Chapter

Mass Spectrometry (Imaging) for Detection and Identification of Cyclic AMPs: Focus on Human Neutrophil Peptides (HNPs)

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Abstract

Antimicrobial peptides (AMPs) are known best for their role in innate immunity against bacteria, viruses, parasites and fungi. However, not only are they showing increasing promise as potential antimicrobial drug candidates, recently, it has been reported that certain AMPs also show a cytotoxic effect against cancer cells. Their possible antitumor effect could make AMPs interesting candidate cancer biomarkers and a possible lead for new anticancer therapy. Due to their cyclic structure, detection and identification of AMPs is challenging, however, mass spectrometry (imaging; MSI) has been shown as a powerful tool for visualization and identification of (unknown) cyclic AMPs. In this chapter, we will discuss how mass spectrometry (imaging), combined with the use of electron-transfer dissociation (ETD) as fragmentation technique, can be used as a reliable method to identify AMPs in their native cyclic state. Using this approach, we have previously detected and identified human neutrophil peptides (HNPs) as important AMPs in cancer, of which a detailed bacterial, viral and cancer-related overview will be presented.

Keywords: human neutrophil peptide 1, 2 and 3 (HNP1-3), proteomics, mass spectrometry (imaging), immunomodulatory function, non-small cell lung cancer (NSCLC)

1. Introduction

In the beginning of last century, the first antimicrobial protein, lysozyme, was reported. A few years later, the best known antimicrobial compound, called penicillin, was discovered, which made research into natural antimicrobial proteins/peptides (AMPs) a very important research domain for therapeutic molecules that can be used against bacterial infections [1]. AMPs are naturally occurring small proteins (or peptides) in different organisms and are produced by many tissues and different cell types, acting as host defense molecules against bacteria, but with some also showing a fast antifungal, antiviral, antiparasitic and antitumor response [1–3].

The largest part of AMPs consists of antibacterial peptides with an inhibitory activity towards bacteria, both Gram-positive and Gram-negative [2]. Studies have revealed that AMPs exhibit an overall positive charge, allowing electrostatic interactions with negatively charged phospholipid groups in the bacterial membrane.

By this attribute, pores can be formed by AMPs to disrupt the membrane integrity. Some AMPs are able to cross the lipid bilayer, followed by disruption of intracellular functions such as blocking enzyme activity or inhibition of protein synthesis, both resulting in bacterial cell lysis [1]. For this reason, AMPs are often referred to 'natural antibiotics'.

AMP activity is not restricted to antimicrobial mechanisms, also AMP activity against parasites has been observed: a few AMPs are reported as antimalarial peptides and can possibly serve as new future drug targets against the malaria parasite. For example, cecropins have been shown to block the development of oocysts into sporozoites, while dermaseptins (and some derivatives) have been found to be able to permeabilize the host cell membrane [4].

Furthermore, a subset of AMPs have shown antifungal characteristics against some fungi commonly found in food and agriculture, but also against the common *Aspergillus* and *Candida albicans* infections [2]. These antifungal peptides can interact with fungal membranes to form pores, comparable to the AMP mechanism in bacteria, but they can also act by targeting the specific fungus cell wall or by acting as nucleic acid inhibitors through direct binding to nucleic acids [5]. A smaller part of AMPs also exhibit antiviral activity, by acting through different mechanisms. A first mechanism includes inhibition of virus attachment and cell membrane fusion. As an example, during the recent COVID-19 pandemic, the antiviral peptide (AVP) EK1C4 has been found to be very effective against S-mediated membrane fusion of the viral particles, thus inhibiting entry of the virus and thereby infection [6]. Another example of inhibition of virus attachment is demonstrated by dermaseptins, which possibly affect the lipid bilayer to alter the fusogenic properties of herpes simplex virus [7]. The virus for host cell infection can also be impeded by the direct action of certain AMPs, such as indolicidin, against enveloped virions, causing membrane instability by destruction of the virus envelope [8]. Combined, there is great potential for future therapeutic development of AVPs for both prevention as treatment of infection [6]. A small number of AMPs are believed to be active as anticancer peptides (ACP). It has been suggested that they specifically target the membrane of cancer cells through interaction with phospholipids, mainly phosphatidylserine, present at the outer leaflet of the cancer cell membrane in higher amounts compared to normal cells. Moreover, the ACP LTX-315 has demonstrated both cytolytic and immunogenic properties towards cancer cells, as LTX-315 induces tumor antigen and danger-associated molecular patterns (DAMPs) release, triggering an immune response towards the cancer cells [9].

AMPs are considered key components of the innate immune system, as shortly after an infection, these are promptly synthesized to neutralize a wide variety of pathogens, but through another mechanism compared to that of cytokines or phagocytes [10]. High concentrations of AMPs are usually required to exert an optimal pathogen killing activity, but *in vivo*, lower concentrations of AMPs are reported, in this case possibly acting as potent immune regulators, also leading to pathogen killing but rather through an indirect mechanism [11]. Besides permeabilizing lipid membranes and bacterial walls, their primary role as antimicrobicidal agents, other targets of AMPs are thus reported. Recently, some AMPs are found to also modulate immune responses in vertebrates, through chemotactic activity, attraction, activation and differentiation of leukocytes and monocytes, influencing Toll-like receptor (TLR) recognition and through secretion of proinflammatory cytokines and chemokines, although their underlying mechanisms have not been fully characterized yet [1].

The best studied AMPs include the human defensins and cathelicidins and both have been shown to be chemotactic: β defensins (hBD) recruit (memory) T cells and immature dendritic cells through their chemotactic activity, suggesting

they promote cellular immune responses via interactions with the G protein-coupled receptor CCR6 [11]. Another example of direct AMP chemotaxis includes cathelicidin LL-37, an AMP that has been proven to be chemotactic for neutrophils, monocytes and T cells, but not dendritic cells [11]. Additionally, an indirect chemotactic effect is possible by AMPs through inducing the release of pro-inflammatory cytokines and chemokines, to further refine and activate the innate, and eventually the adaptive, immune response [1, 11]. In synergy with particular immune mediators, LL-37 has been shown to enhance IL-6 and IL-10 cytokine production, even as the production of macrophage chemoattractant proteins (MCP-1 and MCP-3) chemokines, resulting in an strengthened (innate) immune response [12]. Toll-like receptors (TLR) are key players in innate immunity by recognizing microbe-associated molecular patterns (MAMPs). TLR activation leads to secretion of AMPs, but some AMPs, including cathelicidins, can modulate TLR-mediated inflammatory responses by strongly reducing LPS-induced TLR activation, mostly by inhibiting TLR4 [11, 13]. Lastly, AMPs, e.g. cathelicidins and β defensins, also exert a regenerative function by affecting wound healing, by stimulating migration, proliferation and tube formation of endothelial cells, through a cascade of activated pathways [11].

Overall, AMPs are important key players in host protection. Due to increasing antibiotic resistance, several AMPs have good potential therapeutic purposes, ranging from antimicrobial, anti-inflammatory and immunomodulatory properties. Also co-administration of AMPs with existing therapies can have good clinical outcomes [14]. Recently, the ACP LTX-315 which has been described earlier, demonstrated in phase I human clinical studies to be an effective drug, due to its immunostimulatory effect resulting in tumor necrosis [9]. Currently, a phase I clinical trial for transdermally accessible tumors is ongoing to evaluate the efficacy of LTX-315 monotherapy or in combination with immune checkpoint inhibitor immunotherapy [9]. Still, some limitations for the therapeutic use of AMPs need to be resolved: high proteolytic degradation of AMPs (i.e. susceptibility to proteases) is commonly observed, unpredicted toxicity is known to occur, chemical synthesis is costly and delivery of AMP targets to the site of infection can be very difficult [9, 14, 15]. As an example, LL-37 has proven to be very effective against Ebola virus infection, but its use as therapeutic molecule is limited as LL-37 is rapidly degraded and can lose its activity under certain conditions. These limitations were overcome with the design of an engineered LL-37 which prevents cell entry of the virus. The therapeutic outcome of these AMPs in animal models is ongoing, possibly combined with other small molecules that interfere with viral replication or together with virus-neutralizing antibodies [16].

AMPs mostly consist of 10 to 60 amino acids, including mainly basic and hydrophobic residues, resulting in positively charged molecules [1, 2]. They can be classified, based on their structure, into four categories; 1) linear extension structure, 2) α-helical AMPs, 3) AMPs consisting of β-strands stabilized by disulfide bonds and 4) both α-helical and β-sheet structures [2]. Due to their cationic properties, AMPs are easily detectable by mass spectrometric analysis. In addition, structural information of AMPs can be obtained by tandem mass spectrometry in which fragmentation spectra are obtained. Based upon this, the corresponding amino acid sequence can be determined and the precursor ion can be identified [17]. Even if the AMPs have a cyclic nature, due to their cysteine bond formation, mass spectrometry can be used as an identification tool, although a specific approach is needed [18–21]. The added benefit of using proteomic approaches to study AMPs is the fact that the majority of AMPs are post- or co-translational proteolytically processed from their large polyprotein precursor, resulting in the release of the active AMP. This is important as it allows for the identification of the active AMP in physiological

conditions. It overcomes the limitations of a genomics approach, as it can be complicated to predict the configuration of the exact active peptide from genomic sequences alone [22, 23]. In addition, due to their high positive charge, distribution of the AMP in a tissue can be analyzed using mass spectrometry imaging (MSI). MALDI MSI is a multiplexed analysis that allows the screening of hundreds of analytes simultaneously in a single tissue section without *a priori* knowledge of the present biomolecules [24–26]. Using MSI, a mass spectrum on every pixel of the tissue is recorded, representing all measured analytes by their mass-to-charge (m/z) ratio. The thousands of generated mass spectra can be combined. This thus provides not only structural information about the AMP, it also retains its spatial distribution and information on the relative abundance throughout the tissue [27]. This can overcome the limitation of antibody-related visualization with AMP-specific antibodies that are in some cases designed towards the non-active part of the AMP.

Overall, mass spectrometry (imaging) is an useful approach for the detection and identification of AMPs in their native and active form.

2. Mass spectrometric detection and identification of human neutrophil peptides 1: 3 (HNP1-3)

2.1 Detection of AMPs in their native state

In the search for positive response patterns towards immune checkpoint inhibitors in non-small cell lung cancer (NSCLC) patients, we recently applied matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) on pretreatment tumor tissue biopsies. Since no prior knowledge of the molecules is required for MSI analysis, new unknown response patterns and biomarkers can be revealed. An additional advantage of using this visualization technique is that new, unknown biomolecules can be detected in their active and native state.

Using this approach, three peptides *m/z* 3369.5, *m/z* 3440.6 and *m/z* 3484.6 have found to be discriminative between a responding and a nonresponding NSCLC patient towards anti-PD-(L)1 immunotherapy, shown in **Figure 1** [21].

2.2 Identification of (cyclic) AMPs

A major bottleneck of the direct analysis of tissues with MALDI-based MSI is the lack of a reliable identification of the visualized molecules, but it has been proven earlier that peptide/protein identification can be performed by using top-down proteomics. This is a major challenge for identification of cyclic proteins or peptides, due to their intramolecular cysteine bridges.

Mass spectrometry followed by *de novo* sequencing has been described as a highly sensitive analytical technique to detect and characterize AMPs that are present in low concentrations within different species. Structural information of the intact AMP can be obtained by tandem mass spectrometry in which peptide ions are dissociated, resulting in a MS/MS spectrum from which the amino acid sequence can be derived. If required, the identification can be confirmed by comparing MS/MS spectra with those of the corresponding synthetic AMP [28]. Shotgun proteomics was performed on both short- and medium-sized antimicrobial peptides, generated by simulated gastrointestinal digestion, from yellowfin tuna samples. This has led to the identification of in total 572 sequences, followed by subjection to antimicrobial activity assays to unravel their AMP properties to evaluate their possible use as new future antimicrobial drugs [29]. In another study, antimicrobial proteins and peptides were extracted from different parts

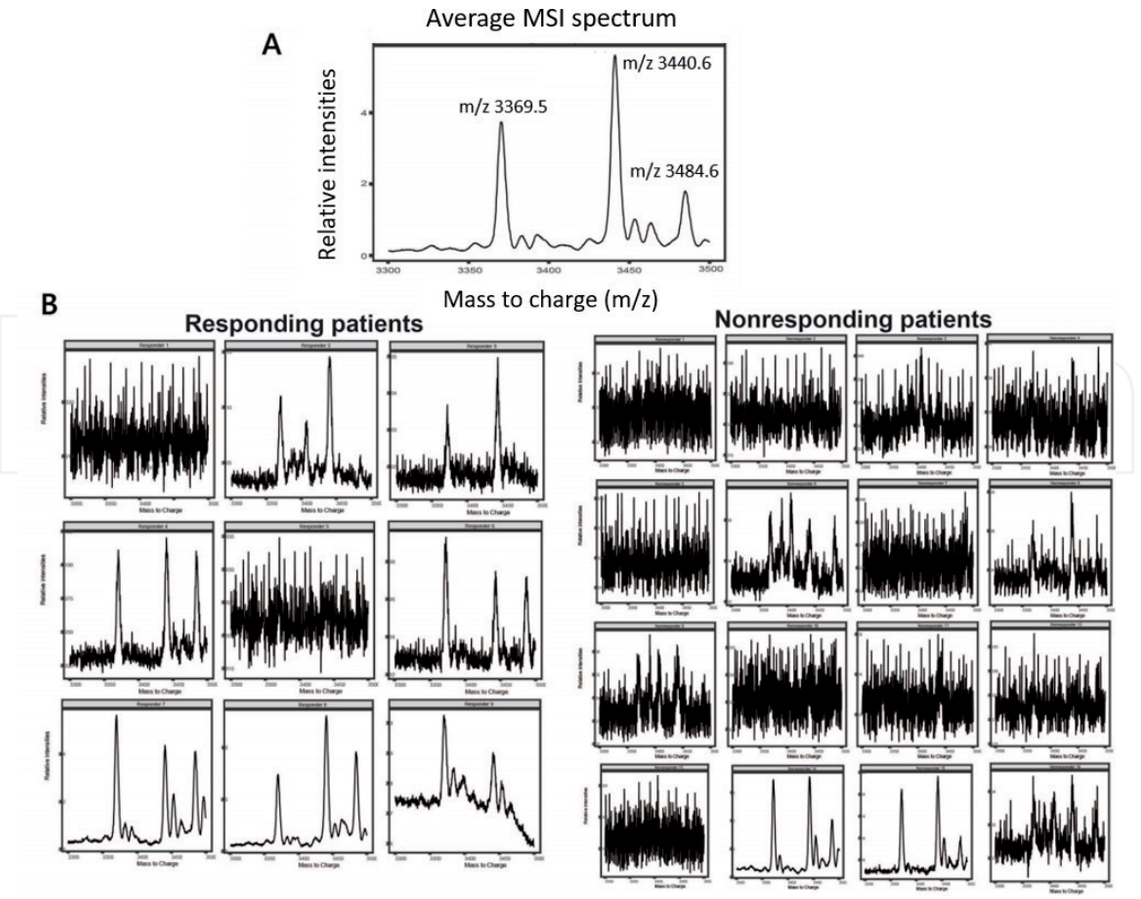

Figure 1.
Average MALDI MSI spectra obtained with mass spectrometry imaging (MSI) analysis of whole formalin-fixed paraffin-embedded (FFPE) tumor biopsies of NSCLC patients. (A) an example of a resulting average mass spectrum with three peptides m/z 3369.5, m/z 3440.6 and m/z 3484.6 of interest. (B) Average MSI spectra of 25 pretreatment tumor FFPE biopsies from NSCLC patients that received anti-PD-(L)1 immunotherapy. From this small patient cohort, nine patients received clinical benefit from the therapy (responders), from which seven patients showed expression of the three interesting peptides. The other 16 NSCLC patients did not derive any clinical benefit from immunotherapy treatment (non-responders), from which 14 show no (or very low) peptide expression. From the nonresponding patients, two NSCLC patients showed interesting peptide expression. Figure adapted from [21] with permission.

of *Charybdis pancration*, a plant used in traditional medicine. These extracts are subsequently tested for their AMP activity against different antibiotic-resistant pathogenic strains. The extracted fraction that has displayed AMP activity, was further analyzed by using mass spectrometry leading to the discovery and identification of seventeen novel peptides with AMP activity [30].

Identification of the cyclic peptides (shown in **Figure 1**) by collision-induced dissociation (either with CID or HCD), a routine approach in top-down peptidome analysis, is mostly not successful as fragmentation of the peptide backbone will not result in multiple fragments of different lengths. Rather, a long fragment with a mass close to the mass of the original parent (minus the loss of H_2O) will be generated, irrespective of where the fragmentation occurred. Hence, these MS/MS spectra cannot be used to deduce a sequence tag that can be used to identify the peptide [31]. However, it has been shown that electron-transfer dissociation (ETD) can be used successfully for the identification of naturally occurring peptides [32], so, ETD has been applied as a fragmentation technique instead of HCD. Rather than generating fragments of the peptides by colliding them with an inert gas in CID, ETD induces fragmentation of large, multiply-charged cations by transferring electrons to them. ETD can be used effectively not only to break the peptide backbone (typically into C and Z ions), but also to reduce any cysteine bridges in the peptide [18–20]. This is nicely demonstrated in **Figure 2B**. An extract of the NSCLC tissue was prepared

and analyzed with LC–MS/MS on a LTQ Velos Orbitrap (Thermo Fisher, Waltham, MA, USA) equipped with ETD. This type of instrument combines a dual stage linear ion trap with an orbitrap analyzer, an HCD cell and an ETD source. This allows for a very flexible use of different fragmentation techniques in the ion trap. In this case, the peptide is fragmented by using ETD and the resulting fragments were measured in the Orbitrap. The fragmentation of any of the three target peptides showed a neutral loss of 3 Da in each peptide (**Figure 2B**), corresponding to a reduction of three disulfide bridges between 6 cysteine residues [21].

The reduction of the disulfide bonds in effect turns the circular peptide into a linear one (**Figure 2A** and **B**). In a subsequent experiment, the resulting reduced and thus no longer circular peptide is selected and trapped in the ion trap for an additional fragmentation with CID (**Figure 2C**). In this way, multiple fragments are generated, measured this time in the ion trap, from which an amino acid sequence can be deduced. This leads to the identification of the peptide with mass 3440.6 as human neutrophil peptide 1 (HNP1), presented in **Figure 2D**. The two other peptides of interest were analyzed in a similar way and identified as human neutrophil peptides 2 and 3. These three peptides have an almost identical amino acid sequence, only differing in the first amino acid residue [21].

To conclude, the combined approach of MSI and top-down proteomics using both ETD and CID has revealed human neutrophil peptide 1, 2 and 3, also known as neutrophil defensin 1, 2 and 3, as putative discriminative markers between a responding and a nonresponding NSCLC patient towards immunotherapy [21], highlighting a possible broader role for these AMPs than just a function as

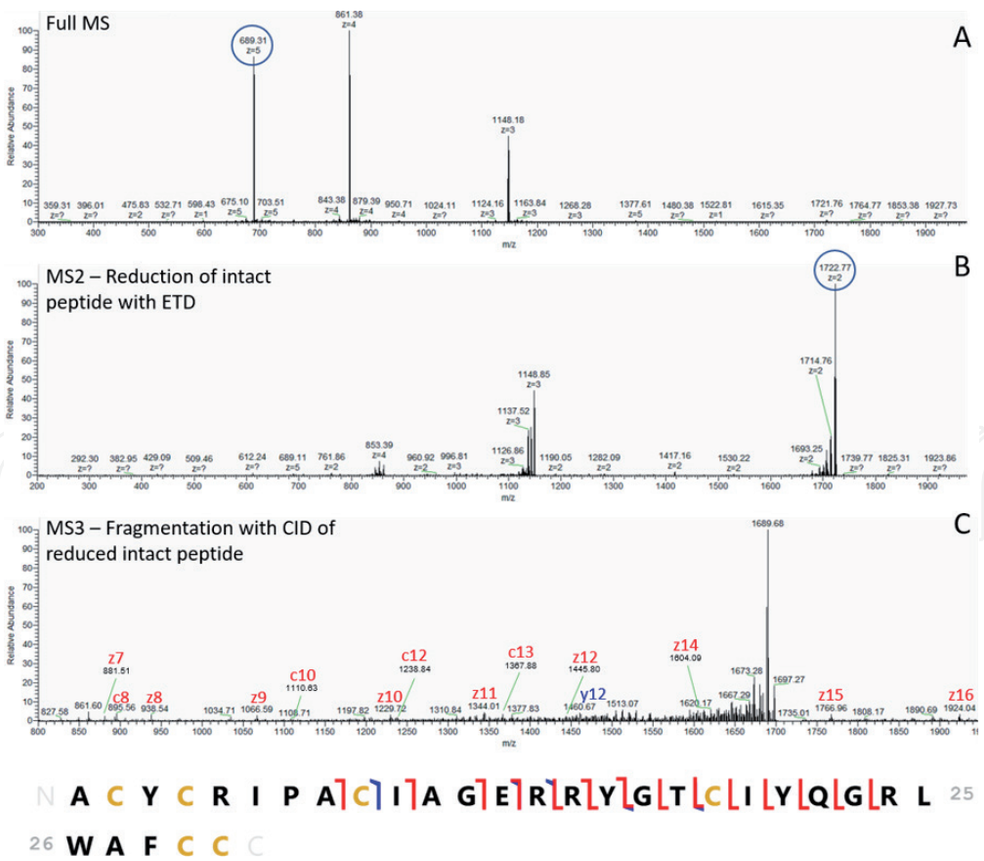

Figure 2.
Mass spectra and annotated sequence of synthetic peptide corresponding for human neutrophil peptide 1 (HNP1). A) Full MS spectrum of intact HNP1, in three different charge states. The five charged ion with m/z 689.31 (mass 3441.6 Da) is selected for reduction of the three internal disulfide bridges with ETD; B) the resulting intact peptide m/z 1722.77 after reduction of three disulfide bridges. This reduced peptide is immediately selected for fragmentation with CID; C) the resulting fragmentation spectrum with c, y and z type ions; D) annotated sequence of human neutrophil peptide 1. Figure adapted from [21] with permission.

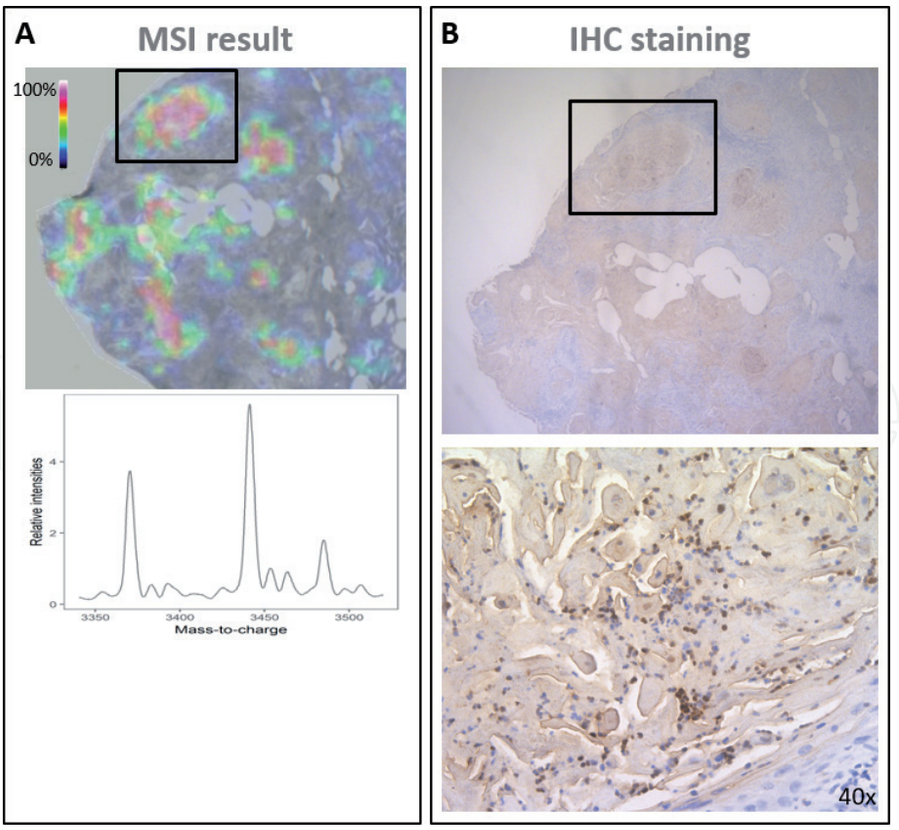

Figure 3.
Distribution of human neutrophil peptides 1-3 in FFPE tissues after MSI and IHC analyses. A) Distribution of HNP1-3 obtained with MSI and the corresponding mass spectrum; B) validation of the presence of HNP1-3 in the same FFPE tissue, prior MSI analyzed, with IHC. The region indicated with the box in the MSI result was compared with the same tissue region after IHC analysis with a defensin 1/3 antibody. Figure adapted from [21] with permission.

antimicrobial peptides. Additionally, these results were verified with immunohistochemical (IHC) analyses on the same pretreatment biopsies with a defensin 1/3 polyclonal antibody. In **Figure 3**, it is illustrated that IHC is feasible on FFPE tissue sections, previously MSI analyzed, without an apparent change in staining intensity [21].

This combined approach has been proven to be very useful in the detection and identification of interesting AMPs, especially in their native, processed form in a clinical context [21].

2.3 The biological activity of HNPs

The previously described observations were a starting point to explore the possible role of neutrophil defensins in cancer immunology.

Defensins, together with cathelicidins, are widely studied, as they were early recognized as important components of the antimicrobial mechanisms of leukocytes. The defensins can be classified in human α-defensins, which are composed of β-strands, and human β-defensins, consisting of both α-helical and β-strands structures [33]. The sequences of these two defensin types include six cysteine residues and their cyclic structure is stabilized by formation of three intramolecular disulfide bonds, from which the cysteine bonding pattern makes a differentiation between α-defensins and β-defensins [33]. These cysteine residues are of major importance for their cationic antimicrobial characteristics [22]. A third class of defensins is also reported, called θ-defensins, and although mRNA expression of θ-defensins has been observed in humans, no functional AMPs from the θ-defensin family are reported to be produced in humans [22].

Currently, 31 β-defensins have been identified and are being studied. They are mainly expressed by epithelial cells and keratinocytes, but can also be secreted from macrophages, neutrophils and lymphocytes, suggesting a broader role in immune responses besides antimicrobial activity [22]. More importantly for this review part are the α-defensin family, more specifically the human neutrophil peptides 1, 2 and 3 (HNP1-3), as previously described. As the name suggests, these AMPs are predominantly produced by neutrophils, but they have also been detected in macrophages, natural killer (NK) cells, immature monocyte-derived dendritic cells and some classes of T and B cells. The fourth α-defensin is also found in these cell types, while α-defensins 5 (HD5) and 6 (HD6) are secreted by intestinal Paneth cells, with the main function to provide intestinal host defense towards pathogens and to control and maintain homeostasis of the intestinal microbiome [22, 34]. HD5 and HD6 deficiency is associated with Crohn's disease, possibly due to the reduced antimicrobial defense capacity by lower HD5 and HD6 expression, leading to an altered microbiome composition [1, 35].

HNP1-3 have an almost identical amino acid sequence, only differing in a single amino acid residue, while this does not hold true for HNP4, HD5 and HD6 (**Figure 4**), although all six α-defensins are characterized by the same cysteine residues [36]. HNP1 and HNP2 (the same is true for HNP2 and HNP3) are released from the same precursor, which is cleaved by a signal peptidase at position 19 leaving a propeptide, which will be further processed by proteolysis in the developing granulocyte. The resulting mature peptide is then packaged into azurophilic granules of the neutrophils, with the HNPs representing more than 30% of the total protein content in these granules [37]. Neutrophils are first-line defense immune cells against different pathogens and are directly recruited to sites of infection, followed by engulfment of the pathogen. Upon neutrophil activation, degranulation of azurophilic granules takes place, thus leading to abundant HNP release as a first-line of response to invading organisms [37–39]. These HNP1-3 have been shown to have antibacterial, antiviral, anticancer and even immunomodulatory activities, which will be discussed further in more detail.

2.3.1 Antibacterial and antiviral activity of HNP1-3

HNP1-3 have a well described antibacterial activity, with demonstrated effectivity towards *S. aureus*, *E. coli*, *P. aerugnosa* and *C. albicans* through interaction of the positively charged HNPs with the anionic bacterial membrane. This can lead to different possible mechanisms, depending on the type of bacterium itself: the most common mechanism of HNPs includes destruction of the bacterial membrane, which is the case for *P. aerugnosa* and *C. albicans.* Lipid II, a bacterial wall precursor, is also a target for HNPs. Such example has been observed in *S. aureus*, in which HNP is responsible for lipid II restriction. In the case of *E. coli,* the disruption by HNP1 happens through intracellular targeting, by inhibiting the bacterium's protein

Figure 4.
Alignment of the amino acid sequences of all 6 human α defensins. Conserved cysteine residues are presented in green and their corresponding cysteine bonding pattern is indicated.

synthesis [40]. Furthermore, HNP1 has demonstrated direct activity towards *Mycobacterium tuberculosis*, the pathogen that can cause the infectious disease of tuberculosis, responsible for over a million deaths per year. The direct killing capacity of HNP1 includes permeabilization of the membrane, followed by pore formation [41]. HNP1 has even been considered as a lead compound in combating methicillin-resistant strains of *Staphylococcus aureus* (MRSA). When applied together with antibiotics, the HNPs show a synergistic effect towards different MRSA isolates, paving the way for a new therapeutic approach to overcome the increasing antibiotic resistance [42].

HNP1-3 have not only proven an effective response towards both Gram-positive and Gram-negative bacteria, but antiviral activity is also a well-known characteristic of HNPs, including human immunodeficiency virus (HIV), human papilloma virus (HPV), herpes virus and influenza A virus (IAV) [2]. These HNPs have also been reported as anti-HIV peptides: high production of HNP1-3 by immature dendritic cells have a host protective role against progression of HIV-1, due to the direct HNP damage capability towards the virions, followed by the virion internalization by the immature dendritic cell, leading to viral processing and presentation to HIV-specific CD4+ T cells [43]. In addition, HNP1-3 are identified in the female genital tract acting as host defense forming a natural barrier to HPV [44]. HNP1-3 can inhibit herpes simplex virus (HSV) entry by directly binding to its target receptor and these defensins even exhibit post entry antiviral activity, leading to reduced viral replication after HSV infection [22]. As a last example worth mentioning, these HNPs have an anti-IAV activity by direct interaction with the virus, leading to destabilization of the viral envelope and thus leading to virus inactivation. HNP1 has also been suggested to bind the protein kinase C (PKC) receptor, in this way avoiding both IAV entry and replication [45, 46].

2.3.2 The role of HNP1-3 in cancer (immunity)

Already a direct antitumor effect has been described for human neutrophil peptides in a variety of tumor cells [47]. Furthermore, HNP1 has been reported as a potential prognostic biomarker in cancer [48–50]. In addition, the HNPs are suggested to induce tumor necrosis [48, 49]. Although, despite the reported anticancer activity, defensins HD5 and HD6 are known markers of development and contribution to colorectal tumor growth [51].

In the same study in which we showed an association of the presence of HNPs and a response towards anti-PD-(L)1 immunotherapy in NSCLC, a possible immune stimulatory effect of HNPs towards lung cancer cells has been reported, while no such activity could be shown against non-tumoral cells [21]. *In vitro* data revealed that NSCLC cell proliferation is significantly reduced when cocultured with peripheral blood mononuclear cells (PBMC) from healthy donors. A conceivable explanation for this observation is immune activation towards cancer cells, as an effect of addition of HNPs to the cancer cells was only observed in the presence of PBMC. Direct addition of HNPs to the three NSCLC cell lines in the absence of immune cells did not result in a significant decrease of tumor cell proliferation, and even an increase in tumor cell proliferation was observed in a certain case [21]. Furthermore, IFN-γ secretion was clearly increased in the PBMC and tumor cocultures after HNP-treatment. Surprisingly, treatment of HNPs to PBMC from healthy donors in the absence of NSCLC cells did not result in an increase of IFN-γ release, indicating that HNPs contribute to activate the immune response, although not directly activating the immune cells. Also, non-tumoral cells remain insensitive to the action of HNPs. Neither was the effect explained by treatment with HNPs on the NSCLC tumor cells directly, as HNP treatment of only A549 cells did not result in IFN-γ release [21].

Although earlier studies suggest a direct cytotoxic anticancer activity of the human neutrophil peptides [47–49], in this study, a possible immune–stimulatory effect of HNP1-3 towards lung cancer is suggested. The question raised by these findings is how these HNPs can act as immune-stimulatory effector, not directly on tumor cells nor on the immune cells, while leaving non-tumoral cells intact. A hypothesis is that α-defensins specifically target tumor cells by interaction with phosphatidylserine (PS) exposure. This interaction has been shown in a recent *in vivo Drosophila* model [52]. In general, cell membranes consist of PS that normally faces the inside of the cell due to the activity of phospholipid flippases. In apoptotic cells, PS can be exposed to the outer surface of the cells by loss of flippase activity and by activation of scramblases. In Drosophila, externalized PS serves as a marker for engulfment by macrophages leading to phagocytosis [53]. This mechanism seems to be preserved in human cells, including lung cancer cells [54], and cancerous cells often increase exposure of the negatively charged PS on the outer leaflet of the cell membrane [55]. This would allow for the positively charged HNPs to interact with the negatively charged tumor cell surface PS, resulting in their cell death, while healthy cells remain insensitive to the HNP action, as PS is still present on the inner leaflet of the cell membrane by flippase activity [52].

PS exposure by the tumor cells have been driven in *Drosophila* through tumor necrosis factor (TNF) expression, while this exposure has not been observed on the normal cells [52, 54]. TNF-α concentrations have also been measured in the supernatants of the PBMC and tumor cocultures treated with HNP1-3. For the NSCLC cell lines, an increase in TNF-α release was observed when the coculture was HNP treated, which was not observed for solely PBMC cultures nor for human bronchial epithelial cells BEAS-2B cocultured with PBMCs of three healthy donors [17]. This can possibly explain phosphatidylserine exposure on NSCLC cell lines, making the NSCLC cell membrane sensitive for interaction with the positively charged HNP to induce transmembrane permeability, a typical AMP characteristic [1]. In this way, tumor-associated antigens (TAAs) could be exposed to activate cell-mediated immunity by provoking an immunogenic response, resulting in a significant decrease in tumor cell proliferation [17, 21].

It is conceivable that HNPs play an immune-stimulatory role towards (lung) cancer cells. Due to their direct antimicrobial activity characteristics, HNPs are considered to be part of the innate immune response, just as neutrophils, their main cellular source, as approximately 9% of the neutrophil protein content includes HNPs [56]. Neutrophils have Jekyll and Hyde properties in relation to cancer, as they have been shown to elicit both antitumoral as protumoral activities [57, 58]. Tumor-associated neutrophils (TANs) have been linked with poor prognosis in late-stage tumorigenesis [58, 59]. Although, recent findings indicate antitumor properties of neutrophils in early-stage human tumors; neutrophils have been shown to present antigens, resulting in T cell interaction leading to a proper T cell activation and response. Furthermore, neutrophils are able to attract and activate these T cells through cytokine secretion [60]. It has also been proven that HNPs enhances adaptive immunity, however, their mechanisms remain largely unknown. Nevertheless, studies have shown that HNPs have immunostimulatory characteristics through chemoattraction of naïve T cells [61], $CD8^+$ T cells [37], monocytes [37], maturation and differentiation of immature dendritic cells [43, 61] and by inducing pro-inflammatory chemokine and cytokine production, such as IFN-γ, IL-8 and IL-2 [62].

Chemoattraction of monocytes by HNP has been proven by De Yang *et al.* by analyzing interleukin-8 induced neutrophil-derived T cell attraction. HNP selectively attracted CD4+ naïve T cells and cytotoxic CD8+ T cells for an effective immune response, but not memory CD4+ T cells [61]. In addition, HNP promotes

an antigen-specific immune response by chemoattracting dendritic cells, the most potent antigen-presenting cells (APCs). When administered together with antigens, HNPs are able to recruit immature dendritic cells and T cells to microbial infection sites, leading to maturation of these monocytes responsible in promoting an adaptive immunity towards microorganisms [61]. It has also been proven *ex vivo* that human monocyte-derived dendritic cells (moDCs) undergo maturation and differentiation as response to HNPs, but the DC maturation procedure remains unknown [56]. This mechanism seems to be HNP dose dependent: high concentrations (micromolar range) of HNP are able to directly disrupt cell membranes of microorganisms or some tumor cells, through formation of pores in the cell membrane or by interacting with negatively charged molecules [61, 63]. Lower concentrations of HNP (nanomolar range) are thought to bind specific cell receptors, responsible for chemotactic activities and thus resulting in a immunostimulatory effect [56, 61].

Monocyte-derived dendritic cells seem to play an important role in this HNP-driven immunity: moDCs can internalize and process antigens in their immature state, followed by maturation of moDCs by upregulating MHC class II molecules that react with naive CD4 and CD8 T cells to induce their activation, leading to induction of adaptive immunity [43, 56]. HNPs are thus thought to form a link between innate and adaptive immunity, by serving as chemoattractants and immune cell activators [37]. It is demonstrated that HNP-driven DC activation leads to an increased DC capacity to stimulate T cells, explaining the possible HNP interplay in adaptive immunity [61, 64, 65]. Also in an *in vitro* cancer-related context, HNPs have been produced at the tumor site and showed to be chemoattracting for moDCs and to promote the production of stimulatory cytokines [65]. In this way, an antitumor immune response can be exerted, as an enhanced antigen presentation is established by the HNP-driven DC maturation. This shows promising potential for the use of HNPs in anticancer therapy [66].

3. Conclusions

During the last decades, it became more clear that antimicrobial peptides (AMPs) are not restricted in characteristics as being antibacterial, but that AMPs are also serving as a first-line of defense against fungi, viruses, and even tumor cells. Furthermore, these small cationic molecules have been shown to exhibit potent immune regulatory activities, including chemoattraction, activation and differentiation of leukocytes and monocytes to initiate and further enhance adaptive immunity. Combined, we believe that AMPs may hold great potential to be used as lead for new (co-)therapeutic agents.

With proteomic approaches, more in particular mass spectrometry, it is feasible to identify (cyclic) AMPs in their active form after their corresponding proteolysis. While emphasis has been put strongly on HNP identification in a tumorimmunology-related context, it is also important to stress out that mass spectrometric analyses allows for the detection and identification of native AMPs in different specimens, even when present at low concentrations. These unbiased analyses can lead to the detection and identification of new AMPs, important for future drug development.

In this way, human neutrophil peptide (HNP) 1, 2 and 3 were identified in a non-small cell lung cancer (NSCLC) related context, of which the presence have been shown to be discriminative between a responding and a nonresponding NSCLC patient towards anti-PD-(L)1 immunotherapeutics. Although the biological activity of HNPs is well described against bacteria and viruses, little is known about their antitumor characteristics. Some studies suggest a direct antitumoral activity

of HNP1-3, while it has also been proven that these HNPs do not show a direct cytolytic activity towards NSCLC, but a reduced NSCLC proliferation was observed in the presence of HNP and peripheral blood mononuclear cells (PBMC) *in vitro.* In the latter case, increasement of IFN-γ was observed, referring a cell-mediated immunity. The question raised by these findings is; what is the mechanism of HNP-mediated immunity? Do the HNPs attack the NSCLC cells directly through interaction with the negatively charged phosphatidylserine on the outer leaflet of the tumor cells? This may result in tumor-associated antigen release responsible for activation of a proper immune response. Also, it is increasingly evident that these HNPs are responsible for an enhanced antigen-specific immune response by activating dendritic cells: upon HNP stimulation, dendritic cells are recruited to the tumor site, followed by maturation and differentiation of these dendritic cells, leading to an enhanced adaptive immune response. However, the exact HNP-driven mechanisms are still largely unknown. Further investigation will reveal if HNPs could be a promising approach in future anticancer therapeutics, possibly in synergy with immunotherapy.

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