normal tissue radiation morbidity requires prospective studies with sufficient number of patients followed over many years. Such profiles might be combinations of nutritional status with regard to lipids and antioxidant status.

Prediction of disease courses are of interest both before start of RT and for patients diagnosed with RA. RF is used as a biomarker predictive of a more serious disease course of RA. New therapeutic options with TNF-α-receptor inhibitors might change the prognosis for RA patients with respect to destruction of joints. A possible usefulness of S100A12 in predicting joint destruction should be studied. Morbidity and mortality from ExRA and CV disease might be reflected by S100A12 levels. Future clinical studies with rheumatic diseases should incorporate adequate measurements and examinations of the patients with regard to CV disease or risk of such, in order to correlate this with concentrations of calprotectin and S100A12 in blood.

6. Conclusions

Although gastrointestinal symptoms and histopathological inflammation in mucosa may share common features in IBD and acute radiation proctitis, the severity and extent of acute radiation proctitis is limited compared to IBD, and common biomarkers of the two disease states are more likely to be found in stool samples than in blood. Fecal lactoferrin and possibly calprotectin seems promising candidate biomarkers.

Time profiles during radiation therapy differ when comparing histopathology, symptoms, fecal lactoferrin and vitamin E concentrations in blood. This timing might be of importance when designing future studies and planning prophylactic interventions to mitigate acute radiation proctitis.

Calprotectin and S100A12 both correlate with disease activity of RA. Calprotectin can be measured in either serum or plasma samples with the aim of assessing disease activity in RA. S100A12 was associated with RF as well as with CV disease. Calprotectin might be more useful for monitoring disease activity, while the role of S100A12 as a biomarker might be related to prognosis in RA.

S100A12 should be analyzed in serum samples, and preferably analyzed within a day, before or after freezing. Neither EDTA nor heparin plasma can be recommended for the estimation of S100A12 concentration.

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Errata

Page 10: Paper IV, second last sentence: "Submitted to The Journal of Rheumatol Sept 2006" has been changed to the correct "Submitted to Scand J Rheum Dec 2006" before printing of PhD-thesis.

Page 39: third and fourth paragraph: " ...stool samples in Paper I and II" has been changed to the correct "...stool samples in Paper I and III" before printing of PhD-thesis.