ENRICHMENT AND IDENTIFICATION OF CITRULLINATED PROTEINS IN BIOLOGICAL SAMPLES

Thesis for the degree of Philosophiae Doctor

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Last, but certainly not least, I would like to thank family and friends for their continuous support and never giving up on me, even though the answer to their invitations often was "sorry, I have to work".

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Oslo, January 2014

Astrid Elisabeth Voorham Tutturen

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ABBREVIATIONS

ACPA	anti-citrullinated protein/peptide antibody
BPG	biotin-PEG-GBA
CID	collision induced dissociation
CRB	citrulline reactive beads
DAMO	diacetylmonoxime
ESI	electrospray ionisation
ETD	electron transfer dissociation
GBA	4-glyoxalbenzoic acid
GFAP	glial fibrillary acidic protein
HCD	higher energy collision dissociation
HLA	human leukocyte antigen
HPG	4-hydroksyphenylglyoxal
HSA	human serum albumin
IgG	immunoglobulin gamma
LC	liquid chromatography
MALDI	matrix-assisted laser desorption ionization
MBP	myelin basic protein
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NET	neutrophil extracellular trap
PAD	peptidylarginine deiminase
PADI	*nomenclature for genes encoding PAD proteins
PAGE	polyacrylamide gel electrophoresis
PEG	polyethyleneglycol
PEP	posterior error probability
PTM	posttranslational modification
RA	rheumatoid arthritis
SDS	sodium dodecylsulfate
TOF	time of flight

INTRODUCTION

Citrullination – a posttranslational modification

Proteins and posttranslational modifications

Proteins are macromolecules essential to life, playing a variety of vital roles in all living organisms. They determine the form and function of cells and provide them with the ability to communicate. Proteins are composed in a process called protein synthesis (Figure 1). Genes in the DNA are transcribed into mRNA, which are transported out of the nucleus into the cytoplasm where ribosomes translate mRNA into polypeptides chains.

Figure 1: Protein synthesis. DNA is transcribed into mRNA, which subsequently is translated into proteins by ribosomes in the cytoplasm.

The natural building blocks for proteins are encoded by DNA and consist of 20 amino acids, which may be incorporated during protein synthesis to become a protein. However, after protein translation these amino acids may undergo modifications, so called posttranslational modifications (PTMs). PTMs considerably increase the diversity of proteins present in living cells. These modifications may be either permanent or transient and may result from either targeted, enzymatically catalysed reactions or spontaneous, chemical reactions in the cell [1]. PTMs occur on nearly all proteins and are crucial because they may alter physical and chemical properties, folding, distribution, stability, activity and consequently the functions of the targeted proteins. More than 200 PTMs are known and most amino acids can undergo several PTMs during the life time of a protein.

PTMs and autoimmunity

The immune system is an intricate network of organs, cells and molecules that protects our body from infectious agents and disease. To do so it is conclusive that the mechanisms driving the immune system manage to discriminate between infectious agents and self. Though, in some cases autoimmunity may occur. Autoimmunity occurs when a failure in the mechanism of immunological tolerance leads to an immune response towards antigens derived from self. During the development of the immune system in an individual, the maturing lymphocytes in the bone marrow and the thymus are strictly selected and deleted if they react too strongly to self-peptides. Peripheral self-peptides are expressed in a wide variety in the thymus and bone marrow to make this selection possible. However, in the peripheral organs proteins may undergo PTMs which may alter a protein's physical and chemical properties, including primary and tertiary structure and proteolytic degradation. It has been shown that between 50 % and 90 % of proteins in the human body may acquire PTMs [2]. Modifications occurring in the periphery may not occur in an identical manner in the thymus and therefore toleration of these modified versions of the proteins during lymphocyte maturation is not allowed [2]. Some PTMs may affect the degradation of proteins by blocking specific proteolytic cleavage sites, generating a new immunogenetic or tolerogenic repertoire of self-peptides. Consequently, self-peptides generated from proteins that have undergone modification in various cellular processes may be taken up by antigen presenting cells and presented to autoreactive B and T cells and an immune response will be initiated [2]. This loss of tolerance towards self may lead to autoimmune disorders. In the autoimmune disease rheumatoid arthritis (RA), immune response is initiated towards proteins with the PTM citrullination. The presence of anti-citrullinated protein antibodies (ACPAs) is in fact used as a diagnostic marker for this disease [3]. The strong link between citrullinated proteins and RA was the driving force behind this thesis.

Citrullination

Protein citrullination is a PTM which is associated with autoimmunity and pathophysiology, but also occurs in biological processes in healthy physiology. Citrullination, also called deimination, is a process where the imine group of the guanidinium group of a peptidylarginine residue is enzymatically substituted with an oxygen atom, to form an ureido group resulting in the amino acid citrulline (Figure 2) [4]. Citrulline has its name from the Latin word for watermelon, *Citrullus vulgaris*, which contains large amounts of this amino acid. In the context of protein synthesis citrulline is

a non-standard amino acid, meaning it is not encoded by DNA and thereby not incorporated into proteins during translation. Consequently, citrulline can only occur in proteins when arginine is posttranslationally modified. This PTM results in a mass increase of 0.984 Da and the loss of one positive charge per converted arginine residue [5]. The loss of a positive charge affects the acidity of the amino acid residue, changing the isoelectric point from 11.41 for arginine to 5.91 for citrulline [6]. Arginine is an important amino acid in the context of protein structure and function due to its structurally unique side chain. The positively charged guanidinium group of the side chain enables ionic and hydrogen bond formation, with both proteins and nucleic acids. Consequently, the loss of charge also influences the proteins ability to participate in bonding networks [7,8]. This may alter the tertiary structure of the protein and has a pronounced effect on intra- and intermolecular interactions. Modifications of arginine in general have emerged as important PTMs, that impact multiple cellular processes [9]. These include methylation, phosphorylation, citrullination, ADP-ribosylation, carbonylation and glycation (reviewed in [9]).

Our focus is on protein citrullination, which has been known to be present in proteins since 1939 [10], but the enzymes responsible for this modification, the peptidylarginine deiminase (PAD) family, was first described in 1977 [11].
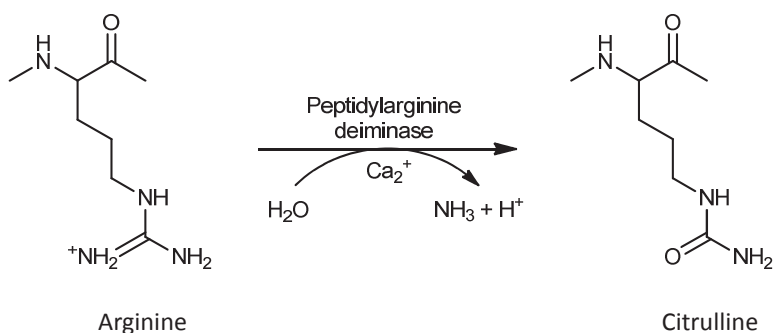
Figure 2: Citrullination. Peptidylarginine is converted into peptidylcitrulline by the calcium dependent enzyme family peptidylarginine deiminases in a posttranslational process.

Peptidylarginine deiminase

Protein citrullination is catalysed by the calcium dependent enzymes of the peptidylarginine deiminase (PAD) family (EC 3.5.3.15) [11,12]. In mammals, the PAD family consists of five isoforms, PAD 1-4 and 6, which share a highly conserved sequence with 59-71 % sequence identity [13].

Although PAD activity is regulated by pH, with an optimum of pH 7.6, the strongest known regulator of PAD activity is calcium concentration. A concentration of 40-70 µM Ca^{2+} is required to obtain half of maximum PAD activity, whereas the lower limit for a minimum PAD activity is 10 µM, according to kinetic data from Kearny *et al.* [14]. Calcium concentration in the cytosol and the nucleus is, however, much lower and fluctuates around 200 nM in resting cells and 1 µM in activated cells [15-17]. Extracellularly the calcium concentration is about 1.2 mM, but the plasma membrane is virtually impermeable for calcium [18]. However, by influx of calcium through plasma membrane channels or from the endoplasmatic reticulum or mitochondria, elevated calcium concentrations can occur locally in the cells (reviewed in [18]). Nevertheless, how PAD is activated *in vivo* is still not fully understood.

The PAD enzymes are naturally widely distributed in many different cell and tissue types. They participate in physiological processes, however, PAD activity is also associated with pathology [12,19,20]. Some of the natural substrates of PAD and possible disease associations are listed in Table 1. All the PAD isoforms are found in the cytoplasm of the cell, with PAD4 as an exception, which carries a nuclear localisation signal, and is thus the only PAD isoform that can be transferred into the nucleus, where it is known to deiminate histones [19]. Recently it has been suggested that also PAD2 might play a role in histone deimination, but this needs more research [21]. PAD activity was also detected in autophagosomes in dendritic cells, macrophages and B cells, which resulted in the presentation of citrullinated self-peptides to T cells [22]. The PAD enzymes may also autocitrullinate, and autocitrullination of two arginine residues close to the active site, R372 and R374, have been shown to inactivate PAD4 [23]. However, the physiological meaning of autocitrullination is still unclear.

In the bacteria *Porphyromonas gingivalis*, which is the causative agent of periodontitis, a prokaryotic PAD enzyme that can citrullinate proteins has been identified [24]. This enzyme (AAF06719) is not calcium-dependent and can convert both peptidylarginine and free L-arginine into citrulline. It is not evolutionary related to vertebrate PAD enzymes, but shares sequence homology with several arginine deiminases [25]. Interestingly, bacterial PAD activity in periodontitis has been linked to RA [26].

The PAD enzymes have caught increasing interest during the recent years since PAD overexpression and up regulated enzyme activity have been observed in several diseases, of which some will be described in a following section.

Table 1: PAD isoforms in mammals, their natural substrate and disease association. Modified from [12].

PAD isoform	Protein distribution	Natural substrate	Biological functions	Diseases
PAD1	Epidermis, uterus	Keratin K1, Filaggrin	Cornification of epidermal tissues	Psoriasis [27]
PAD2	Broadly expressed: skeletal muscle, brain, spleen, secretory glands, spinal cord, pituitary gland	Myelin basic protein, vimentin, histones H3 and H4, β- and γ-actins	Plasticity of the central nervous system, transcription regulation, innate immune defence, female reproduction	Multiple sclerosis [28], RA [29], Alzheimers disease [30], Creutzfeldt-Jackob disease [31]
PAD3	Epidermis, hair follicles	Trichohyalin, fillaggrin	Regulation of epidermal functions	Unknown
PAD4	White blood cells (granulocytes and monocytes)	Histones H2A, H3 and H4, ING4, p300/CBP, nucleophosmin, nuclear laminine C	Chromatin decondensation, transcription regulation, tumorigenesis, innate immunity, NET formation	RA [32], multiple sclerosis [33], adenoma-carcinomas [34,35]
PAD6	Egg, ovary, early embryo	Keratin	Oocyte cytoskeletal sheet formation and female fertility, early embryo development (mouse)	Unknown

Citrullination in normal physiology

Citrullination represents only a small change in a protein's chemical structure, but still it has a pronounced impact on the targeted protein. Therefore it has a role to play in several cellular events in normal physiology, of which some are mentioned below.

Cell death - necrosis and apoptosis

Citrullination in normal physiology is best studied in the context of cell death; apoptosis and necrosis. During cell death the cell membrane becomes leaky, allowing an unlimited influx of calcium ions leading to activation and a higher transcription of PADs [36]. During this process several cytoskeleton proteins undergo citrullination disrupting their three dimensional structure. One of the first proteins to become citrullinated is vimentin, which is important for the structural integrity of the cell. Deimination of vimentin impairs its function and ability to form intermediate filaments and finally changes the phenotype of the dying cell dramatically [37]. Other intracellular proteins becoming rapidly citrullinated during cell death are histones, which are basic proteins essential for chromatin structure. The loss of positive charges caused by citrullination results in a change of charge state in the nucleosomes, impairing the interaction with the negatively charged DNA backbone, which is highly dependent on positively charged histones. Therefore, the reduction of net charge, together with conformational changes, causes the nucleosomes to open up, making DNA more susceptible for degradation [38].

Citrullination in gene regulation

Protein deimination of histones may also play a role in regulating various chromatin functions, including gene transcription, repair of DNA damage and condensation/decondensation [39]. In fact, histones may undergo several PTMs, and the repertoire of PTMs occurring on histones is called the "histone code". The function of deimination as part of the "histone code" is to alter the interaction between histones and DNA backbone by neutralising the positive charges. Further, PAD4 may function as a histone H3 specific arginine deiminase, which has the potential to repress transcription by blocking methylation by methyltransferase [40]. Arginine methylation coincide with activation of transcription, but when arginine is citrullinated methylation cannot take place [40]. In addition, PAD4 has been shown to demethylate mono-methylated arginine and convert the arginine residue into citrulline, which also represses transcription [40].

Cytoskeletal proteins

Cytoskeleton proteins are known to be targeted for citrullination during epithelial terminal differentiation. Proteins important in this process are (pro)filaggrin and the keratins K1 and K10 [5]. Citrullination allows binding between these proteins due to reduction of the isoelectric point [27]. Further, citrullination enables the protease calpain to cleave profilaggrin into filaggrin units, which bundles keratin filaments into an organised matrix [12,41].

Central nervous system

In the central nervous system myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) are deiminated by PAD2, which is expressed by asterocytes, oligodendrocytes and microglial cells [5]. Citrullination of MBP is thought to be essential for the plasticity of the brain at young age since the ratio between citrullinated MBP and total MBP decreases rapidly after postnatal life [42]. The role of physiological citrullination of GFAP is unclear [5].

Immune functions

During the last decade it has been shown that citrullination of the inflammatory chemokine CXCL8 decreases its signal potency by reducing its binding properties and prevent it from proteolytic cleavage into a more potent fragment [43]. In addition, citrullination of CXCL8 abrogated the chemokine's ability to recruit neutrophils into the peritoneal cavity and dampened neutrophil extravasation during acute or chronic inflammation [43]. On the other hand, citrullination of CXCL8 significantly increased this chemokine's ability to mobilize neutrophils into the blood circulation [44]. Citrullination has also been shown in other chemokines such as CXCL12, which was gradually weakened as leukocyte chemoattractant and HIV inhibitor depending on the degree of citrullination [45], and CXCL10 and CXCL11, which also reduced their chemoattractive and signalling capacity upon citrullination [46]. These results indicate that through citrullination of chemokines PADs can directly modulate immune reactions.

Further, hypercitrullination of histones mediates decondensation of chromatin and facilitates the formation of neutrophil extracellular traps (NETs) [47]. NET formation is a feature of the innate immune response towards bacterial infection and inflammation, where decondensed chromatin nets are used to trap pathogens.

Citrullination in disease

The presence of citrullinated proteins was initially considered specific for the synovium of patients with RA [48]. However, later studies reported the presence of citrullinated proteins in non-RA tissues [49,50]. In fact, citrullinated proteins were found to be present in a wide range of inflamed tissues and citrullination is now known to occur in almost all sites of inflammation; being an inflammation dependent process rather than disease specific [51]. Nevertheless, citrullination does play a role in several diseases (reviewed in [5,52]), of which some are mentioned below. Elucidation of citrullination patterns in these diseases may reveal knowledge of the disease mechanisms and could potentially lead us closer to possible treatments.

Cancer

In recent years, citrullination in tumourigenesis has attracted an increased attention. Researchers have found a pronounced higher expression of PADI4 genes in various malignant tumours, but not in benign tumours or surrounding healthy tissues [34]. Elevated serum levels of PAD4 and citrullinated antithrombin were also observed in patients with malignant tumours [35]. Further, Li *et al* found that PAD4 was involved in repressing the expression of a subset of tumour suppressor p53 target genes [53]. And recently, expression of the PADI2 genes in human mammary gland epithelial cells and MCD-7 breast cancer cells was detected [54].The high expression of PADI genes in cancer cells indicates that citrullination may promote tumourigenesis.

Multiple sclerosis

Multiple sclerosis is a chronic disorder of the central nervous system characterised by local T-cell and macrophage infiltration, leading to demyelination and loss of neurological function [55]. Hypercitrullination of MBP, resulting in an increased overall ratio of citrullinated MBP versus total MBP and in the number of citrullines within the citrullinated MBP molecules, has been described in multiple sclerosis [12,42,56]. Citrullination of MBP results in lower affinity to negatively charged myelin phosphatidylserine residues, as overall positive charge of MBP is reduced, making MBP much more prone to degradation by cathepsin D [57]. GFAP, which is an asterocyte-specific intermediate filament, has also been shown to undergo deimination and

increased amounts of citrullinated GFAP are present in the brain of patients with multiple sclerosis [58]. Therefore citrullination is thought to play an important role in this disease.

Psoriasis

The autoimmune disease psoriasis is characterised by an increased mitotic activity in the epidermis and this rapid proliferation results in an abnormal cornification [5]. A decreased level of citrullination of keratin 1 (CK1) has been shown in the psoriatic epidermis [27], indicating the role of citrullination in this disease.

Rheumatoid arthritis

Protein citrullination in the context of disease is, however, most often linked to the autoimmune disease RA. RA is affecting around 0.5-1 % of the world's population [59], and as for many other autoimmune diseases, women are more likely to develop RA than men, with a female:male ratio of 3:1 [60]. Although RA can occur at any age, the typical age for disease onset is between 30 and 50 years. The disease course is characterised by chronic inflammation of the synovial lining in diarthrodial joints causing bone erosion and destruction and may lead to severe disabilities [61,62].

RA is further characterised by the presence of *anti-citrullinated protein antibodies* (ACPAs) [63] in 62 % of early arthritis patients and 75 % of established arthritis patients, with a specificity of 95-99 % [64]. ACPA-positive status is strongly associated with the development of more erosive disease course [65]. ACPA is a collective term including several antibodies with distinct citrullinated antigen-binding specificity, although a minority of these may show cross-reactivity with multiple citrullinated epitopes [66,67]. A continuously growing number of citrullinated proteins present in RA synovium have been shown to be autoantigenic. These includes citrullinated forms of fibrin [68], vimentin [69], collagen type II [70], α-enolase [71], which all are well-established autoantigens. Recently citrullinated forms of fibronectin [72], and myeloid nuclear differentiation antigen, apolipoprotein E and actin [73] were found to be autoantigenic. While citrullination is occurring in most inflammatory sites, the immune response towards citrullinated proteins seems to be restricted to RA [74].
RA is a complex disease, strongly associated with both genetic and environmental risk factors, equally contributing to the disease [75,76]. The main genetic contributions are certain human leukocyte antigen-DRB1 (HLA-DRB1) allelic variants; amongst these are

DRB1*0101 (DR1), DRB1*0401 (DR4) and DRB1*0404 (DR4) [77]. These DRB1 alleles encode similar amino acid sequences in the hypervariable region of the HLA class II DRβ chain, especially in position 70 through 74, called the "shared epitope" [78]. The shared epitope amino acids contribute to the peptide anchoring pocket P4 and makes it positively charged [79]. Hill et al. demonstrated that the conversion of positively charged arginine to neutral citrulline in a vimentin derived peptide, dramatically increased the affinity for this peptide to DR4 [79], which was later confirmed by Snir et al. [80]. Other citrullinated epitopes have also showed the same increase in affinity and thereby association with ACPA-positive RA and the shared epitope alleles [81-83]. However, for a number of fibrin derived peptides this could not be reproduced [84]. Moreover, recently, Scally et al. presented high resolution crystal structures of HLA-DR4 in complex with citrullinated aggrecan and vimentin epitopes [85]. They showed that the binding of citrulline within the electropositive P4 pocket of HLA-DRB1*0401/04 was conserved. However, van Steendam et al. found that citrulline-dependent T-cell response is not restricted to RA, as they could not detect any differences in number of citrulline reactive T-cells when comparing peripheral blood monocytes from healthy individuals, spondylarthritis ancylopoetica and RA patients, indicating that citrullination as such gives rise to a universal break in tolerance [86]. Consequently, T-cell response in RA has to be further investigated.

The main environmental risk factor for developing RA is tobacco smoking [76,87,88]. In fact, the combination of smoking and the presence of double copies of the shared epitope genes increase the relative risk for developing RA more than 20 times, compared to a non-smoker without the shared epitope genes [87]. Other potential environmental risk factors include silica dust [89,90], mineral oil [91] and other airway exposures [92].

The trigger or causative agent of RA is, however, not known, but increased citrullination in the lungs as a result of tobacco smoking [93,94], or increased citrullination in the oral cavity due to periodontitis and activity of bacterial PAD from P.gingivalis [26,95], have been hypothesised to initiate RA in genetically susceptible individuals.

Interestingly, ACPA-negative RA seems to be a complete different subset of the RA, as both genetic and environmental risk factors differs from the ones associated with ACPA-positive RA [96].

Methods for the detection of citrullination

As citrullination only increases the mass of the targeted protein by 1 Da per converted residue this PTM is challenging to investigate. Historically direct MS-based identification of citrullinated proteins present in a biological sample has not been possible due to limitations in resolution and sensitivity of MS instrumentation [97]. Therefore several methods have been developed in order to allow a deeper investigation of protein citrullination, and to enlighten the role of this modification in various biological processes. Most of the proteins known to be citrullinated today have been identified by combining immunodetection after SDS-PAGE and Western blotting followed by MS analysis for identification. However, many additional methods have been developed in order to increase citrulline detection and make high throughput approaches. An overview of existing methods is given in Table 2.

Modification of the citrulline ureido group

The unique ureido group of the citrulline side chain has been the target for various chemical reactions in order to specifically detect and identify citrullination. Initially this modification was based on the highly specific reaction between the ureido group of citrulline and diacetylmonoxime (DAMO) under strong acidic conditions. However, DAMO is thought to hydrolyse to 2,3-butandione prior to reaction [98] and in more recent approaches DAMO was replaced by 2,3-butanedione. Based on the modification with DAMO, Fearon presented in 1939 a colorimetric assay for detection of citrulline where modified citrulline resulted in a pink/orange colour and could be detected by absorbance at 530 nm [10], an assay that later has been improved by several research groups [99-104]. The reaction product was later found to be highly reactive with nucleophiles and antipyrine is now normally added to the reaction mixture for DAMO/2,3-butanedione modification of citrulline, resulting in a more stable reaction product and a yellow colour (Figure 3).

The 2,3-butanedione and antipyrine modification of citrulline residues is the basis for the most commonly used method for detection of citrullinated proteins today. Senshu and co-workers generated the so-called *anti-modified citrulline antibodies* [105] and presented a method where citrulline residues in proteins were covalently modified on-blot by 2,3-butanedione and antipyrine after separation by SDS-PAGE and being transferred onto a polyvinylidenedifluoride membrane by Western blotting. The modified citrulline residues were visualised by immunodetection using polyclonal antibodies

against the modified citrulline. This allowed specific detection of citrulline residues regardless of the protein backbone and flanking amino acids. Although high specificity and sensitivity were obtained in this method, it cannot provide information about the identity of the citrullinated protein or its modification site(s). Therefore, immunodetection was combined with MS by running two parallel 2D-SDS-PAGEs; one for Western blotting, to designate spots containing citrullinated proteins, and one for conventional staining followed by MS analysis of the equivalent spot with the same electrophoretic profile [71,106,107]. This modification technique followed by immunodetection has also been used in immunohistochemistry to visualise the presence of citrullinated proteins in tissue sections [51,71,108,109].

Antibodies against unmodified citrulline have also been raised, however, these antibodies have shown to have poorer recognition rate than e.g. the anti-modified citrulline antibodies (reviewed in [110]).

Another glyoxal derivative, phenylglyoxal has also been shown to specifically modify the citrulline ureido group under acidic conditions [111,112]. Bicker and colleagues showed that protein citrullination could be visualised by treating a protein sample with a rhodamine-phenylglyoxal probe followed by separation by SDS-PAGE and fluorescent imaging analysis [113]. The sensitivity of this method was shown to be equal to the "Senshu-technique". It further allowed determination of K_m values for high affinity protein substrates. However, identification of citrullinated proteins was not obtained and the method has been found to be unsuitable for detection of citrullination in complex samples since strong background reactivity was observed [110].

Table 2: Overview of the currently available methods for the detection of citrulline residues, with their advantages and disadvantages (adapted and modified from [114])

Method	Advantages	Disadvantages
Immuno detection		
Antibodies against modified citrulline [115]	Sensitive Specific Applicable to *in vivo* samples	Time consuming No location of citrulline within protein No identification
MS analysis with modification		
2,3-butanedione + antipyrine. Detection by UV+ MALDI-MS [98]	Specific	Large sample amounts
2,3-butanedione + antipyrine. Detection through signature ion at 201.1 *m/z* after CID + ETD [111]	Specific	Large sample amounts Specialized equipment
Selective enrichment after modification with 4-hydroxyphenylglyoxal [112]	Specific Reduction of sample complexity	Large sample amounts
2,3-butanedione and LC-MS detection [116]	Specific Widely available equipment	Large sample amounts
Stable isotope labelling with H_2O^{18} [117]	Specific	Inapplicable for analysis of *in vivo* citrullination
Br signature by modification with 4-bromophenyl glyoxal [118]	Specific	Time consuming
Selective enrichment after modification with biotin-PEG$_2$-glyoxalbenzoic acid (BPG). Detection by LC-MS [119]	Specific Reduction of sample complexity Sensitive	Fragmentation of the BPG structure during MS/MS fragmentation
MS analysis without modification		
1 Da gain when compared to non-citrullinated peptide [120]	Applicable to *in vivo* samples	Non-distinguishable from deamidation of N or Q Accurate equipment Incomplete deimination Charge state distribution
No trypsin cleavage after citrulline [71,121,122]	Applicable to *in vivo* samples	Possible missed cleavage
Accurate mass and retention time [120]	Applicable to *in vivo* samples	Non-distinguishable from deamidation of N or Q Time consuming
Neutral loss of 43 Da after CID [123]	Specific Applicable to *in vivo* samples	Specialised equipment
Direct LC-MS/MS analysis [73,124] [Tutturen *et al.*, submitted]	Specific Straightforward	High resolution instrumentation
Colorimetric detection		
2,3-butanedione + antipyrine Detecting absorbance [103,104]	Specific Sensitive	No identification No location of citrulline sites
Rhodamine-phenylglyoxal modification and UV detection [113]	Specific Sensitive Determine kinetics	No identification No location of citrulline sites

Modification of citrulline followed by mass spectrometric analysis

Although several approaches have been developed based on the specific modification of the citrulline residue with 2,3-butanedione and antipyrine, the exact chemistry behind this reaction was not revealed until Holm and co-workers elucidated the reaction mechanism using nuclear magnetic resonance (NMR, Figure 3) [98]. Further, they applied these modification conditions to synthetic citrulline-containing peptides and used UV absorption at 464 nm for detecting the modification structure and determine the presence of citrulline. This was combined with analysis of the modified peptides by matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) MS. In this analysis a +238 Da mass shift of the peptide could be observed, corresponding to the expected mass shift for this modification.

As a next step, Stensland and colleagues developed a method for targeted analysis of protein citrullination using the 2,3-butanedione and antipyrine modification followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis applying alternating collision induced dissociation (CID) and electron transfer dissociation (ETD) fragmentation [111]. They discovered the presence of a modification derived signature ion at *m/z* 201.1 in the mass spectra after CID fragmentation (Figure 3-A). Furthermore, when applying ETD fragmentation the modification structure remained intact and MS/MS spectra allowed identification of the peptides and their citrullination sites. This method was demonstrated to be applicable at realistic sample amounts, but nevertheless, required specialised MS equipment which allows both CID and ETD fragmentation.

Another MS-based approach was presented by De Ceuleneer and co-workers [116]. This approach is based on the specific modification of citrulline using 2,3-butandione alone, which gives a mass increase of 50 Da per modified residue. They showed that by subjecting a whole protein digest to modification conditions followed by MS analysis of both the modified and unmodified version of the same sample, citrullinated peptides could be identified by comparing the peptide mass fingerprints. This method requires large sample amounts, but has the advantage of being applicable for a wide variety of mass spectrometers.

Further, Choi *et al.* have presented a technique based on phenylglyoxal modification [118]. They modified the citrulline residue in a peptide by the molecule 4-bromophenylglyoxal followed by MALDI-TOF MS analysis. Due to the unique isotopic distribution of Br (almost 50/50 between ^{79}Br and ^{81}Br) a Br signature was observed in the mass spectrum showing the first and the third isotopic peaks with similar heights. This signature facilitated identification of the citrullination site by studying the

fragmentation spectrum. However, for identification of citrullination in biological samples the sensitivity may be a limitation.
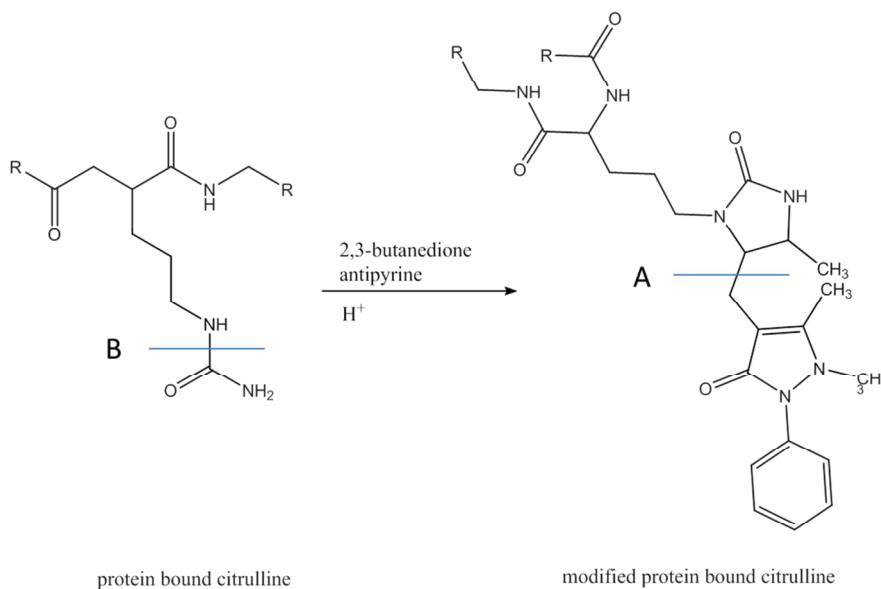
Figure 3: Specific modification of citrulline by 2,3-butanedione and antipyrine (adapted from [98,114]). (A) Indicates the fragmentation site resulting in a signature ion at m/z 201.1 during CID fragmentation [111], and (B) indicates the fragmentation site within the ureido group of the citrulline side chain, resulting in a 43 Da neutral loss representing the loss of isocyanic acid [123].

Mass spectrometric detection without modification

A specific feature of the citrulline residue itself, which happens during LC-MS/MS analysis of citrullinated peptides was discovered by Hao *et al.* [123]. They observed that in CID fragmentation the structure of the citrulline side chain was labile and resulted in a neutral loss of 43 Da. They further demonstrated that this neutral loss represented the release of an isocyanic acid moiety from the citrulline ureido group (Figure 3-B). For characterisation of citrullinated peptides present in a sample, the sample was analysed by LC-MS/MS and CID spectra were extracted for the neutral losses of 43 Da (singly charged) and 21.5 Da (doubly charged) for peptide identification.

This unique fragmentation feature was taken advantage of by Jin *et al.,* who presented a study where the neutral loss of citrulline in CID fragmentation was used as a signature ion and a trigger for a second fragmentation of that peptide, using higher energy dissociation (HCD) fragmentation [125]. HCD fragmentation does not suffer from low mass cut-off and therefore gives a higher coverage of the peptide sequence than obtained by CID fragmentation. Whereas CID fragmentation is the dominant fragmentation pathway for isocyanic acid, and neutral loss fragmentation was abundantly observed in this fragmentation mode, the abundance of neutral loss fragmentation was significantly reduced in HCD fragmentation. The authors therefore found CID triggered HCD fragmentation to be the optimal approach in their investigation of citrullination of the recombinant proteins MBP and GFAP and in brain tissue samples.

Also other differences in specific features between citrulline and arginine have been utilised in the investigation of citrullinated proteins. Hermansson *et al.* presented a method based on accurate mass and retention time employed in LC-MS/MS analysis [120]. Based on this method they propose that once the retention time and the mass of a synthetic citrullinated peptide is known, the same peptide normally occurring in *in vivo* samples may be targeted specifically for MS acquisition. The authors could detect citrullinated fibrinogen peptides extracted from RA synovial samples by determine the accurate mass and retention time from a previous run of *in vitro* citrullinated and non-citrullinated fibrinogen. However, peptides containing asparagines and glutamines had to be excluded from the analysis, because deamidation of these amino acids result in the same mass shift as deimination of arginine, namely 0.98 Da. The double analysis makes this a time consuming approach, and *in vivo* identifications are limited to peptides present in the *in vitro* sample, whereas all other potential citrullinated peptides will be ignored during acquisition.

Kubota *et al.* developed a method to determine citrullination sites using ^{18}O stable isotope labelling followed by MS analysis [117]. This method is applicable for *in vitro* studies of citrullination sites after performing PAD treatment of proteins in 50 % $H_2^{18}O$, which will result in a characteristic isotope pattern in the mass spectra of citrullinated peptides.

During the last decades MS technology has improved dramatically, offering high resolution and high speed data acquisition. The increased sensitivity and mass accuracy obtained by these new instruments have been shown, by a few studies, to enable identification of citrullinated proteins by direct MS analysis. Some research groups have applied direct MS analysis in their attempt to elucidate citrullination patterns in RA synovial fluid. Van Beers and colleagues separated synovial fluid by SDS-PAGE and

identified citrullinated proteins by direct MS analysis after in-gel digestion [73]. In the third publication presented in this thesis, direct MS analysis was performed of RA synovial fluid after a simple fractionation step performing depletion of immunoglobulin gamma (IgG) and human serum albumin (HSA), in order to reach citrullinated proteins of low abundance (Tutturen *et al.*, 2014, submitted). However, also direct MS analysis of total, unfractionated, in-solution digested synovial fluid, has been published [124]. All three strategies resulted in the identification of numerous citrullinated proteins, but due to individual variations in degree of citrullination between patients, as shown by Van Beers [73], and differences in MS instrumentation applied, the strategies cannot be compared based on number of identifications.

Enrichment of citrullinated peptides followed by MS analysis

So far, only two strategies have been developed for specific enrichment of citrullinated peptides from complex mixtures, which both are based on specific modification of the citrulline ureido group. Both strategies are presented in publications included in this thesis.

The first method represents a bead-based approach in which beads were functionalised with the citrulline reactive glyoxal derivative 4-hydroxyphenylglyoxal via a base labile linker, generating "citrulline reactive beads" (CRBs) [112]. During acidic incubation of a peptide mixture with CRBs, citrulline residues are covalently modified and thereby immobilized onto the beads, allowing unbound non-citrullinated peptides to be washed away. Citrullinated peptides are subsequently released by applying basic conditions in the presence of guanidinium followed by identification by MS analysis. Although the specificity is remarkable the sensitivity is still a limitation of that approach.

The second enrichment strategy has both high specificity and sensitivity and represents an in-solution-based modification approach in which citrulline residues in a complex mixture are specifically biotinylated using the molecule biotin-PEG$_2$-4-glyoxalbenzoic acid (BPG) [119]. Thereby their selective enrichment is rendered possible by incubation with streptavidin beads. After extensive washing of the streptavidin beads in order to remove non-citrullinated peptides, citrullinated peptides are eluted from the beads by excess of free biotin and analysed by MS.

Quantitative determination of degree of citrullination

Although many methods have been developed for the detection and identification of citrullinated proteins only a few of these can acquire information about the exact citrullination site and allow quantitation of degree of citrullination. Degree of citrullination is the percentage of a given arginine residue residing in its citrullinated state.

MS-based quantitation of degree of citrullination is challenging, as citrullination leads to a mass increase of only 1 Da. For a partially citrullinated peptide the native and citrullinated isotopic distribution patterns will overlap, complicating the calculation. However, based on a method for calculation of deamidation described by Robison *et al.* [126] Stensland *et al.* determined degree of citrullination of PAD4-treated synthetic citrulline-containing peptides based on isotopic distribution patterns obtained by MALDI-TOF MS analysis [127]. Calculation was performed by subtracting the centroid mass of the native peptide from the centroid mass of the PAD4-treated peptide, resulting in a centroid mass corresponding to the average degree of citrullination. Further, De Ceuleneer *et al.* based their analysis on the isotope mixture model described by Dasari *et al.* [128] and also calculated degree of citrullination of peptides based on their skewed isotopic distribution pattern [129]. The overlap of isotopic distribution patterns for partially citrullinated peptides results in a deviation from the theoretical isotopic distribution pattern for the native peptide which was the basis for calculation. They found that the skewed isotope distribution pattern of the lowest charge state of the peptide linearly corresponded to the percentage of citrullination of that peptide. Using this method they also quantified *in vivo* degree of citrullination in peptides derived from synovial fluid samples.

However, the first research group able to quantify *in vivo* citrullinated peptides was Hermansson *et al.* who used the accurate mass and retention time method described above [120]. The degree of citrullination in RA tissue was calculated as the ratio of the chromatographic peak areas of the citrullinated and non-citrullinated version of the same peptide.

In the third publication presented in this thesis individual peptide quantitation was also obtained using an area under curve approach based on calculations from the software MaxQuant. Degree of citrullination in proteins identified in RA synovial fluid was calculated by dividing the sum of intensities of peptide identifications in a particular citrullination state by the sum of intensities of all identifications of that peptide independently of modifications.

AIM OF STUDY

The aim for this thesis was to detect citrullinated peptides by mass spectrometry and to develop strategies for specific and sensitive enrichment of citrullinated peptides from complex biological samples.

SUMMARY OF PAPERS

Paper I

A technique for the specific enrichment of citrulline-containing peptides

In this study we developed a bead-based technique for the selective enrichment of citrullinated peptides from complex peptide mixtures. First we show that the ureido group of citrulline can be selectively modified by glyoxal derivatives under strong acidic conditions, resulting in a covalently modified citrulline residue. Further this glyoxal derivative was immobilized onto solid phase via a base-cleavable linker, generating the Citrulline Reactive Beads (CRB). The beads were applied for enrichment of synthetic citrulline-containing peptides from increasingly more complex samples. The method was validated by enrichment citrullinated peptides form a digest of enzymatically deiminated myelin basic protein. Results showed a remarkable specificity.

Paper II

Specific biotinylation and sensitive enrichment of citrullinated peptides

In this study we present a sensitive in-solution-based technique for the specific modification and enrichment of citrullinated peptides from complex biological samples. The technology is based on an in-house synthesised molecule, biotin-PEG-GBA (BPG), consisting on a 4-glyoxalbenzoic acid (GBA) group, which reacts specifically with the citrulline ureido group at low pH, and a biotin moiety that binds streptavidin with a very high affinity. Consequently, upon modification biotin is covalently attached to the citrullinated peptides present in a biological sample and the modified peptides are selectively enriched using streptavidin and characterised by MALDI-TOF/TOF MS. The specificity and sensitivity of this enrichment strategy were demonstrated by enrichment of synthetic citrulline-containing peptides or enzymatically deiminated peptides spiked into a heterogeneous biological digest.

Paper III

Assessing the citrullinome in rheumatoid arthritis synovial fluid with and without enrichment of citrullinated peptides

In this study we assess the citrullinome of RA synovial fluid by direct LC-MS/MS analysis and by the use of the enrichment strategy presented in Paper II. Synovial fluid was depleted for abundant proteins and non-depleted and depleted fractions were analysed. From direct MS analysis, frequencies of citrullinated peptides together with the degree of citrullination in individual sites, in addition to a novel *in vivio* autocitrullination site in PAD4, were determined. Further, by the use of specific enrichment the number of citrullinated peptides, estimated by detecting a modification specific signature ion, was more than thirteen times higher than the number of citrullinated peptides identified by direct MS analysis. This result strongly indicates that only a fraction of the citrullinated peptides present in the synovial fluid was identified by direct MS analysis and that the use of specific enrichment tools most likely will provide greater insight into the synovial fluid citrullinome.

METHODOLOGICAL CONSIDERATIONS

Proteases used for sample preparation

Trypsin is by far the most used protease applied for proteolytic digestion of proteins prior to MS analysis. Trypsin cleaves C-terminally to arginine and lysine residues with high specificity [130]. However, citrullination of arginine is expected to block tryptic cleavage at this site because the positive charge needed for enzyme catalysed hydrolysis is removed [71,121,122]. Missed cleavages will therefore be more often observed for samples containing citrulline residues than for those which don't. In some studies this difference is taken advantage of because it facilitates comparison of chromatograms and enables identification of citrullination sites. Nevertheless, some studies have reported C-terminal citrulline in tryptic peptides [23,131], therefore these comparisons must be carried out very carefully. To avoid this we have used the proteolytic enzyme endoproteinase LysC (or lysylendopeptidase) in the three publications presented in this thesis. As this enzyme only cleaves after lysine residues, cleavage occurs independently of the presence of arginine or citrulline. The disadvantage of using endoproteinase LysC is that the generated peptide fragments become longer, with an average size of 20.5 amino acids [132].

Enrichment of citrullinated peptides from complex samples

In proteomic-based studies of PTMs reduction of sample complexity has been a requirement. Therefore several enrichment techniques have been developed for a variety of PTMs, which extensively have been applied for the elucidation of their patterns in biological processes. However, for citrullination no enrichment technology was available until the first article presented in this thesis was published.

Bead-based enrichment

In Paper I we generated "citrulline reactive beads" for the specific enrichment of citrullinated peptides from complex peptide mixtures. Beads were functionalised with 4-hydroxyphenylglyoxal (HPG) via a base-labile linker. Incubation of the citrulline reactive beads with a peptide mixture at low pH allowed selective immobilisation of citrullinated

peptides, whereas non-citrullinated peptides were easily washed away. Washing had to be carried out at acidic conditions to avoid a neutral or slightly basic environment, which would allow arginine modification to occur leading to immobilisation of non-citrulline-containing peptides [133]. Further, the presence of guanidinium as a scavenger during basic cleavage of immobilised citrullinated peptides was required to avoid carboxymethyl-HPG moieties to modify arginine residues present in the released citrulline-containing peptides.

Even though this enrichment technique showed a remarkable specificity its sensitivity was limited. The immobilisation of citrulline reactive glyoxal groups onto relatively big beads could reduce the chance for citrulline residues present in peptides to encounter the glyoxal moieties, and thereby result in lower reaction efficiency compared to an in-solution-based approach. Further, the beads applied (PL-DMA Resins) were porous and peptides could be trapped inside the beads. Tracking of citrullinated peptides, e.g. by using radioactive labelled peptides would clarify this. The PL-DMA Resin was chosen due to its stability during the harsh conditions required for modification (50 % trifluoroacetic acid and 45 °C). However, ideally, small, non-porous beads, with resistance to the required modification conditions, and preferably magnetic, should be used. The use of such beads would most probably significantly increase the sensitivity.

Synthesis of GBA and BPG

In Paper II we synthesised a citrulline reactive molecule, 4-glyoxalbenzoic acid (GBA), by selenium dioxide mediated oxidation of 4-acetyllbenzoic acid [134]. The purity of the product was evaluated by thin layer chromatography (TLC), where 4-acetylbenzoic acid and GBA ascend the TLC plate at different rates. UV light was used for detection of aromatic compounds and results showed only one spot when analysing the product. However, the obtained yield was rather low, approximately 15 %, indicating that the synthesis procedure could be optimised. On the other hand, since we obtained a product free from 4-acetylbenzoic acid and the reagents were of low cost, optimising the synthesis yield was not prioritised.

Further, GBA was coupled to the commercially available molecule amine-PEG$_2$-biotin, generating the citrulline reactive and biotin labelled molecule, biotin-PEG-GBA (BPG). After synthesis, BPG was purified using reversed phase chromatography. The eluates after purification were pooled and divided into aliquots. The identity of BPG was confirmed by MS and by functional tests where synthetic citrulline-containing peptides

were modified and analysed by MALDI-TOF/TOF MS. The yield of the purified product, however, was not determined.

Mass spectrometry in the investigation of citrullination

In the last decades mass spectrometric instrumentation has presented enormous advances in terms of sensitivity and resolution and thereby increasing mass accuracy and identification rate. Studies on protein characterisation, including identification of modification sites for PTMs, have greatly benefitted from such improvements and MS is extensively used in this field.

MALDI-TOF MS in the analysis of carboxymethyl-HPG and BPG-modified peptides

The use of MALDI-TOF MS enables fast and accurate analysis of simple peptide mixtures. In Paper I and Paper II MALDI-TOF MS was used to confirm correct modification of citrulline residues by analysing native and modified peptides in TOF mode. The modification site was pin-pointed by generating a fragmentation spectrum of the modified peptide in LIFT mode (TOF/TOF) followed by interpretation of the acquired spectrum. Further, MALDI-TOF MS was employed in the optimisation of the specificity of the enrichment strategies. E.g. for the enrichment of a known synthetic citrulline-containing peptide spiked into a complex mixture, the enriched sample was also evaluated for the presence of other, non-citrullinated peptides derived from unspecific binding. Sensitivity was also addressed using MS analysis by detecting the enrichment of a citrulline-containing peptide spiked into a complex sample in decreasing amounts. When validating the methods enriched peptides from PAD-treated MBP were analysed by recording TOF and TOF/TOF spectra. In addition to manual interpretation TOF/TOF spectra were subjected to database search using the Mascot search engine in order to identify enriched peptides.

In MALDI, however, basic and aromatic peptides seem to be favoured [135], and especially arginine-containing peptides are known to ionise better than non-arginine containing peptides [136,137]. Stensland *et al.* also showed that citrullination of the basic and easily protonated arginine residue significantly reduce the response factor, or ionisation properties, of the citrullinated product when using MALDI [127]. In the development of the enrichment strategies we compared arginine-containing peptides

with modified citrulline-containing peptides, which we would expect to have the same difference in response factor as arginine and citrulline. However, the synthetic citrulline-containing peptides applied also contained an arginine residue, and therefore the difference in ionisation properties was not an issue in the analysis of these. For the enriched PAD-treaded MBP peptides, on the other hand, a difference in response factor could have contributed to low signal intensities of the enriched non-arginine-containing peptides.

LC-MS/MS in the analysis of citrullination

For mass spectrometric analysis of complex biological samples LC-MS/MS is the method of choice. Peptides are separated by reversed phase chromatography by high performance liquid chromatography (HPLC) before entering the mass spectrometer via an electrospray ionisation (ESI) source.

The MS instrument used in Paper III, a hybrid quadrupole-orbitrap mass spectrometer (QExactive) with HCD fragmentation mode, provides high resolution, which is a requirement for direct analysis of citrullination in complex samples. Only high resolution instruments obtain the mass accuracy needed to distinguish between the citrullinated and non-citrullinated version of a peptide.

When analysing samples of high complexity, like synovial fluid, a long LC-gradient is needed to obtain good separation of peptides resulting in high identification rates. As part of Paper III we investigated synovial fluid by direct LC-MS/MS analysis and used a 260 minutes multi stepped gradient optimised for the sample. Further, a long gradient also allows separation of citrullinated peptides from their non-citrullinated counterparts due to the difference in their hydrophobicity. While MALDI favours arginine-containing peptides, ESI is less affected by the presence of this amino acid in a peptide [135-137].

Identification of citrullinated proteins in synovial fluid

In Paper III we present a study where synovial fluid from an ACPA-positive RA patient was either directly analysed by LC-MS/MS or after enrichment of citrullinated peptides, as described in Paper II. A simple sample preparation, including stepwise depletion of IgG and HSA, was performed and obtained synovial fluid fractions were analysed by a high resolution hybrid quadrupole-orbitrap mass spectrometer (QExactive).

From the direct analysis of all synovial fluid fractions, 2595 citrullinated peptides were identified with redundancy, from which 268 were unique identifications. In total 139 proteins were identified to harbour together 298 citrullination sites. The high number of citrullinated proteins identified shows a depth of analysis, which has only been shown by one other, recently published study, where also direct MS analysis was applied [124].

The synovial fluid fractions analysed by the direct approach (except from the *Pellet*) were also analysed using the BPG-enrichment strategy, followed by LC-MS/MS analysis. The obtained raw files were analysed in a similar way as the directly analysed samples; by using the MaxQuant software [138] and the Andromeda search engine [139]. The only difference was the addition of the variable modification Arg->BPGmodCit to the search parameters. This resulted in 109 identifications of BPG-modified citrullinated peptides with redundancy, of which only 24 were unique peptides. The raw files obtained for the enriched samples were initially searched using the search engine Mascot (from Matrix Science), which is the search engine most widely used in proteomics. This search only identified five BPG-modified peptides, of which four were unique identifications. The two search engines Mascot and Andromeda both use a probabilistic scoring model, but different search algorithms. Andromeda is optimised for orbitrap data, and is based on two levels of scoring; peptide identification scoring and posterior error probability (PEP) scoring. Mascot only uses the peptide identification score, which is solely based on MS/MS quality. The PEP score gives the probability of a false hit based on the peptide identification score and the number of theoretical peptide candidates in the database. It has been shown that the use of Andromeda gives an improved identification of heavily modified peptides compared to Mascot [139]. The BPG modification itself implements a big, bulky structure to the peptide with a mass increase of 516.2 Da per modified citrulline residue, which may be the reason for why Andromeda seems to be better suited for characterisation of BPG-modified peptides. As a consequence of these results we only used MaxQuant and Andromeda for data analysis. Nevertheless, when compared to the results obtained in the direct MS analysis of these synovial fluid fractions, the identification rate after BPG enrichment was disappointingly poor.

Therefore, we wanted to demonstrate the specificity of the enrichment technique, when applied to a sample with such high complexity as synovial fluid, by a different strategy. From earlier studies, using both MALDI and ESI, the PEG-spacer within the BPG molecule was known to easily fragment, resulting in the generation of a specific signature ion at *m/z* 270.1 (Figure 4). This signature ion was observed in every MS/MS spectrum recorded of BPG-modified peptides and was therefore taken as an indicator

for the fragmentation of a BPG-modified citrullinated peptide. MS/MS spectra acquired from the non-depleted and the IgG and HSA depleted synovial fluid showed that 95 % of these contained the signature ion. In contrast, m/z 270.1 was only found in less than 1 % of the MS/MS spectra acquired for same fractions analysed by the direct approach, demonstrating that the occurrence of ions with isobaric masses to the signature ion in non-BPG-modified samples are rare. This confirms the remarkable specificity of the enrichment strategy demonstrated in Paper II.

Further, the numbers of unique citrullinated peptides enriched from non-depleted and IgG and HSA depleted synovial fluid fractions were estimated by counting unique monoisotopic precursor masses selected for MS/MS. The numbers were estimated to be approximately 3600 and 2100, respectively. By direct MS analysis of the same fractions the numbers of unique citrullinated peptides identified were 119 and 157, respectively; more than thirteen times lower. This indicates that despite highly increased performance of MS instrumentation there is still a substantial portion of the synovial fluid citrullinome that is currently unreachable for direct MS identification. The use of specific enrichment tools would most likely provide a greater insight into the synovial fluid citrullinome.

Nevertheless, there are obviously still challenges that need to be overcome in the LC-MS/MS analysis of BPG-modified peptides. A closer investigation of the MS/MS spectra acquired of BPG-enriched peptides revealed that only 1.4 % of almost 155 000 MS/MS spectra were of a quality high enough to result in peptide identification. Fragmentation of the BPG-structure during HCD fragmentation resulted in several BPG-derived ions, in addition to the m/z 270.1, which could be observed in the majority of the MS/MS spectra; m/z 227.1, corresponding to the mass of a biotinyl fragment (Figure 4), m/z 286.1, corresponding to a fragmentation site in the PEG-spacer (Figure 4), m/z 246.1 and m/z 332.1, with unknown origin and m/z 517.1 and m/z 535.2, which represents the BPG molecule itself, with the loss of one water molecule for m/z 517.1. The extensive BPG fragmentation seemed to reduce fragmentation yield of the peptide backbone resulting in poor sequence coverage of the analysed peptide and a low quality of MS/MS spectra. To reduce the interference of the numerous BPG-derived fragment ions during data analysis, tailor made bioinformatic tools, subtracting these m/z values from the mass list before submitted to the search algorithms, could be implemented. This could potentially increase the identification rate of BPG-modified peptides to some extent. However, the major reason for low identification rate is poor MS/MS quality due to reduced fragmentation of peptide backbone. Therefore, the optimal solution would presumably be to introduce a cleavage site into the modification structure, e.g. a base-labile site cleaved under basic conditions or an S-S linker cleaved under reducing

conditions. Hereby enriched peptides could be directly cleaved off the streptavidin beads without elution, resulting in a significantly smaller modification structure left on the citrulline side chain and more informative MS/MS spectra could be acquired for enriched peptides leading to their identification.
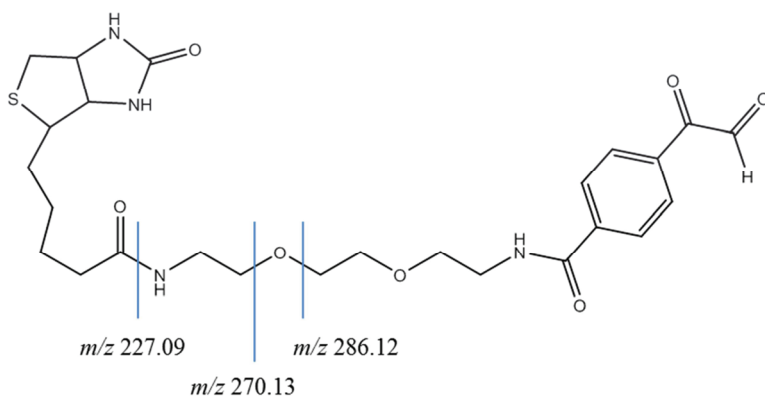

Figure 4: Proposed fragmentation sites of the BPG molecule in HCD fragmentation indicated as blue lines.

GENERAL DISCUSSION

Citrullination in rheumatoid arthritis

In recent years, citrullination has been increasingly recognised for its involvement in both health and disease. In important physiological processes, such as apoptosis and gene regulation, citrullination has been shown to play a role. However, the major focus in the context of citrullination has been its role in the pathophysiology of several diseases, and especially in RA. In RA, citrullination seems to play a key role, as pathology includes both humoral and cellular immune response towards citrullinated proteins. ACPAs are found in 75 % of patients with established arthritis [64] and can be detected up to ten years before disease onset [140,141]. However, ACPAs only appear in patients carrying certain susceptibility genes, with the MHC class II HLA-DRB1 shared epitope alleles [78] as the most important genetic risk factor [142,143]. How the MHC class II influences B-cell activation and ACPA production is still not fully understood, however, interaction between antigen presenting cells and T cells is required [144].

Despite the value of ACPAs as a predictive diagnostic marker, there is still limited knowledge about the ACPA antigens *in vivo*. The importance of citrullination in RA and the need for a comprehensive characterisation of citrullinated proteins in the RA synovium was what motivated us to start this project.

Enrichment strategies in the analysis of PTMs

A wide range of techniques have been developed in order to study PTMs, and several of these are based on specific enrichment of proteins or peptides carrying the PTM of interest. Although MS technology has improved dramatically in recent years, the significance of increasing the relative abundance of PTM-bearing proteins by applying enrichment procedures, was shown to be evident when comparing articles published from 2010 to 2012 presenting PTM identification and characterisation by MS [145].

Strategies to reduce sample complexity in order to increase the number of identified PTMs are often based on affinity purification or chemical labelling techniques. Antibodies specific towards a broad spectrum of PTMs can easily be raised, facilitating

approaches based on immunoaffinity and immunoprecipitation. These approaches are widely used for several types of modifications, including acetylation, methylation, protein acylation and protein ubiquitinylation. Further, several affinity chromatography based techniques have been developed, where proteins or peptides with affinity to a given affinity matrix or ligand are retained, whereas those showing no affinity are washed away. Subsequently, the retained proteins or peptides are eluted using either a competitive ligand or by changing operational parameters, such as pH or ionic strength. Such a technique is combined fractional diagonal chromatography (COFRADIC), which was specifically developed for isolation and characterisation of methionine-containing peptides [146], but can also be used to study protein N-acetylation [147]. Other techniques based on affinity chromatography are immobilized metal ion affinity chromatography (IMAC) [148] and titanium dioxide (TiO$_2$) [149] (reviewed in [150]), which are the most commonly used enrichment strategies for phosphoanalysis. Immobilisation strategies have also been developed. E.g. for enrichment of N-glycoproteins hydrazide chemistry is mostly used, where carbohydrates in the glycoproteins are oxidised allowing their subsequent immobilisation onto hydrazide-activated beads [151].

Enrichment strategies for citrullination, however, were not available at the time this project was initiated. For enrichment of citrullinated proteins or peptides immunoaffinity purification has been difficult because the molecular change resulting from the conversion of arginine to citrulline, where a nitrogen and a hydrogen atom are being substituted by an oxygen atom, is very small. It is therefore challenging to obtain highly specific antibodies that discriminate between arginine and citrulline within its epitope. Yet, a few antibodies against unmodified citrulline have been raised (reviewed in [110]). The most known is an IgM monoclonal antibody designated as F95 [152]. This antibody was raised against a decacitrullinyl peptide and was further selected for its reactivity against citrullinated MBP. It has been used for immunohistochemistry and staining on Western blot, but, to the authors knowledge, not for enrichment.

Due to difficulties in raising specific antibodies against citrulline itself, modification based techniques would be more suited for the enrichment of this small PTM. Historically, approaches based on chemical labelling of the citrulline residue have shown remarkable specificity, as described more detailed in the chapter Introduction. Modification with DAMO/2,3-butandione and/or antipyrine have been extensively used for citrulline modification, followed by either on-blot immunostaining with modification specific antibodies raised in rabbits [115] or MS analysis [98,111,116]. However, neither of these two variants of the modification allowed enrichment. Therefore, we tested different

glyoxal derivatives, which contained a second functional group in addition to the citrulline reactive dicarbonyl group [112]. We found that phenylglyoxal derivatives modified the ureido citrulline group with the same remarkable specificity as 2,3-butanedione, however, the modification reaction needed more harsh conditions, such as 50 % trifluoroacetic acid. Encouraged by promising results, we followed that way, what has resulted in two enrichment strategies. In Paper I we applied the glyoxal derivative 4-hydroxyphenylglyoxal (HPG) as citrulline reactive group [112]. The hydroxyl group of HPG was utilized for coupling the molecule onto solid phase, resulting in a bead-based approach. In Paper II we synthesised the molecule 4-carboxyphenylglyoxal; or 4-glyoxalbenzoic acid (GBA), in which the carboxyl group allowed coupling to the amine group of the biotinylated molecule amine-PEG$_2$-biotin, forming a stable amide bond and resulting in a citrulline reactive biotin-label [119]. This soluble molecule allowed in-solution modification of citrulline residues followed by streptavidin based enrichment. Both methods were shown to be highly specific for the enrichment of citrulline-containing peptides. A drawback, however, for both methods, might be the chemical aspects in preparing the CRBs and synthesis of BPG, as both protocols consist of quite many steps.

The major focus of the discussion of these two methods is of a technical nature, and has therefore been considered in the chapter Methodological Considerations.

Identification of citrullinated proteins in rheumatoid arthritis

Due to the extreme complexity and the dynamic range of protein composition in body fluids, like synovial fluids, the analysis of these samples is known to be challenging. Identification studies of most *in vivo* PTM patterns therefore usually require enrichment before analysis. Hence, it was an obvious choice of direction to head for the development of enrichment strategies to enable identification of *in vivo* citrullination. However, in the last years, substantial improvements in MS instrumentation have enabled identification of citrullination through direct MS analysis [73,124]. On the other hand, a recent review states that the significance of applying enrichment strategies still is evident [145]. Therefore, in Paper III we present a study where RA synovial fluid is analysed in a direct proteomic-based approach and after specific BPG enrichment in order to assess the *in vivo* citrullination pattern. The results of this study confirmed that analysis of small PTMs in complex samples clearly benefits from the advances in MS

technology, which was demonstrated by the high number of identified citrullinated proteins. However, despite unexpected challenges in the LC-MS/MS analysis of BPG-modified peptides, the enrichment strategy appeared to be superior to the direct approach, when it comes to depth of analysis. This strongly indicates that further development of specific enrichment tools would gain more knowledge and a greater insight into the *in vivo* citrullinome. This is discussed in detail in the chapter Methodological Considerations.

Further, most citrullinated proteins present in RA synovium have been identified by analysing synovial tissue and synovial fluid. This is in line with the suggestion that citrullination of intracellular proteins may occur extracellularly, after breakdown of the membrane of dying cells leading to the release of these proteins, including PADs [74]. This suggestion was proposed since millimolar concentrations of calcium was shown to be required for PAD activation *in vitro* [153], whereas intracellular calcium concentrations normally do not rise above low micromolar levels, even though elevated calcium concentrations could occur locally in the cells by efflux of calcium from the endoplasmatic reticulum, mitochondria or through plasma membrane channels [18]. However, it may seem that there still are mechanisms of PAD activation that remain to be discovered. Recently, Romero and colleagues presented a study where they demonstrated that citrullination in the RA joint is cell-associated and since it was characterised by prominent citrullination of a broad range of proteins, they termed this pattern "hypercitrullination" [124]. By examination of neutrophils and monocytes, which are largely presented in the RA synovium [154] and also have been shown to be major sources for PADs in the RA joint [155], they show that hypercitrullination is prominently induced by the immune-mediated membranolytic pathways, perforin and membrane attack complex. In another recent study, Darrah *et al.* from the same group, demonstrated that the dependency of PAD4 to calcium could be significantly decreased through interaction with an antibody [153]. This indicates that PAD activity may not be as dependent on high calcium concentrations as previously thought. Romero *et al.* therefore suggests that PAD activity causing hypercitrullination within neutrophils and monocytes could be induced by interaction with a binding partner, still of unknown identity, which could decrease the requirement for high calcium concentrations and thereby allow intracellular citrullination to occur [124]. This opens up for new areas to be studied in the search of the origin of citrullinated autoantigens in RA.

Several citrullinated autoantigens in the pathogenesis of RA have been identified. These include citrullinated forms of vimentin [69,156], fibrin (fibrinogen α-and β-chains) [68], collagen type II [70,157] and α-enolase [71,157,158], which all are well-established

autoantigens in RA (reviewed in [159]). Further, more recently, also citrullinated forms of apolipoprotein E, myeloid nuclear differentiation antigen and actin [73] in addition to citrullinated fibronectin [72] have been found to be autoantigenic when screened with ACPA-positive RA sera. Immune response towards citrullinated proteins, in form of ACPA production, is restricted to RA [74], even though citrullination occur in most, if not all, inflamed tissues. Therefore, it would be of great interest to elucidate whether it is the nature of one or a few specific antigens or the antigen load that may initiate the immune response. Knowledge about this could be achieved by a comprehensive, in-depth comparison of citrullinated proteins present in synovial samples from different patient groups; ACPA-positive and ACPA-negative RA and e.g. osteoarthritis. Further, the majority of citrullinated proteins identified to be autoantigenic have been found by selecting several citrullinated candidate peptides which were screened with RA sera, and the ones that were reactive were defined as autoantigenic. However, a more direct strategy, which would reflect the *in vivo* situation, could be obtained by eluting antigens from IgGs isolated from ACPA-positive RA synovial fluid. By mass spectrometric analysis of eluted antigens quantitative data could be obtained, giving an indication of which autoantigens are playing a central role in the act of ACPA response.

Homocitrullination - carbamylation of lysine

The enrichment strategies presented in Paper I and Paper II may also be applied in the analysis of homocitrullinated proteins, which was demonstrated by the enrichment of synthetic homocitrulline-containing peptides in unpublished results.

Homocitrulline resembles citrulline in structure and physical properties, as both amino acids carry an ureido group. Several of the techniques developed for detection of citrullination presented in the introduction, as well as the enrichment strategies presented in the Paper I and Paper II, are based on modification of the ureido group, leading to modification of both citrulline and homocitrulline residues present in the sample [160]. Techniques based on visualisation without identification of modified proteins will not allow distinguishing between citrulline and homocitrulline. However, when MS is applied for identification the difference in side chain length by one CH_2-molecule, between these two amino acids, can be implemented in the search parameters and both citrullinated and homocitrullinated peptides will independently be identified.

Homocitrullination occur by carbamylation of lysine in a non-enzymatic spontaneous chemical reaction of urea-derived cyanate with the free NH_2-group on lysine residues. In normal physiology urea concentration is too low to allow carbamylation, however, in inflammation sites thiocyanate is converted to cyanate by the enzyme myeloperoxidase, which is released from neutrophils [161].

Both citrullination and homocitrullination results in the loss of a positive charge, therefore inducing the same biochemical consequences for their targeted proteins. The striking similarity between these two amino acids, in structure, physical properties and occurrence in inflammation sites, has raised the question of the role of homocitrullination in the pathogenesis of RA, and if ACPAs also would recognise homocitrullinated/carbamylated peptides. Recently, it was shown that carbamylated proteins were present in the RA joints [162], which were also the presence of anticarbamylated protein (anti-CarP) antibodies [163]. However, anti-CarP antibodies and ACPAs were found to be two distinct set of autoantibodies, which did not show cross reactivity [163]. Nevertheless, the presence of another autoantibody system recognising carbamylated proteins, in addition to the autoantibody system that recognises citrullinated proteins, adds new insight into the pathogenesis of RA, and maybe particularly ACPA-negative RA [163].

FINAL COMMENTS

In RA, where an immune response is initiated towards citrullinated proteins, the characterisation of proteins that undergo citrullination and which of these becomes autoantigenic, could potentially reveal underlying mechanisms in the pathogenesis of the disease.

Mass spectrometric instrumentation has the last decades improved dramatically now allowing the identification of several citrullinated proteins present in synovial fluid by direct MS analysis. However, the use of citrulline specific enrichment strategies have generated evidence for that many citrullinated peptides present in such a complex sample go underreported in the direct analysis. This finding strongly supports further development and optimisation of specific enrichment tools to enable a comprehensive elucidation of *in vivo* citrullination.

Characterisation of *in vivo* citrullination patterns in ACPA-positive RA compared to ACPA-negative RA and other inflammatory conditions could gain insight into if the immune response towards citrullinated proteins in ACPA-positive RA is caused by the presence of certain autoantigens.

Recently, also homocitrullination has been found to play a role in the pathogenicity of RA. Specific enrichments tools based on chemical modification of the ureido group, followed by identification of enriched peptides by mass spectrometry, allow characterisation of both citrullinated and homocitrullinated proteins. This might bring new insight into our understanding of RA.

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ERRATA

Thesis

Page 10, line 5

The sentence "Autoimmunity is when the immune system's mechanism is bypassed and immune response is elicited by antigens derived from self." has been changed to "Autoimmunity occurs when a failure in the mechanism of immunological tolerance leads to an immune response towards antigens derived from self."

Page 12, line 13

The sentence "However, by efflux of calcium through plasma membrane channels or from the endoplasmatic reticulum or mitochondria, elevated calcium concentrations can occur locally in the cells (reviewed in [18])." has been changed to "However, by influx of calcium through plasma membrane channels or from the endoplasmatic reticulum or mitochondria, elevated calcium concentrations can occur locally in the cells (reviewed in [18])."

Page 25, last paragraph

The first sentence "The second enrichment strategy represents an in-solution-based modification approach…" has been changed to "The second enrichment strategy has both high specificity and sensitivity and represents an in-solution-based modification approach…".

The last sentence "This method showed both high specificity and sensitivity." has been removed.

Paper II

Third page, line 26 in Preparation of biotin-PEG-GBA in Experimental section

The correct wave lengths are 210, 254 and 280 nm

I

II