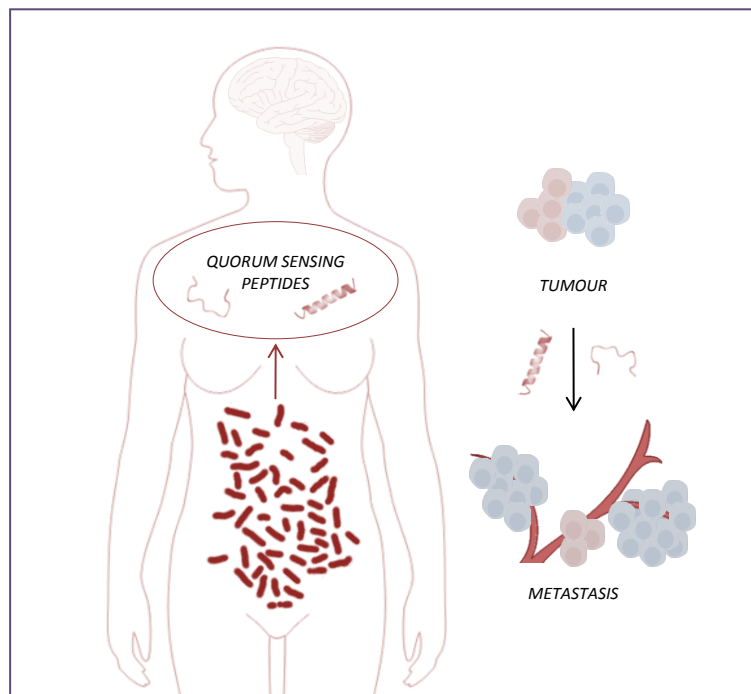# Exploration of quorum sensing peptides:
the missing link between microbiome
and cancer outcome?

Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

Evelien WYNENDAELE

Promotor
Prof. Dr. Bart DE SPIEGELEER

UNIVERSITEIT
GENT

EXPLORATION OF QUORUM SENSING PEPTIDES:
THE MISSING LINK BETWEEN MICROBIOME
AND CANCER OUTCOME?

Evelien WYNENDAELE
Master of Science in Drug Development

Promoter
Prof. Dr. Bart DE SPIEGELEER

2014

Thesis submitted to obtain the degree of
Doctor in Pharmaceutical Sciences

COPYRIGHT

Ghent, 22 December 2014

The promotor   The author

Prof. Dr. Bart De Spiegeleer   Evelien Wynendaele

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of many people.

First of all, I would like to thank my promoter, *prof. Dr. Bart De Spiegeleer*, for his new, controversial research idea at the start-up of this project, as well as his valuable supervision and numerous discussions on the project strategies, which undoubtedly contributed to this work. I really admire his endless drive for research, while at the same time being mindful of his family.

I also want to thank the members of the *reading and examination committee* (*i.e.* prof. Dr. Tom Coenye as chairman, prof. Dr. Ann Van Schepdael, prof. Dr. Marc Bracke, prof. Dr. Christian Burvenich, prof. Dr. Ralf Hoffmann, prof. Dr. Stefaan De Smedt, prof. Dr. Olivier De Wever, Dr. Kathelijne Peremans, prof. Dr. Serge Van Calenbergh and prof. Dr. Christophe Van De Wiele) for reading this work with a critical view; the instructive discussions really improved the quality of this thesis. Special thanks go to *prof. Dr. Christophe Van De Wiele*, who sprouted the idea of studying quorum sensing molecules in humans for inflammation. I am also thankful to *prof. Dr. Marc Bracke and prof. Dr. Olivier De Wever* for offering me their facilities and sharing their knowledge concerning cell-based assays. 'Thank you' as well to *prof. Dr. Christian Burvenich*, for sharing his ideas on this topic and encouraging us when manuscripts are not accepted as smoothly as desired.

I also acknowledge Ghent University and the *Special Research Fund* (BOF number 01J22510) for giving us the financial support to make this research possible.

Next, I am grateful to my former *DruQuaR colleagues* Dr. Jente Boonen, Dr. Sylvia Van Dorpe, Dr. Bram Baert, Dr. Valentijn Vergote, Elien Vangheluwe and the previous lab technicians, who sparked my interest in pharmaceutical research and taught me the necessary skills. I really enjoyed being in the DruQuaR lab, and this is largely due to them and my present colleagues. Matthias D'Hondt, Mathieu Verbeken, Nathalie Bracke, Sultan Suleman, Sofie Stalmans, Kirsten Vandercruyssen, Lieselotte Veryser, Lien Taevernier, Bert Gevaert, Frederick Verbeke, Han Hao, Xiaolong Xu and Marianne Lauwers: thank you for the nice moments during lunch, our organized diners, game nights and of course our truly unforgettable weekend. Let it be the start of a long-lasting friendship!

Moreover, I would like to thank the people I worked with during our collaborations. I am indebted to *prof. Dr. Guy De Tré*, *Dr. Antoon Bronselaer* and *Joachim Nielandt* (UGhent, Department of Telecommunications and Information Processing, Telin) for their help in the accomplishment of the Quorumpeps® database. I am very thankful to all the members of the *LECR group* (UGhent,

Department of Radiation oncology and experimental cancer research) for guiding me through the different techniques, with a special 'thank you' to *Dr. An Hendrix* who introduced me to the operational world of experimental cancer research. For the constructive cooperation in one of the many parts during my PhD and related projects, I am also grateful to many other persons like *prof. Dr. Johan Svenson* (University of Tromsø, Department of Chemistry), the group of *prof. Dr. Dirk Elewaut* (UGhent, Department of Internal Medicine) or *prof. Dr. Gottfried Alber* (University of Leipzig, Institute of Immunology).

Last but foremost, I wish to thank my *family and friends* for their support and help. The distracting diners and joyfull Christmas fairs certainly created the necessary destressing moments I sometimes needed. Steven, thank you for your patience in times of 'deadlines' and for being there at the right moments...

Evelien

LIST OF ABBREVIATIONS AND SYMBOLS

Agr: Accessory gene regulator

AHL: *N*-acyl homoserine lactone

AIP: Autoinducing peptide

Akt: Protein kinase B

AROM: Aromaticity index

ARR: Aromatic ratio

ATCC: American Type Culture Collection

ATS: Broto-Moreau autocorrelation of a topological structure

BBB: Blood-brain barrier

BEH: Highest eigenvalue of Burden matrix

BEL: Lowest eigenvalue of Burden matrix

BFCA: Bifunctional chelating agent

BIP: Bacteriocin Inducing Peptide

BSA: Bovine serum albumin

CagA: Cytotoxin-associated gene A

CAM: Chick Chorioallantoic Membrane

CSF: Competence and Sporulation Factor

CSP: Competence stimulating peptide

DADS: Diamidedithiols

DADT: diaminedithiols

DMSO: Dimethyl sulfoxide

DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

DTPA: Di-ethylene triamine pentaacetic acid

EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDF: Extracellular Death Factor

EDQM: European Directorate for the Quality of Medicines and HealthCare

EGFR: Epidermal growth factor receptor

EMA: European Medicines Agency

EMT: Epithelial to mesenchymal transition

ErbB: Erythroblastosis oncogene B

ESI: Electrospray ionization

FA: Formic acid

Fab: Fragment antigen-binding

FBS: Foetal bovine serum

FDA: Food and Drug Administration

Fmoc: Fluorenylmethyloxycarbonyl

GATS: Geary autocorrelation

GBAP: Gelatinase Biosynthesis-Activating Pheromone

H&E: Hematoxylin & Eosin

HBSS: Hanks' balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER: Human epidermal growth factor receptor

hERG: Human Ether-à-go-go Related Gene

HGF: Hepatocyte growth factor

HILIC: Hydrophilic-Interaction Chromatography

HIV: Human immunodeficiency virus

HOMA: Harmonic Oscillator Model of Aromaticity index

HPLC: High Performance Liquid Chromatography

HYNIC: 6-hydrazinonicotinamide

ID: Identifier

IFN-γ: Interferon gamma

IgG: Immunoglobulin G

IL-6:   Interleukin 6

IUPAC: International Union of Pure and Applied Chemistry

LC: Liquid chromatography

LCL: Lower control limit

LoD: Limit of detection

LR: Lactated Ringer

MAMA: Monoamidemonoaminedithiols

MAPK: Mitogen-activated protein kinase

MEK: Mitogen/Extracellular signal-regulated Kinase

MES: 2-(N-morpholino)ethanesulfonic acid

MS: Mass spectrometry

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

nHAcc: number of H-bond acceptor atoms

nHDon: number of H-bond donor atoms

NHS: *N*-hydroxysuccinimide

NOTA: 1,4,7-triazacyclononane-1,4,7-triacetic acid

Opp: Oligopeptide permease

pAzpa: *p*-azidophenylalanine

pBO$_2$pa: *p*-boronophenylalanine

PC: Principal Component

PCA: Principal Component Analysis

PDA: Photodiode array

PEG: Polyethylene glycol

PEI: Polyethylenimine

PET: Positron emission tomography

Ph. Eur.:   European Pharmacopoeia

Phr: Pheromone

Phr: Phosphatase regulator

PI3K: Phosphatidylinositol 3-kinase

pI: Isoelectric point

pNO$_2$pa: *p*-nitrophenylalanine

PUMA: p53 upregulated modulator of apoptosis

QC: Quality control

QS: Quorum sensing

QSAR: Quantitative structure-activity relationship

QSPR: Quantitative structure-property relationship

R&D: Research and development

Raf: Rapidly Accelerated Fibrosarcoma

RAP: RNAIII-activating protein

Rap: Receptor aspartyl phosphatase

Ras: Rat sarcoma

RDF: Radial Distribution Function

RGD: Arginyl-glycyl-aspartic acid

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RP: Reversed-phase

RP: Rapid prototypes

RRT: Relative retention times

RTK: Receptor tyrosine kinase

SAW:	Surface acoustic wave
SDF-1α:	Stromal cell-derived factor 1
SEM:	Standard error on mean
SFB:	*N*-succinimidyl-4-fluorobenzoate
SIB:	*N*-succinimidyl-3-iodobenzoate
SIM:	Selected ion monitoring
SIPC:	*N*-succinimidyl-5-iodo-3-pyridinecarboxylate
SMILES:	Simplified Molecular Input Line Entry Specification
SPECT:	Single photon emission computed tomography
spp:	Species
SPPS:	Solid-Phase Peptide Synthesis
SPR:	Surface plasmon resonance
SSTR:	Somatostatine receptor
STAT:	Signal transducer and activator of transcription
TAT:	Trans-activating transcriptional activator
TFA:	Trifluoroacetic acid
TGF-α:	Transforming growth factor alpha
Th1/2:	Type 1/ 2 helper T cells
TNF-α:	Tumour necrosis factor alpha
TPSA:	Topological polar surface area
TRAP:	Target of RNAIII-activating protein
TRIS:	Tromethamine
TQ-S:	Triple quadrupole mass spectrometer
UCL:	Upper control limit
uPA:	Urokinase-type plasminogen activator
UPLC:	Ultra Performance Liquid Chromatography
USP:	United States Pharmacopeia
UV:	Ultraviolet
VacA:	Vacuolating cytotoxin A
VEGF:	Vascular endothelial growth factor
VEGFR:	Vascular endothelial growth factor receptor
WHIM:	Weighted Holistic Invariant Molecular
WHO:	World Health Organisation

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

"All thruth passes through three stages. First, it is ridiculed. Second, it is violently opposed.
Third, it is accepted as being self-evident."

Arthur Schopenhauer
(°1788 - †1860; German philosopher)

Parts of this chapter were published:

CHAPTER I

INTRODUCTION

1. THE HUMAN MICROBIOME

The human microbiome, the collection of trillions of microbes in and on the human body, has recently attracted renewed attention through the Human Microbiome Project and the Metagenomics of the Human Intestinal Tract. A full characterisation of the microbial communities found at different human body sites was aimed, next to the exploration of possible correlations between the microbiome composition and health [1]. Studies of the human microbiome revealed that the composition of the microbiome primarily depends on the anatomical location (Figure 1) [2]. Secondly, the microbes occupying different habitats of the human body can differ remarkably between healthy individuals: diet, environmental conditions, host genetics and early microbial exposure were found to play a key role in these personal differences, with other factors still to be evaluated [3].
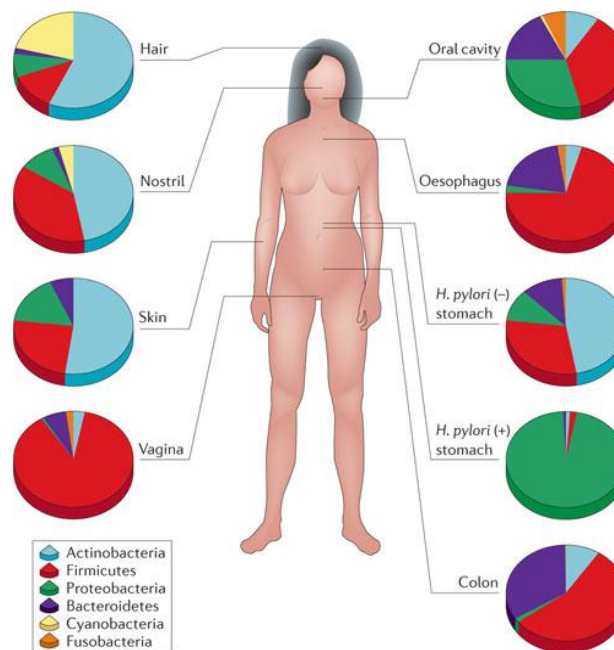

Figure 1. Microbiome composition at different anatomical locations.
The figure indicates percentages of sequences at the taxonomic phylum level,
which are temporarily stable in individuals, at specific body sites [3].

A person's microbiome is believed to originate since birth (vaginal delivery), with the microbial population closely resembling that of the mother's vagina [4]. The initial gastrointestinal microbiome is then enriched by the consumption of mother's milk, which mainly contains *Lactococcus*, *Staphylococcus*, *Streptococcus* and *Weisella* bacterial species [5]. The adult intestinal microbiome is stable over time in healthy persons, but changes with aging (> 65 years). Moreover, inter-individual differences are highly observed within this older population [6].

The microbiome has been indicated to be beneficial for health. Commensal gut bacteria for example supply nutrients, help metabolizing indigestible compounds and defend against colonization by opportunistic pathogens or damage by dietary toxins and carcinogens [7]. These beneficial effects of the gastrointestinal microbiota are elaborated using probiotics, *i.e.* live microorganisms which confer a health benefit on the host when administered in adequate amounts. Clinical evidence indicates their efficacy in the treatment or prevention of *i.a.* acute viral gastroenteritis, post-antibiotic-associated diarrhea and inflammatory bowel disease (IBD) [8].

Besides, the alteration of the human microbiome composition can be associated with different diseases as well, *e.g.* psoriasis [9], gastric adenocarcinoma [10], colorectal cancer [11], inflammatory bowel disease [12,13] and obesity [14]. These malignancies can be initiated by *i.a.* the bacterial-dependent production of genotoxic agents (*e.g.* reactive oxygen species) and toxins (*e.g.* CagA), the increased metabolisation of compounds (*e.g.* oestrogen) or the activation of the adaptive immune response against specific pathogens [11, 15].

2. QUORUM SENSING

Many (commensal and pathogenic) bacteria produce a variety of signal molecules which facilitate them to sense their environment and interact socially, thereby coordinating activities to function as a multicellular unit. This process, called 'quorum sensing', enables bacterial cells to establish cell-cell communication and to regulate the expression of specific genes in response to local changes in cell density [16,17]. Quorum sensing chemicals up till now identified as key components in bacterial cell-cell communication can broadly be divided in three main groups [18]: *N*-acyl homoserine lactone derivatives (AHLs, auto-inducer-1), larger quorum sensing oligopeptides and boron-furanone derivatives (auto-inducer-2). The larger quorum sensing oligopeptides are recently recognized as important bacterial pheromones, especially in Gram-positive organisms [19-22].

In order to be referred to as quorum sensing signals, it is generally accepted that a few criteria should apply. First, its production occurs during specific stages of growth, in response to environmental changes or under certain physiological conditions. Second, an extracellular accumulation of the quorum sensing signal is recognized by a specific receptor. Third, at a critical threshold concentration of this quorum sensing signal, a concerted response is triggered. An important fourth criterion outlines that this response should entail more than just the metabolisation and detoxification of the quorum sensing signal. It enables the population of cells to prepare for problems connected to a high cell density or to take advantage of the opportunities that arise from it [23]. The quorum sensing signals thus serve as a type of 'compartment sensing', informing through diffusion about spatial separation of subpopulations, the number of cells, their physiological state and the environmental conditions they abide in [24].

In this dissertation, the emphasis is only on one group of quorum sensing signal molecules, namely the quorum sensing oligopeptides. A detailed description of the other groups of quorum sensing molecules is already extensively described in several recent review papers [25,26].

The quorum sensing oligopeptide space shows a distinct diversity in structure (Figure 1): cyclic peptides containing a (thio)lacton linkage (*e.g.* AIP-1 in *Staphylococcus aureus*), as well as short linear peptide fragments (*e.g.* PhrC in *Bacillus subtilis* and *Bacillus mojavensis*) are found [21,27]. Moreover, also a posttranslational isoprenylation can occur: for example, the signalling molecule for genetic competence in *Bacillus subtilis*, ComX, is a six amino acid peptide whose tryptophan residue has been modified and a geranyl group attached [28].
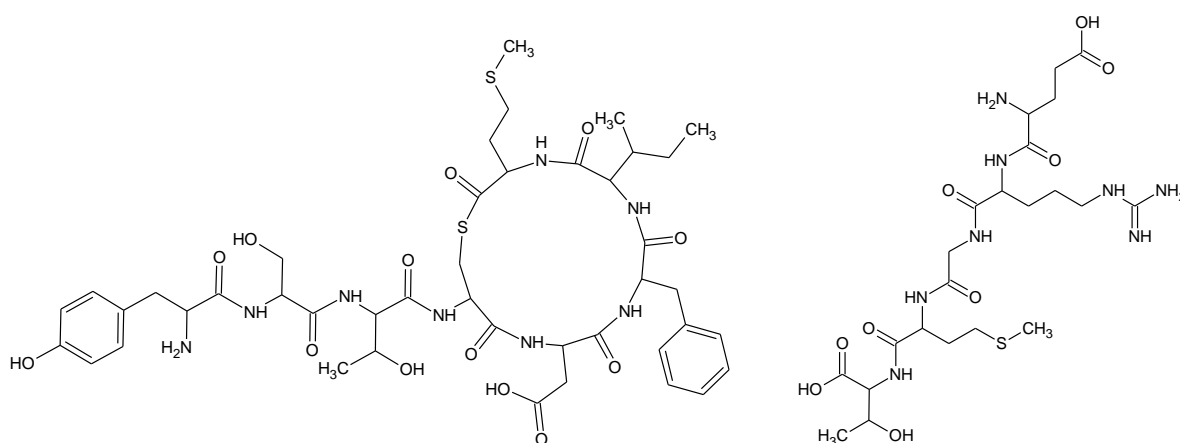

Figure 1. Quorum sensing peptides with diverse structures: AIP-1 from *Staphylococcus aureus* (left) and PhrC from *Bacillus subtilis* (right).

The quorum sensing peptides bind to (1) cell-surface-bound signalling proteins, or (2) cytoplasmic receptors, after which the transcription of one or more target genes is activated (Figure 2). The quorum sensing ligands interact with quorum sensing receptors in a concentration-dependent manner, consistent with traditional concepts of receptor binding [27]. Also consistent with the concepts of receptor binding is the observation that autoinducer ligands acting at the same quorum sensing receptor compete with each other. For example, a series of structural analogs of *N*-3-oxo-dodecanoyl homoserine lactone compete with autoinducer binding to LasR and have varying degrees of agonist activity. Experimental evidence has also shown that cells respond to extremely low concentrations of the quorum sensing signal molecules: only one or two molecules per cell are sufficient, so that low nM concentrations are expected to be active [29].
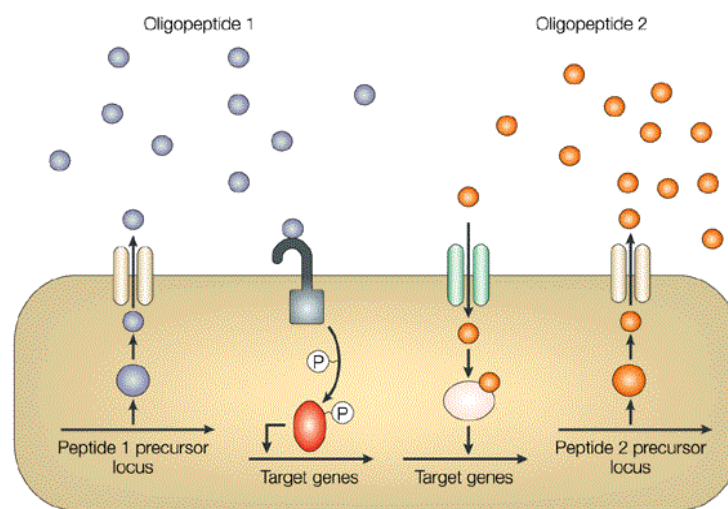
Figure 2. A model for oligopeptide-mediated quorum sensing in Gram-positive bacteria. Oligopeptide signals are synthesized as precursor peptides that are processed, modified and exported using ABC export systems. For some oligopeptides (oligopeptide 1), a cellular two-component signal-transduction system detects the oligopeptide signal and transmits it into the cytosol through phosphorylation-mediated signal transduction from the two-component sensor kinase to the two-component response regulator. The phosphorylated response-regulator protein is thereby activated and regulates the transcription of target genes. For other oligopeptides (oligopeptide 2), the peptides are actively transported into the bacterial cell by a specific oligopeptide permease. The peptide then binds to a cognate regulatory protein, which in turn regulates the transcription of its target genes [30].

The best-studied quorum sensing pathway in Gram-positive bacteria is the system used by *Staphylococcus aureus*. The regulation of its virulence phenotype is mediated by an RNA molecule (RNAIII), whose expression level is controlled by the accessory gene regulator (*agr*) locus. This gene cluster also contains the operon that encodes the genes for the production and detection of the peptide signal AgrD (autoinducing peptide, AIP). Once a threshold concentration of AgrD is reached, the autoinducing peptide binds to a cognate receptor protein, AgrC, located on the cell exterior,

resulting in the activation of a two-component intracellular signalling system: a histidine kinase phosphorylates the intracellular response regulator AgrA, which leads to the regulated gene expression of AgrBDCA and RNAIII [31,32]. In *Staphylococcus aureus*, strains can be divided into four *agr* specific groups, resulting in four markedly different AgrC molecules (AgrC1-4) [33,34]. The integral membrane protein AgrB is involved in the processing and secretion of the autoinducing peptide as a thiolactone-modified cyclic oligopeptide [31,32].

A second quorum sensing system in *i.a. Staphylococcus aureus* has been described as well, modulating the activity of *agr* and consequently, its virulence. This system is proposed to operate upstream of *agr*, controlling the activity of RNAIII via its own autoregulatory mechanism. Once the extracellular concentration of RAP (RNAIII-activating protein) has achieved a sufficient density, it activates its target protein, TRAP, and induces the phosphorylation of three conserved histidine residues. From here, TRAP interacts with the *agr* system [35,36].

Like for *S. aureus*, the ComX-derived peptide pheromones in *e.g. Bacillus subtilis* are mediated by a sensor histidine kinase (ComP) and a response regulator (ComA) protein as well. After binding of the peptide pheromone to ComP, ComA is activated via signal transduction, which ultimately results in the development of genetic competence and the transcription of quorum sensing controlled genes. The synthesized ComX peptide is then cleaved and modified by ComQ before export to the extracellular environment occurs [37,38].

The second type of peptide signalling pathway in *i.a. Bacillus subtilis* involves the cell-penetration of CSF (Competence and Sporulation Factor) peptides through an oligopeptide permease (Opp) and the subsequent interaction with the cytoplasmic receptor Rap (receptor aspartyl phosphatase). The *Bacillus subtilis* family of Rap phosphatases comprises 11 members, of which RapA, RapC, RapE and RapF are most frequently observed [39]. Intracellularly, the phosphatase regulator (Phr)-derived peptide inhibits the phosphatase activity of the Rap receptor, resulting in a phosphorylated ComA protein and sporulation induction [38,40].

The quorum sensing signal molecules were originally found as intra-species communication tools in bacteria, but recent evidence indicates interspecies and host signalling as well [41,42]: AHLs have been found to be directly recognised by eukaryotic cells and even to influence the behaviour of eukaryotic organisms. For example, 3-oxo-dodecanoyl homoserine lactone exhibits potent immune modulatory activities by suppressing the interleukin- (IL-) 12 and tumour necrosis factor alpha (TNF-α) secretion [43]. Another example indicates that some AHLs can have cardiovascular effects by inducing relaxation of blood vessels, next to the induction of apoptosis in several breast cancer cell lines by 3-oxo-dodecanoyl homoserine lactone, the quorum sensing molecule secreted by

Pseudomonas aeruginosa [17, 44]. A third example concerns the quorum sensing peptide antagonist, siamycin, which seems to qualify as an HIV-cell fusion inhibitor. This molecule exerts a strong inhibitory effect on syncytium formation, targeting the HIV envelope glycoprotein gp41 [45,46]. In addition, the *Bacillus subtilis* quorum sensing peptide CSF (Competence and Sporulation Factor) activates p38 mitogen-activated protein kinase and protein kinase B in host intestinal epithelial cells and induces cytoprotective heat shock protein synthesis [47]. Despite these interesting 'crosstalk' observations between bacteria and eukaryotes, research mainly focuses on the bacterial intra- and interspecies signalling cascades as a tool to develop new antibiotic therapeutics. Quorum sensing inhibition, the mechanisms developed to interfere with the bacterial cell-to-cell communication pathways, has used quorum sensing antagonists to inhibit (virulence) gene expression or enzymes that interact with quorum sensing molecules ('quorum quenching'), leading to deactivation of the quorum sensing process [48].

3. PEPTIDES

Therapeutic peptides have taken a long time to come of age, with the first chemical synthesis of a therapeutic peptide, *i.e.* oxytocin, in 1953. All-in-all, in 2012, about 65 peptide-based drug products have reached approval, with over 75% of these coming in the last three decades; 100-200 peptides are currently in clinical trials and 400-600 in pre-clinical studies (Figure 3). The number of new peptide-based drug products achieving approval, together with the widening range of medical indications, thus underlines the increasing maturity of peptides as a class of pharmaceutical actives. Therapeutic peptides contribute significantly to the treatment of diabetes, osteoporosis, oncologic malignancies, gastroenterologic diseases, cardiovascular disorders, immune suppression, acromegaly, enuresis, obesity, or have antiviral, antibacterial or antifungal indications [49-51].
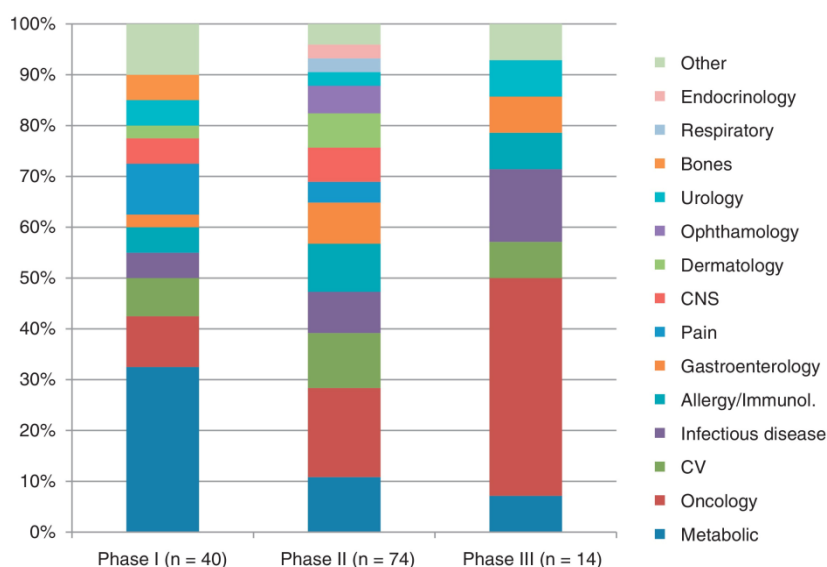
Figure 3. Overview of disease classes in which peptides are currently investigated for their therapeutic potential (clinical studies, situation in 2013) [52].

Peptide-based research, diagnostic and therapeutic compounds have attracted great attention owing to their high affinity, strong selectivity for their targets and low toxicity. In contrast to proteins and antibodies, they have a good penetration of tissues because of their small size, are less immunogenic, show more specific tissue binding and are chemically more under consistent control, thus leading to more functional quality [53,54]. The advantages over small molecules include the higher selectivity for their targets, the greater efficacy, the lower toxicity of their degradation products and the limited tissue-accumulation. Despite these attractive features, peptides have an increased proteolytic instability compared to small molecules and monoclonal antibody therapeutics. To meet this main drawback, peptides can be cyclised or chemically modified at both the amide bonds and side-chains, rendering peptidomimetics that are resistant to this enzymatic degradation. Moreover, peptides show a poor ability to cross physiological barriers, including the intestinal mucosa, because of their general hydrophilicity. Bioavailability is therefore optimised using novel peptide delivery technologies for different routes of administration, *e.g.* dermal patches, nasal spray or protease inhibitors for oral delivery [55,56].

4. ONCOLOGY

Cancer is one of the leading causes of death worldwide and accounts for approximately 8 million deaths per year. This disease can affect everyone and represents a tremendous burden on patients, families and society [57,58]. Lung (1.59 million), liver (745 000), stomach (723 000), colon (694 000)

and breast (521 000) cancer cause the most cancer deaths each year. The changes in cell properties are mostly the result of the interaction between a person's genetic factors and several categories of external agents, including physical (*e.g.* radiation), chemical (*e.g.* tobacco smoke) and biological (*e.g.* infections) carcinogens [57].

Conventional cancer therapies, such as surgery or radiation to achieve local control and chemotherapy to exert systemic effects, are currently the methods of choice for cancer management [59,60]. However, there is still a clear need for the development of innovative preventive, diagnostic and therapeutic strategies, maximizing cure rates with minimal toxicity that exploit the emerging insights in tumour biology and the increased pharmaceutical developmental capabilities [61,62]. During the last decades, different peptides were investigated for their antitumour characteristics in order to fill this therapeutic and diagnostic gap.

The underlying action mechanisms of antitumour peptides are diverse, with multiple actions for some peptides demonstrated, offering the advantage of combination therapy in a single compound [53]. For example, some of the necrosis-inducing peptides and apoptosis-inducing peptides have also additional anti-angiogenic properties, *e.g.* the lactoferricin-derived peptides [63-65].

Necrosis-inducing peptides are a group of cell membrane lytic peptides. The outer membranes of cancer cells over-express negatively charged phosphatidylserine and O-glycosylated mucins in comparison with normal cells, favouring their selective attack by necrosis-inducing peptides, which are mainly short, positively charged amphipathic structures. Upon binding of the necrosis-inducing peptides to cell-membranes, these are disrupted via micellization or through pore formation, leading to the necrotic death of cancer cells. Necrosis-inducing peptides have a higher degree of selectivity for cancer cells than traditional chemotherapeutic drugs. Moreover, these peptides kill chemoresistant cancer cells by membrane lysis, bypassing the cellular mechanisms responsible for multidrug resistance. Cecropins, melittins and defensins are examples of currently known necrosis-inducing peptides [53,63].

Another action mechanism is based on the knowledge that cancer cells have an elevated apoptotic threshold: several peptides such as magainin-2, bovine lactoferricin and cathelicidin derived peptides (*e.g.* BMAP-28, hCAP$_{109-135}$), as well as several plant-derived peptides have been shown to induce mitochondria-dependent intrinsic apoptosis [61]. Other apoptotic pathways, such as the p53-controlled apoptosis regulation or through extrinsic death receptors like Tumour necrosis factor receptor (TNFR), are targeted by peptides as well, *e.g.* Ala-Val-Pro-Ile tetrapeptide [66].

As tumours express increased proteolytic enzymes facilitating invasion and metastasis, *e.g.* cathepsins, urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs), selective protease inhibitor peptides have been shown to suppress migration and inhibition, *e.g.* His-

Trp-Gly-Phe tetrapeptide [67]. Other peptides have anti-angiogenic properties, *e.g.* CVX15, due to their interaction with cancer cell-surface selective receptors [68]. Some peptides have been shown to bind to cell-adhesion proteins as well, *e.g.* the Arg-Gly-Asp tripeptide derivatives like cilengitide are able to inhibit αvβ3 integrin-mediated cell adhesion to extracellular matrix proteins, thereby blocking tumour invasion and angiogenesis [69,70]. Interactions with laminin receptors or neural-cadherin glycoproteins are other cell-adhesion targets for peptide drugs, *e.g.* Tyr-Ile-Gly-Ser-Arg pentapeptide [71]. Protein kinases are commonly activated in cancer cells, and have hence become an attractive target for anticancer therapy as well, whereby small peptides have been developed to inhibit the interaction of kinases with their substrates [53]. Last, peptides with immunostimulatory activity have been identified [72,73], activating the innate and acquired immune system, *e.g.* muramyl dipeptide, and its analogues like romurtide, or bestatin which has in addition shown also protease inhibitor and anti-angiogenic activity. These peptides stimulate macrophages, natural killer cells and T-lymphocytes to release proinflammatory molecules like IFN-γ [74,75].

Recently, attempts have been started to integrate the different peptide functionalities: antimicrobial peptides, which have been found in bacteria, fungi, plants and animals, are investigated [76] and indeed emerging as anticancer peptides as well, belonging to the apoptosis-inducing, necrosis-inducing, anti-angiogenic or immune-stimulating peptides [61,77-80]. In conclusion, while the exact inhibitory mechanisms of the different antitumour peptides are far from being elucidated, current data at least show great potential and different possibilities, depending upon the peptide structure and the heterogeneous tumour biology.

Already now, many peptides are used in the treatment and diagnosis of various cancers, while the development of nutraceuticals containing cancer-preventive peptides is a very promising area as well [81]. Chronic administration of Luteinizing Hormone-Releasing Hormone agonists (*e.g.* Decapeptyl, Leuprolide, Buserelin and Goserelin) is being utilized to induce the regression of prostate and breast cancer [62]. Somatostatin analogues (Octreotide, Lanreotide) are used for the treatment of neuroendocrine tumours of the gastroenteropancreatic system [62,82]. The molecular basis for this clinical application is the presence of a high density of somatostatin receptors, whereby the subtypes (sst_1, sst_2, sst_3, sst_4 and sst_5) are differently expressed in the various types of cancer [83]. Moreover, the radiolabelled peptidic compound ^{111}In-DTPA-[D-Phe^1]octreotide has become the most widely used tracer for somatostatin receptor scintigraphy, while ^{90}Y-DOTA-Tyr^3-octreotide is the most frequently used analogue for radiotherapy [84]. Currently, 21 peptide drugs are marketed for cancer therapy, and the total peptide drug pipeline (in oncology) contains 232 peptide drugs in various stages of clinical development, applied in 587 developmental cancer projects [85].

5. STUDY OBJECTIVES

Despite the increasing evidence of the human microbiome influencing health, up till now, no research has been performed describing the role of quorum sensing peptides on tumour progression. However, important parallels are seen between the bacterial quorum sensing mechanisms and the biochemical and biological mechanisms metastatic cancer cells use to function as a society of malignant cancer cells. The process of biofilm formation has many of the characteristics of metastatic colonization, including motility of cells towards appropriate surfaces, attachment and interaction of cells with each other, surface adhesion and colonization, formation of a complex, heterogeneity,... [86].

We surely believe this information can be of high importance, not only from a fundamental point of view (*i.e.* "Do quorum sensing molecules interact with cancer cells and how is this interaction achieved?"), but also for health in general (*i.e.* "Does this interaction affect cancer fate, either positively or negatively?"). Therefore, **the goal of this thesis is to explore the effect of quorum sensing peptides on tumour cell behaviour**. Obviously, it is impossible to obtain a complete and full explanation of this crosstalk phenomenon within the given time and resource boundaries. However, the goal is to have some foundations laid down for the first time where upon future research (*e.g.* in-depth fundamental and biomedical investigations) can be built.

In order to explore this interaction, different questions are put forward as objectives:

(1) **Which quorum sensing peptides are produced by which bacteria and what is their current bacterial functionality?**

A comprehensive quorum sensing peptide database will be developed to obtain a structured overview of these peptides and their synthetic peptide analogues. Their species origin, as well as the functional information currently available, will also be included.

(2) **How is the chemical space of these quorum sensing peptides organized?**

Structural information of the quorum sensing peptides will be used to cluster these peptides and to select model compounds.

(3) **Can a possible effect on tumour cells be caused by peptide impurities originating from peptide synthesis?**

In order to correctly assign functional properties to the quorum sensing peptides, the purity of chemically synthesized quorum sensing peptides will be investigated using chromatographic techniques. As some peptide impurities can be biomedically active or even alter the main peptide's activity, it is important to explore this (often neglected) quality

parameter, already in this very early, exploratory basic biomedical research. The investigation towards the functional quality of (peptide) pharmaceuticals is the main research topic of DruQuaR, so this third objective fits perfectly the strategic ambition of our laboratory.

(4) Do quorum sensing peptides interact with colorectal cancer cells and can they influence the metastatic processes involved in tumorigenesis?

Seen the increasing evidence of microbiome-related development of colorectal cancer, selected quorum sensing peptides will be investigated for their crosstalk behaviour with human colorectal adenocarcinoma cells, *i.e.* HCT-8/E11. Both tumour invasive and pro-angiogenic properties will be studied to explore the metastatic potential of these molecules.

(5) Can the observed crosstalk between quorum sensing peptides and tumour cells be elaborated to breast cancer?

The very recent identification of different Gram-positive bacteria in the breast tissue excited the research in the microbiome's influence on breast cancer cells. To this end, we will investigate the effect of quorum sensing peptides, synthesized by these bacteria, on breast adenocarcinoma cells (*i.e.* MCF-7/AZ).

(6) Do quorum sensing peptides, when present in the blood circulation, penetrate the blood-brain barrier and what is their tissue distribution?

One of the narrowest barriers in the human body is the blood-brain barrier (BBB), protecting the central nervous system from potentially harmful chemicals while regulating transport of essential molecules and maintaining a stable environment. Moreover, there is increasing evidence that different central nervous system disorders are microbiome-related. Up till now however, no data are available concerning the blood-brain barrier permeability characteristics of quorum sensing peptides which may be present in the blood.

(7) Can quorum sensing peptides, when present in the intestines, reach the blood circulation and what is their possible toxicity on different cell types?

The human gut consists of trillions of microorganisms, with some of these bacterial species capable of producing quorum sensing peptides. Therefore, in this thesis, the permeability of some quorum sensing peptides through the intestinal barrier will be investigated in order to evaluate the possible tumour-influencing effects elsewhere in the human body. When quorum sensing peptides reach the blood circulation, it is important to explore their toxicity as well. Hence, the effect on red blood cell haemolysis will be investigated, next to their direct toxicity on both healthy and cancerous cells. Moreover, the stability of the quorum

sensing peptides will be investigated in cell medium as well as in human plasma, in order to support the *in vitro* and *in vivo* significance of the biological functionality results.

(8) How can radiolabelled peptides be developed for use in biomedical research, as diagnostics or therapeutics?

In the last chapter, consistent with the DruQuaR tradition, a more general regulatory-quality oriented overview is presented. We reviewed the quality aspects of radiolabelled peptides as used not only in the biomedical research but also as diagnostics or therapeutics in the current shift towards personalized medicine.

6.   THESIS OUTLINE

As this exploratory research covered different aspects in parallel, the chapters of this thesis are not chronologically ordered, but rather go from *in silico* over quality control to cell functionality, pharmacokinetics and toxicity, ending with a more formal regulatory overview in line with DruQuaR tradition. Therefore, each chapter is written and presented so that it can be read as a "stand-alone" text, with the introduction giving the specific context of the chapter.

In **Chapter II**, the Quorumpeps® database is presented, which gives a structured, literature-based overview of the currently known quorum sensing peptides. In this database, information concerning their chemical characteristics, their microbial origin (species) and their bacterial functionality (receptor and activity) is summarized, thereby serving as a central information point about quorum sensing peptides. Moreover, this database serves as a useful tool to rationalize peptide choices for evaluating different responses or to study quantitative structure-property relationships (QSPR) of these quorum sensing molecules.

In **Chapter III**, the chemical information, obtained from the Quorumpeps® database, is used to explore the chemical space of quorum sensing peptides. Using multivariate techniques, a number of chemically distinct peptide groups can be distinguished, which serves as a basic tool for 'model peptide' selection. Besides, the species distribution is investigated as well, demonstrating the diversity of synthesized peptide structures by specific bacterial species.

In **Chapter IV**, the purity of a set of chemically synthesized quorum sensing peptides is screened using U/HPLC-UV and the identity of main peak and related impurities examined using U/HPLC-ESI/MS. This study intends to demonstrate the importance of a thorough characterization of peptides when used during functional assays, even in basic biomedical research settings.

The main research question "Do quorum sensing peptides influence tumour cell behaviour?" is partly answered in **Chapter V**. In this chapter, the metastatic potential of selected quorum sensing peptides on colorectal cancer cells is investigated both *in vitro* and *in vivo* by collagen type I invasion and Chick Chorioallantoic Membrane (CAM) assays, respectively. Moreover, an initial pathway-map is developed to elucidate the effects of quorum sensing peptides on these cancer cells. The same approach is used for breast cancer cells, with the results of this study described in **Chapter VI**. Seen the recent findings of a possible breast (cancer) tissue microbiome, the effects of the quorum sensing peptides on breast cancer progression can confirm the microbiome-tumour crosstalk observations. By using the word 'crosstalk', emphasis is placed on bacterial to mammalian communication. Both studies (Chapter V and VI) intend to indicate, as an initial proof-of-principle, that the human microbiome, through their quorum sensing peptides, is one of the factors influencing cancer metastasis.

In **Chapter VII**, the permeability of chemically diverse quorum sensing peptides through the blood-brain barrier (BBB) is investigated. Although no effect of the quorum sensing peptides on brain (cancer) tissue is yet described, the permeability kinetics of these peptides can guide future functionality assays: only quorum sensing peptides that cross this physiological barrier should be studied for their effect on the central nervous system and its disorders.

In **Chapter VIII**, the *in vitro* stability of selected quorum sensing peptides is investigated in cell medium as well as in human plasma, in order to correctly interpret the *in vitro* and *in vivo* functionality results obtained in Chapter V, VI and VII. Moreover, the intestinal permeability of some quorum sensing peptides is studied as well, of which the results can indicate the possibility of crosstalk behaviour at distant sites of the human body. Finally, the toxicity of the quorum sensing peptides is initially explored. This study thus intends to correlate the obtained functionality results with initial peptide stability and toxicity information.

Chapter IX gives an overview of the currently available techniques that are used in the development of radiolabelled peptides and proteins. Moreover, the importance of thorough quality control measurements after radiolabelling within a regulated context is emphasized. Based on our exploratory findings of quorum sensing peptide interaction with cancer cells, the information presented in this chapter can be applied to more efficiently develop radiolabelled analogues.

7.   REFERENCES

[1]   http://www.hmpdacc.org/ (September 2014).

[2]   Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* 2012; **13**: 260-270.

[3]   The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* 2012; **486**: 207-214.

[4]   Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. U.S.A.* 2010; **107**: 11971–11975.

[5]   Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am. J. Clin. Nutr.* 2012; **96**: 544-551.

[6]   Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HMB, Coakleu M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RR, O'Toole PW. Gut microbiota composition correlates with diet and health in the elderly. *Nature* 2012; **488**: 178-185.

[7]   Neish AS. Microbes in gastrointestinal health and disease. *Gastroenterology* 2009; **136**: 65-80.

[8]   Park J, Floch MH. Prebiotics, probiotics, and dietary fiber in gastrointestinal disease. *Gastroenterol. Clin. North. Am.* 2007; **36**: 47–63.

[9]   Gao Z, Tseng CH, Strober BE, Pei Z, Blaser MJ. Substantial alterations of the cutaneous bacterial biota in psoriatic lesions. *PLoS ONE* 2008; **3**: e2719.

[10]   McColl KE. Clinical practice. *Helicobacter pylori* infection. *N. Engl. J. Med.* 2010; **362**: 1597–1604.

[11]   Plottel CS, Blaser MJ. Microbiome and malignancy. *Cell Host Microbe* 2011; **10**: 324–335.

[12]   Mondot S, Kang S, Furet JP, Aquirre de Carcer D, McSweeney C, Morrison M, Marteau P, Doré J, Leclerc M. Highlighting new phylogenetic specificities of Crohn's disease microbiota. *Inflamm. Bowel Dis.* 2011; **17**: 185–192.

[13]   Bibiloni R, Schiffrin EJ. Intestinal host-microbe interactions under physiological and pathological conditions. *Int. J. Inflam.* **2010**; 386956.

[14]   Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022–1023.

[15]   Schwabe RF, Jobin C. The microbiome and cancer. *Nat. Rev. Cancer* 2013; **13**: 800-812.

[16]   Martin CA, Hoven AD, Cook AM. Therapeutic frontiers: preventing and treating infectious diseases by inhibiting bacterial quorum sensing. *Eur. J. Clin. Microbiol. Infect. Dis.* 2008; **27**: 635-642.

[17]   Diggle SP, Crusz SA, Cámara M. Quorum sensing. *Curr. Biol.* 2007; **17**: 907-910.

[18]   Konaklieva MI, Plotkin BJ. Chemical communication – Do we have a quorum? *Mini Rev. Med. Chem.* 2006; **6**: 817-825.

[19]   Ibrahim M, Guillot A, Wessner F, Algaron F, Besset C, Courtin P, Gardan R, Monnet V. Control of the transcription of a short gene encoding a cyclic peptide in *Streptococcus thermophilus*: a new quorum-sensing system. *J. Bacteriol.* 2007; **189**: 8844-8854.

[20]   Griffith KL, Grossman AD. A degenerate tripartite DNA-binding site required for activation of ComA dependent quorum response gene expression in *Bacillus subtilis*. *J. Mol. Biol.* 2008; **381**: 261-275.

[21]   Pottathil M, Jung A. CSF, a species-specific extracellular signaling peptide for communication among strains of *Bacillus subtilis* and *Bacillus mojavensis*. *J. Bacteriol.* 2008; **190**: 4095-4099.

[22]   Nishiguchi K, Nagata M, Tanokura M, Sonomoto K, Nakayama J. Structure-Activity Relationship of Gelatinase Biosynthesis-Activating Pheromone of *Enterococcus faecalis*. *J. Bacteriol.* 2009; **191**: 641-650.

[23]   Winzer K, Hardie KR, Williams P. Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr. Opin. Microbiol.* 2002; **5**: 216-222.

[24]   Dunny GM, Leonard BA. Cell-cell communication in gram-positive bacteria. *Annu. Rev. Microbiol.* 1997; **51**: 527–564.

[25]   Galloway WRJD, Hodgkinson JT, Bowden SD, Welch M, Spring DR. Quorum sensing in gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem. Rev.* 2011; **111**: 28-67.

[26]   Garg N, Manchanda G, Kumar A. Bacterial quorum sensing: circuits and applications. *Antonie Van Leeuwenhoek* 2014; **105**: 289-305.

[27]   Ni N, Li M, Wang J, Wang B. Inhibitors and antagonists of bacterial quorum sensing. *Med. Res. Rev.* 2009; **29**: 65-124.

[28]   Okada M, Sato I, Cho SJ, Iwata H, Nishio T, Dubnau D, Sakagami Y. Structure of the *Bacillus subtilis* quorum-sensing peptide pheromone ComX. *Nat. Chem. Biol.* 2005; **1**: 23-24.

[29]   Von Bodman SB, Willey JM, Diggle SP. Cell-cell communication in bacteria: united we stand. *J. Bacteriol.* 2008; **190**: 4377-4391.

[30]   Lazdunski AM, Ventre I, Sturgis JN. Regulatory circuits and communication in Gram-negative bacteria. *Nat. Rev. Microbiol.* 2004; **2**: 581-592.

[31]   Gorske BC, Blackwell HE. Interception of quorum sensing in *Staphylococcus aureus*: a new niche for peptidomimetics. *Org. Biomol. Chem.* 2006; **4**: 1441-1445.

[32]   Sifri CD. Quorum sensing: bacteria talk sense. *Clin. Infect. Dis.* 2008; **47**: 1070-1076.

[33]   Ji G, Beavis R, Novick RP. Bacterial Interference Caused by Autoinducing Peptide Variants. *Science* 1997; **276**: 2027-2030.

[34]   Ni N, Li M, Wang J, Wang B. Inhibitors and Antagonists of Bacterial Quorum Sensing. *Med. Res. Rev.* 2008; **29**: 65-124.

[35]   Shaw LN, Jonsson I, Singh VK, Tarkowski A, Stewart GC. Inactivation of trap has no effect on the agr quorum-sensing system or virulence of *Staphylococcus aureus*. *Infect. Immun.* 2007; **75**: 4519-4527.

[36]   Korem M, Sheoran AS, Gov Y, Tzipori S, Borovok I, Balaban N. Characterization of RAP, a quorum sensing activator of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 2003; **223**: 167-175.

[37]   Lazazzera BA, Solomon JM, Grossman AD. An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*. *Cell* 1997; **89**: 917-925.

[38]   Comella N, Grossman AD. Conservation of genes and processes controlled by the quorum response in bacteria: characterization of genes controlled by the quorum-sensing transcription factor ComA in *Bacillus subtilis*. *Mol. Microbiol.* 2005; **57**: 1159-1174.

[39]   Perego M, Brannigan JA. Pentapeptide regulation of aspartyl-phosphate phosphatases. *Peptides* 2001; **22**: 1541-1547.

[40] Bischofs IB, Hug JA, Liu AW, Wolf DM, Arkin AP. Complexity in bacterial cell-cell communication: Quorum signal integration and subpopulation signaling in the *Bacillus subtilis* phosphorelay. *Proc. Natl. Acad. Sci. U.S.A.* 2008; **106**: 6459-6464.

[41] Gitzinger M, Parsons J, Reski R, Fussenegger M. Functional cross-kingdom conservation of mammalian and moss (*Physcomitrella patens*) transcription, translation and secretion machineries. *Plant Biotechnol. J.* 2009; **7**: 73-86.

[42] Williams P. Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology* 2007; **153**: 3923-3938.

[43] Pritchard DI. Immune modulation by *Pseudomonas aeruginosa* quorum-sensing signal molecules. *Int. J. Med. Microbiol.* 2006; **296**: 111–116.

[44] Li L, Hooi D, Chhabra SR, Pritchard D, Shaw PE. Bacterial N- acylhomoserinelactone-induced apoptosis in breast carcinoma cells correlated with down-modulation of STAT3. *Oncogene* 2004; **23**: 902-4894.

[45] Nakayama J, Tanaka E, Kariyama R, et al. Siamycin Attenuates fsr Quorum Sensing Mediated by a Gelatinase Biosynthesis-Activating Pheromone in *Enterococcus faecalis*. *J. Bacteriol.* 2007; **189**: 1358-1365.

[46] De Clercq E. Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. *Med. Res. Rev.* 2000; **20**: 323-349.

[47] Fujiya M, Musch MW, Nakagawa Y, Hu S, Alverdy J, Kohgo Y, Schneewind O, Jabri B, Chang EB. The *Bacillus subtilis* quorum-sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. *Cell Host Microbe* 2007; **1**: 299-308.

[48] Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* 2005; **21**: 319-346.

[49] Ali R, Rani R, Kumar S. New peptide based therapeutic approaches. In *Advances in Protein Chemistry* (Eds: Ashraf G, Sheikh IA), OMICS eBook group, **2013**, pp. 3-15.

[50] Global Business Intelligence Research. Protein and peptide-based therapeutics – Trends in drug discovery and development. *Research and Markets* **2012**; GBIHC226MR.

[51] Sun L. Peptide-based drug development. *Mod. Chem. Appl.* 2013; **1**: 1000e103.

[52] Kaspar AA, Reichert JM. Future directions for peptide therapeutics development. *Drug Discov. Today* 2013; **18**: 807-817.

[53] Bhutia SK, Maiti TK. Targeting tumors with peptides from natural sources. *Trends Biotechnol.* 2008; **26**: 210-217.

[54] Aina OH, Liu RW, Sutcliffe JL, Marik J, Pan CX, Lam KS. From combinatorial chemistry to cancer-targeting peptides. *Mol. Pharm.* 2007; **4**: 631-651.

[55] Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. *Drug Discov. Today* 2010; **15**: 40-56.

[56] Otvos L, Wade JD. Current challenges in peptide-based drug discovery. *Front. Chem.* 2014; **2**: doi 10.3389/fchem.2014.00062.

[57] World Health Organisation, Cancer, http://www.who.int/topics/cancer/en/ (February 2014).

[58] Alberts B. The challenge of cancer. *Science* 2011; **331**: 1491.

[59] Kitajima K, Murakami K, Yamasaki E, Domeki Y, Kaji Y, Sugimura K. Performance of FDG-PET/CT for diagnosis of recurrent uterine cervical cancer. *Eur. Radiol.* 2008; **18**: 2040-2047.

[60] Clin B, Morlais F, Guittet L, et al. Performance of chest radiograph and CT scan for lung cancer screening in asbestos-exposed workers. *Occup. Environ. Med.* 2009; **66**: 529-534.

[61]   Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. *Biochim. Biophys. Acta* 2008; **1778**: 357-375.

[62]   Schally AV. New approaches to the therapy of various tumors based on peptide analogues. *Horm. Metab. Res.* 2008; **40**: 315-322.

[63]   Mader JS, Hoskin DW. Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment. *Expert Opin. Investig. Drugs* 2006; **15**: 933-946.

[64]   Zhang L, Ming L, Yu J. BH3 mimetics to improve cancer therapy: mechanisms and examples. *Drug Resist. Updat.* 2007; **10**: 207-217.

[65]   Shimura T, Suehiro T, Suzuki H, Mochida Y, Okada K, Mochiki E, Kuwano H. Peptides derived from a soluble molecule of the human leukocyte antigen (HLA) class I cause apoptosis in gastric cancer cell lines. *Dig. Dis. Sci.* 2009; **54**: 63-69.

[66]   Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X, Shi Y. Structural basis of IAP recognition by Smac/DIABLO. *Nature* 2000; **408**: 1008-1012.

[67]   Koivunen E, Arap W, Valtanen H. Tumor targeting with a selective gelatinase inhibitor. *Nat. Biotechnol.* 1999; **17**: 768-774.

[68]   Wu B, Chien YET, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, Stevens RC. Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science* 2010; **330**: 1066-1071.

[69]   Liu Z, Wang F, Chen X. Integrin alpha(V)beta(3)-targeted cancer therapy. *Drug Dev. Res.* 2008; **69**: 329-339.

[70]   Reardon DA, Nabors LB, Stupp R, Mikkelsen T. Cilengitide: an integrin-targeting arginine-glycine-aspartic acid peptide with promising activity for glioblastoma multiforme. *Expert Opin. Investig. Drugs* 2008; **17**: 1225-1235.

[71]   Ardini E, Sporchia B, Pollegioni L, Modugno M, Ghirelli C, Castiglioni F, Tagliabue E, Ménard S. Identification of a Novel Function for 67-kDa Laminin Receptor: Increase in Laminin Degradation Rate and Release of Motility Fragments. *Cancer Res.* 2002; **62**: 1321-1325.

[72]   Jin M, Komohara Y, Shichijo S, Yamanaka R, Nikawa J, Itoh K, Yamada A. Erythropoietin-producing hepatocyte B6 variant-derived peptides with the ability to induce glioma-reactive cytotoxic T lymphocytes in human leukocyte antigen-A2$^+$ glioma patients. *Cancer Sci.* 2008; **99**: 1656-1662.

[73]   Yamanaka R. Cell-and peptide-based immunotherapeutic approaches for glioma. *Trends Mol. Med.* 2008; **14**: 228-235.

[74]   Ueda H, Yamazaki M. Induction of tumor necrosis factor-α in solid tumor region by the orally administered synthetic muramyl dipeptide analogue, romurtide. *Int. Immunopharmacol.* 2001; **1**: 97-104.

[75]   Schorlemmer HU, Bosslet K, Sedlacek HH. Ability of the immunomodulating dipeptide bestatin to activate cytotoxic mononuclear phagocytes. *Cancer Res.* 1983; **43**: 4148-4153.

[76]   Powers JPS, Hancock REW. The relationship between peptide structure and antibacterial activity. *Peptides* 2003; **24**: 1681-1691.

[77]   Kondo E, Seto M, Yoshikawa K, Yoshino T. Highly efficient delivery of p16 antitumor peptide into aggressive leukemia/lymphoma cells using a novel transporter system. *Mol. Cancer Ther.* 2004; **3**: 1623-1630.

[78]   Kondo E, Tanaka T, Miyake T, Ichikawa T, Hirai M, Adachi M, Yoshikawa K, Ichimura K, Ohara N, Moriwaki A, Date I, Ueda R, Yoshino T. Potent synergy of dual antitumor peptides for growth suppression of human glioblastoma cell lines. *Mol. Cancer Ther.* 2008; **7**: 1461-1471.

[79]   Meyer JE, Harder J. Antimicrobial peptides in oral cancer. *Curr. Pharm. Des.* 2007; **12**: 3119-3130.

[80]   Parodi PW. A role for milk proteins and their peptides in cancer prevention. *Curr. Pharm. Des.* 2007; **13**: 813-828.

[81]   De Mejia EG, Dia VP. The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metastasis Rev.* 2010; **29**: 511-528.

[82]   Strosberg J, Kvols L. Antiproliferative effect of somatostatin analogs in gastroenteropancreatic neuroendocrine tumors. *World J. Gastroenterol.* 2010; **16**: 2963-2970.

[83]   Appetecchia M, Baldelli R. Somatostatin analogues in the treatment of gastroenteropancreatic neuroendocrine tumours, current aspects and new perspectives. *J. Exp. Clin. Cancer Res.* 2010; **29**: 19.

[84]   Reubi JC. Peptide Receptors as Molecular Targets for Cancer Diagnosis and Therapy. *Endocr. Rev.* 2003; **24**: 389-427.

[85]   BioSeeker group, Peptides in Oncology Drug Pipeline Update 2014, http://www.researchandmarkets.com/reports/1196702/peptides_in_oncology_drug_pipeline_update_2014 (July 2014).

[86]   Hickson J, Yamada DS, Berger J, Alverdy J, O'Keefe J, Bassler B, Rinker-Schaeffer C. Societal interactions in ovarian cancer metastasis: a quorum- sensing hypothesis. *Clin. Exp. Metastasis* 2009; **26**: 67-76.

CHAPTER II

QUORUMPEPS® DATABASE: CHEMICAL SPACE, MICROBIAL ORIGIN AND FUNCTIONALITY OF QUORUM SENSING PEPTIDES

"Happiness and bacteria have one thing in common; they multiply by dividing!"

Rutvik Oza
(Indian film writer)

Parts of this chapter were published:

ABSTRACT

Quorum sensing peptides are biologically attractive molecules, with a wide diversity of structures and prone to modifications altering or presenting new functionalities. Therefore, the Quorumpeps database (http://quorumpeps.ugent.be) is developed to give a structured overview of the quorum sensing oligopeptides, describing their microbial origin (species), functionality (method, result, receptor), peptide links and chemical characteristics (3D-structure derived physicochemical properties). The chemical diversity observed within this group of quorum sensing signalling molecules can be used to develop new synthetic bio-active compounds.

CHAPTER **II**

QUORUMPEPS DATABASE:

CHEMICAL SPACE, MICROBIAL ORIGIN AND

FUNCTIONALITY OF QUORUM SENSING

PEPTIDES

Main focus in this chapter:

- To construct a database, containing chemical, species-origin and functional data of quorum sensing peptides.

- To present an easy applicable tool to explore quorum sensing peptide-related information.

1. INTRODUCTION

Quorum sensing (QS) enables bacterial cells to establish cell-cell communication and to regulate the expression of specific genes in response to local changes in cell density [1,2]. The concept of intercellular communication within bacterial populations originates from two independent discoveries in the 1960s and 1970s. In 1965, Tomasz stated that a hormone-like extracellular peptide was important for genetic competence in *Streptococcus pneumoniae* [3]. In 1970, Hastings et al. reported that luminescence in the marine bacterium *Vibrio fischeri* was produced only at high cell density, but not in dilute suspensions. They called the responsible component 'autoinducer', which was later identified as *N*-(3-oxohexanoyl)-homoserine lactone [4,5].

Several classes of microbially derived signalling molecules have now been identified. In general, Gram-negative quorum sensing bacteria use acylated homoserine lactones (AHLs) as autoinducers, while Gram-positive bacteria predominantly communicate with each other using oligopeptides that often contain chemical modifications [6]. In addition, a third family of compounds termed as autoinducer-2, derived from the common precursor 4,5-dihydroxy-2,3-pentanedione, has been found to be

widespread in the bacterial world [7]. Using these quorum sensing signalling molecules, bacteria are able to regulate a diverse array of physiological activities in a cell-density-dependent manner. Processes controlled by quorum sensing are usually those that are unproductive when undertaken by an individual bacterium, but become effective when undertaken by a group. Thus, quorum sensing allows bacteria to behave like a multicellular organism. In addition to genetic competence, bioluminescence, conjugation and swarming motility, quorum sensing also controls virulence factor secretion, biofilm formation and sporulation [6,8,9,10].

In the past decade, a significant increase in interest in bacterial quorum sensing is noticed. The discovery of the quorum sensing mediated virulence factor expression in many clinically relevant pathogens, raised the idea of quorum sensing antagonist production. Blocking quorum sensing is now recognized as a viable approach for the development of novel antibiotics [11]. Moreover, the increased antimicrobial resistance, due *i.a.* to the formation of a biofilm in which the micro-organisms are protected against antimicrobial chemotherapy and the immune system of the host, has called the attention to the quorum sensing system [12]. Intriguingly, recent evidence indicates chemical communication not only between bacteria of different species but also between bacteria and host as well. For example, AHLs have been found to be directly recognised by eukaryotic cells and even to influence the behaviour of eukaryotic organisms (immune suppression, blood vessel relaxation) [2,13]. These quorum sensing signalling peptides may thus have diagnostic and therapeutic properties in oncology and other pathologies as well [14].

Peptides have recently attracted renewed attention for their use in research, disease prevention, diagnosis and/or therapy. These physiologically active molecules demonstrate high affinity, strong selectivity and low toxicity, and can be synthetically modified in order to optimize their affinity for a particular receptor and to display a more specific biodistribution pattern [15,16]. The main drawback of the use of peptide-based compounds is their low stability to peptidases and proteases found in most tissues [16]. However, metabolic stability can be increased by substitution of unnatural amino acids or D-isomers, amidation or acetylation of peptide termini, cyclisation..., hereby increasing the probability of obtaining useful drugs, structurally related to the parent lead-peptides [17,18]. Peptides can thus reverse the increased attrition rate observed with small-molecule drugs.

Seen the increased interest in bacterial quorum sensing signalling molecules and the potential of peptides as new therapeutic or diagnostic drugs, we present here a database of (modified) quorum sensing peptides with the acronym 'Quorumpeps' (http://quorumpeps.ugent.be). This database encompasses the structures and microbial origin, as well as functionality responses of the quorum sensing derived signalling peptides, as described in the literature.

2.   ORGANIZATION OF THE DATABASE

Data model

In order to list all relevant data about the quorum sensing signalling peptides, a relational database is constructed [19]. The usage of such a relational model facilitates the insertion of new and/or additional data. Moreover, it avoids the occurrence of duplicate data and data inconsistencies to the largest extent possible. The schematic database model is available through the Quorumpeps website (Figure 1). In this visual representation, each block describes the structure of a table, with the table name mentioned in the header of the block. In each block, the names of the columns are listed. The column names marked in bold font together determine the primary key of the table. This means that the combination of values for these columns is unique for each row in the table. For clarity, the primary key columns are separated from the other columns by means of a dotted line. Relationships between tables are symbolized by an arrow.

The central table in the Quorumpeps database model is 'Experiment', representing the binding-analysis of a peptide (identified by MoleculeID) with a specific receptor (identified by ReceptorName), using a given method (identified by MethodName). The resulting response (result type, measurement and unit) is also given in this table. The table 'Molecule' summarizes the chemical information about the peptides, including the peptide name, the peptide chemical sequence, the Simplified Molecular Input Line Entry Specification (SMILES)-string and some physicochemical properties. Moreover, also the optimized three-dimensional structure is given in the database in .HIN format, acquired using HyperChem 8.0 (Hypercube, Gainesville, FL, USA). The bacterium that produces the signalling molecule is given in the table 'Origin', while derived molecules are connected to the molecules from which they are derived through the table 'Molecule_Modification'. The receptors are listed in the table 'Receptor'. All methods used to analyse the functionality of the peptides and the references used are summarized in the tables 'Method' and 'Publication', respectively. The observed activity (*e.g.* agonist or antagonist) of a peptide, bound to a specific receptor, is stored in the additional table 'Binding'.

The Quorumpeps database has been implemented on a MySQL backend and is publicly available through the website http://quorumpeps.ugent.be. The website is implemented by using the content management system Drupal and provides a simple, keyword-based search interface to access the data available. Several search options are presented: peptide information (including sequence, trivial name, SMILES or molecular formula), origin, functionality method, receptor and literature information (Figure 2). Each performed search results in an overview of peptides that match the query. From this overview page, detailed information about the selected peptides or related literature can be obtained. User-information can be found at the help page of the Quorumpeps website.
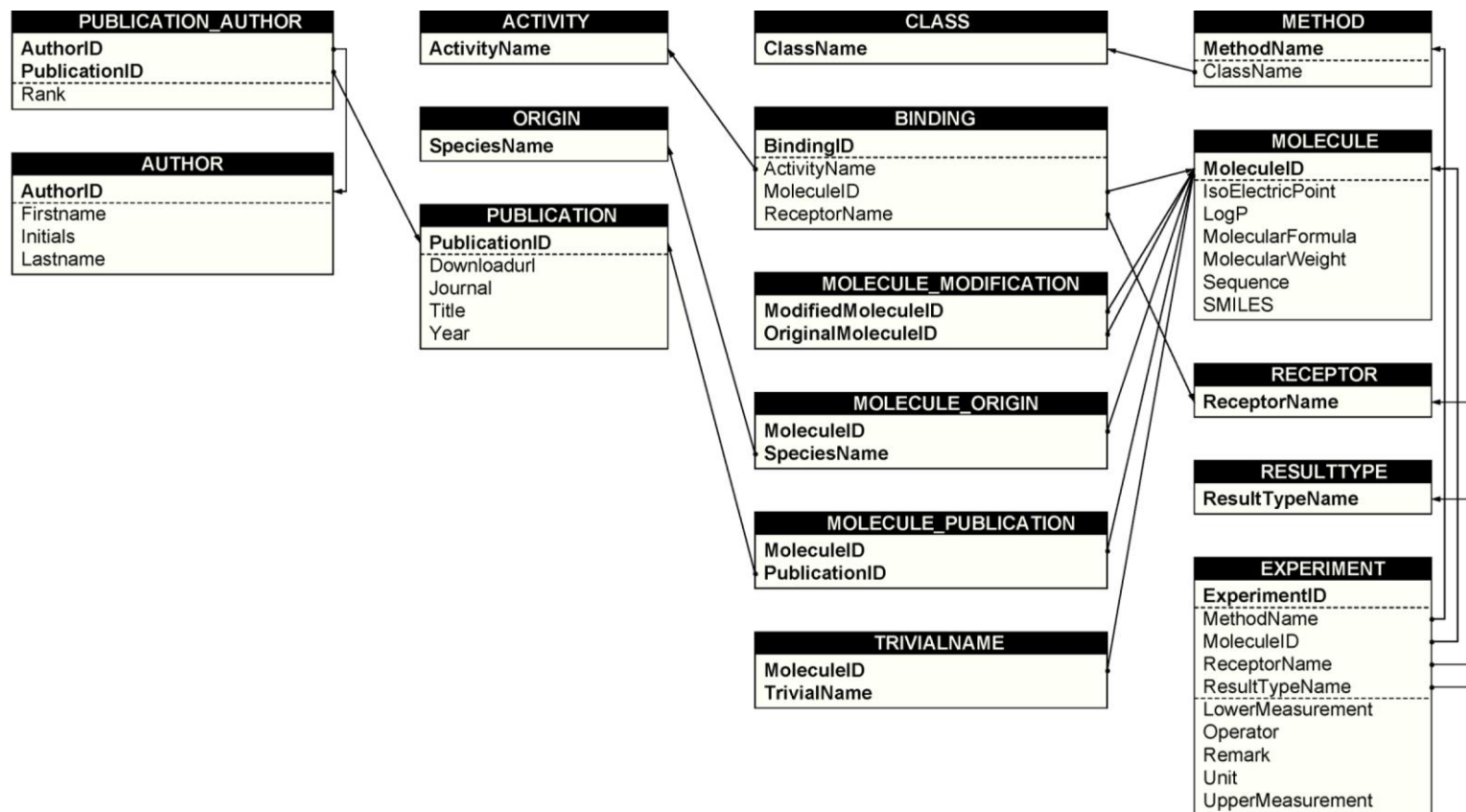
Figure 1. Scheme of Quorumpeps database design

Figure 2. Homepage of the Quorumpeps® database.

Data collection

For loading information in the database, literature data was gathered by using the search engines Web of Knowledge, PubMed and Google, covering the period 1955-2012. The terms 'Quorum sensing', 'autoinducer' and 'pheromone', each separately, as well as 'peptide', 'agonist' and 'antagonist', using the Boolean operation 'AND', were used. The obtained literature was processed manually and all relevant information was stored in the database. The data were used as described by the authors to ensure the data are exactly the same as originally published (data integrity as part of data quality). In order to expand the data available in the database, a data submission page has been constructed on the website allowing researchers to inform us of new information.

3.   QUORUMPEPS IN DETAIL

Chemical information

The chemical information about the quorum sensing peptides includes the IUPAC one-letter code amino acid sequence, with or without any (post-translational) modification, trivial name, SMILES, molecular formula and physicochemical properties (molecular weight, logP and isoelectric point (pI)). The trivial name comprises every name that is given to the specific peptide, thus allowing group names (*e.g.* Autoinducing peptide, AIP) as well as individual peptide names (*e.g.* PhrA).

Before calculating the different physicochemical properties, the geometrical structure of these quorum sensing peptides was optimized using HyperChem 8.0. The geometry optimization was obtained by the molecular mechanics force field method using the Polak–Ribière conjugate gradient algorithm with a root mean square gradient of 0.1 kcal/ (Å×mol) as stop criterion. Afterwards, these Cartesian coordinate matrices were used to calculate the different descriptors, using HyperChem 8.0 and Marvin Beans 5.3.3 (Chemaxon, Budapest, Hungary) software programs. This optimized .HIN structure is available through the Quorumpeps website, together with the 2D-SMILES notation. These formats allow the user to perform any sort of analysis on their various chemical properties, *e.g.* QSAR or multivariate data analysis [20]. Diversity analysis using the diversity index (described by the Tanimoto coefficient) of the 231 quorum sensing peptides currently available in the Quorumpeps database, indicated that this dataset is extremely diverse (DI = 0.21) and thus suitable for QSAR or QSPR studies [21,22].

Species origin

In general, quorum sensing peptides are synthesized by Gram-positive bacteria, *e.g. Staphylococcus aureus* and *Bacillus subtilis*. As numerous different species are reported in literature, an overview of all listed bacteria is available at the Quorumpeps homepage, by using the information-icon: using this scroll-list, rapid directed searches can be performed by the user of Quorumpeps.

Chemical modifications (*e.g.* amino acid substitution) of an original quorum sensing peptide are visualized in the 'Species origin' section by referring to its original counterpart.

Functionality

The functional properties of the quorum sensing signalling peptides are described in the Quorumpeps database. Moreover, an overview of the receptors and methods can be found at the homepage of Quorumpeps, using the information-icon next to the appropriate characteristic.

The functionality of the signalling peptides is characterized by the method used to study their quorum sensing activity. The methods are using either the isolated signalling peptides or the (genetically modified) bacteria to investigate a biological activity. The target genes or their products are then qualitatively or quantitatively measured using antibacterial assays [23,24], biosensors [25-31], biofilm assays [32,33], analytical and immunoassays [34,35], DNA and RNA detection [36,37] or viability measurements [34,38-41]. Moreover, different quorum sensing pathways are reported, which can be divided into mainly cell membrane receptor mediated activation and, to a much lesser amount, cytoplasmic receptor activation after cell-penetration of the peptides. The vast majority of quorum sensing peptides initiate a signalling pathway by activating a membrane-integrated histidine

kinase, after which the cytoplasmic response regulator mediates the output response. This cascade of reactions is species specific, with homology seen between the different organisms. Different pathways can be distinguished, which are given in the Quorumpeps database: AgrC/AgrA and TRAP virulence system [42-47], ComD/ComE competence system [48], ComP/ComA and Rap competence or sporulation system [49-52], FsrC/FsrA virulence system [53] and NisK/NisR system or analogues [54,55]. Furthermore, also the quantitative binding results of the quorum sensing peptides with a specific receptor are given in the Quorumpeps database, in which agonistic or antagonistic properties can be distinguished.

Links

This 'Links' section allows the user of Quorumpeps to link the reported quorum sensing peptides through their binding properties (*i.e.* receptor) or microbial origin. Peptides that bind with the same receptor as the selected peptide are given in the appropriate table, differentiating them based on their agonist or antagonist activity. Moreover, peptides that are synthesized by the same species are again listed in this information section.

Literature

All information gathered to develop this Quorumpeps database is summarized in the 'Literature' section, described by a publication-ID; detailed information is obtained after ID-selection: journal name, title, year and author names in the same order as they appear on the publication. Moreover, the possibility to download this publication is also available, through their PubMed ID-number and related link.

4. CONCLUSIONS

This database (http://quorumpeps.ugent.be) gives an overview of reported quorum sensing signalling peptides and their derivatives, summarizing their chemical and functional properties, together with species information. Therefore, this database can function as a useful tool to justify peptide choices for evaluating different responses or to study quantitative structure-property relationships (QSPR) of these quorum sensing molecules. Quorumpeps will quarterly be updated by the authors, ensuring up-to-date information of this interesting and expanding field for other researchers.

5. REFERENCES

[1]   Martin CA, Hoven AD, Cook AM. Therapeutic frontiers: preventing and treating infectious diseases by inhibiting bacterial quorum sensing. *Eur. J. Clin. Microbiol. Infect. Dis.* 2008; **27**: 635-642.

[2]   Diggle SP, Crusz SA, Cámara M. Quorum sensing. *Curr. Biol.* 2007; **17**: 907-910.

[3]   Tomasz A. Control of the competent state in Pneumococcus by a hormone-like cell product: an example of a new type of regulatory mechanism in bacteria. *Nature* 1965; **208**: 155-159.

[4]   Nealson KH, Platt T, Hastings JW. Cellular control of the synthesis and activity of bacterial luminescent system. *J. Bacteriol.* 1970; **104**: 313-322.

[5]   Eberhard A, Burlingame AL, Eberhard C, Kenyon GL, Nealson KH, Oppenheimer NJ. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochem.* 1981; **20**: 2444-2449.

[6]   Miller MB, Bassler BL. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 2001; **55**: 165-199.

[7]   Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* 2002; **415**: 545-549.

[8]   Kleerebezem M, Quadri LEN, Kuipers OP, de Vos WM. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol.* 1997; **24**: 895-904.

[9]   Bassler BL, Losick R. Bacterially speaking. *Cell* 2006; **125**: 237-246.

[10]   Ni N, Li M, Wang J, Wang B. Inhibitors and antagonists of bacterial quorum sensing. *Med. Res. Rev.* 2009; **29**: 65-124.

[11]   Chen G, Swem LR, Swem DL, Stauff DL, O'Loughlin CT, Jeffrey PD, Bassler BL, Hughson FM. A strategy for antagonizing quorum sensing. *Mol. Cell* 2011; **42**: 199-209.

[12]   Heilmann C, Götz F. Cell-cell communication and biofilm formation in gram-positive bacteria. In *Bacterial signalling* (Eds: Krämer R, Jung K) WILEY-VCH, Weinheim, **2010**, pp. 7-22.

[13]   Pritchard DI. Immune modulation by *Pseudomonas aeruginosa* quorum-sensing signal molecules. *Int. J. Med. Microbiol.* 2006; **296**: 111–116.

[14]   Wynendaele E, Pauwels E, Van de Wiele C, Burvenich C, De Spiegeleer B. The potential role of quorum-sensing peptides in oncology. *Med. Hypotheses* 2012; **78**: 814-817.

[15]   Bhutia SK, Maiti TK. Targeting tumors with peptides from natural sources. *Trends Biotechnol.* 2008; **26**: 210-217.

[16]   Zaccaro L, del Gatto A, Pedone C, Saviano M. Peptides for tumour therapy and diagnosis: current status and future directions. *Curr. Med. Chem.* 2009; **16**: 780-795.

[17]   Adessi C, Soto C. Converting a peptide into a drug: strategies to improve stability and bioavailability. *Curr. Med. Chem.* 2002; **9**: 963-978.

[18]   Svenson J, Vergote V, Karstad R, Burvenich C, Svendsen JS, De Spiegeleer B. Metabolic fate of lactoferricin-based antimicrobial peptides: effect of truncation and incorporation of amin acid analogs on the in vitro metabolic stability. *J. Pharmacol. Exp. Ther.* 2010; **332**: 1032-1039.

[19]   Codd EF. A relational model of data for large shared data banks. *Commun. ACM* 1970; **13**: 377-387.

[20]   Eriksson EJL, Kettaneh-Wold N, Trygg J, Wikstrom C, Wold S. *Multi- and Megavariate Data Analysis: Part I - Basic Principles and Applications*, Umetrics Academy, **2006**.

[21]   Baert B, Deconinck E, Van Gele M, Slodicka M, Stoppie P, Bodé S, Slegers G, Vander Heyden Y, Lambert J, Beetens J, De Spiegeleer B. Transdermal penetration behaviour of drugs: CART-clustering, QSPR and selection of model compounds. *Bioorg. Med. Chem.* 2007; **15**: 6943-6955.

[22]   Yap CW, Li ZR, Chen YZ. Quantitative structure-pharmacokinetic relationships for drug clearance by using statistical learning methods. *J. Mol. Graphics Modell.* 2006; **24**: 383-395.

[23]   Schmitz S, Hoffmann A, Szekat C, Rudd B, Bierbaum G. The lantibiotic mersacidin is an autoinducing peptide. *Appl. Environ. Microbiol.* 2006; **72**: 7270-7277.

[24]   Al-Hussaini R, Mahasneh AM. Microbial growth and quorum sensing antagonist activities of herbal plants extracts. *Molecules* 2009; **14**: 3425-3435.

[25]   Tortosa P, Logsdon L, Kraigher B, Itoh Y, Mandic-Mulec I, Dubnau D. Specificity and genetic polymorphism of the *Bacillus* competence quorum-sensing system. *J. Bacteriol.* 2001; **183**: 451-460.

[26]   Brelles-Marino G, Bedmar EJ. Detection, purification and characterization of quorum-sensing signal molecules in plant-associated bacteria. *J. Biotechnol.* 2001; **91**: 197-209.

[27]   Okada M, Sato I, Cho SJ, Dubnau D, Sakagami Y. Chemical synthesis of ComX pheromone and related peptides containing isoprenoidal tryptophan residues. *Tetrahedron* 2006; **62**: 8907-8918.

[28]   Blomqvist T, Steinmoen H, Havarstein LS. Pheromone-induced expression of recombinant proteins in *Streptococcus thermophilus*. *Arch. Microbiol.* 2006; **186**: 465-473.

[29]   Ji G, Beavis RC, Novick RP. Cell density control of Staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl. Acad. Sci. U.S.A.* 1995; **92**: 12055-12059.

[30]   Lanigan-Gerdes S, Briceno G, Dooley AN, Faull KF, Lazazzera BA. Identification of residues important for cleavage of the extracellular signaling peptide CSF of *Bacillus subtilis* from its precursor protein. *J. Bacteriol.* 2008; **190**: 6668-6675.

[31]   Nishiguchi K, Nagata K, Tanokura M, Sonomoto K, Nakayama J. Structure-activity relationship of gelatinase biosynthesis-activating pheromone of *Enterococcus faecalis*. *J. Bacteriol.* 2009; **191**: 641-650.

[32]   Abraham WR. Controlling biofilms of gram-positive pathogenic bacteria. *Curr. Med. Chem.* 2006; **13**: 1509-1524.

[33]   Burtin E, Yakandawala N, Lovetri K. A microplate spectrofluorometric assay for bacterial biofilms. *J. Ind. Microbiol. Biotechnol.* 2007; **34**: 1-4.

[34]   Cooksley CM, Davis IJ, Winzer K, Chan WC, Peck MW, Minton NP. Regulation of neurotoxin production and sporulation by a putative agrBD signaling system in proteolytic *Clostridium botulinum*. *Appl. Environ. Microbiol.* 2010; **76**: 4448-4460.

[35]   Jiang M, Grau R, Perego M. Differential Processing of Propeptide Inhibitors of Rap Phosphatases in *Bacillus subtilis*. *J. Bacteriol.* 2000; **182**: 303-310.

[36]   Martin M, Showalter R, Silverman M. Identification of a locus controlling expression of luminescence genes in *Vibrio harveyi*. *J. Bacteriol.* 1989; **171**: 2406-2414.

[37]   Weber B, Croxatto A, Chen C, Milton DL. RpoS induces expression of the *Vibrio anguillarum* quorum-sensing regulator VanT. *Microbiology* 2008; **154**: 767-780.

[38]   Zhang K, Ou M, Wang W, Ling J. Effect of quorum-sensing on cell viability in *Streptococcus mutans* biofilm formation. *Biochem. Biophys. Res. Commun.* 2009; **379**: 933-938.

[39] Zhu J, Yin X, Yu H, Zhao L, Sabour P, Gong J. Involvement of quorum sensing and heat-stable enterotoxin a in cell damage caused by a porcine enterotoxigenic *Escherichia coli* strain. *Infect. Immun.* 2011; **79**: 1688-1695.

[40] Derengowski L, De-Souza-Silva C, Braz SV, Mello-De-Sousa TM, Báo SN, Kyaw CM, Silva-Pereira I. Antimicrobial effect of farnesol, a *Candida albicans* quorum sensing molecule, on *Paracoccidioides brasiliensis* growth and morphogenesis. *Ann. Clin. Microbiol. Antimicrob.* 2009; **8**: 1-9.

[41] Kolodkin-Gal I, Hazan R, Gaathon A, Carmeli S, Engelberg-Kulka H. A linear pentapeptide is a quorum-sensing factor required for mazEF-mediated cell death in *Escherichia coli*. *Science* 2007; **318**: 652-655.

[42] Gorske BC, Blackwell HE. Interception of quorum sensing in *Staphylococcus aureus*: a new niche for peptidomimetics. *Org. Biomol. Chem.* 2006; **4**: 1441-1445.

[43] Sifri CD. Quorum sensing: bacteria talk sense. *Clin. Infect. Dis.* 2008; **47**: 1070-1076.

[44] Ji G, Beavis R, Novick RP. Bacterial Interference Caused by Autoinducing Peptide Variants. *Science* 1997; **276**: 2027-2030.

[45] Ni N, Li M, Wang J, Wang B. Inhibitors and Antagonists of Bacterial Quorum Sensing. *Med. Res. Rev.* 2008; **29**: 65-124.

[46] Shaw LN, Jonsson I, Singh VK, Tarkowski A, Stewart GC. Inactivation of trap has no effect on the agr quorum-sensing system or virulence of *Staphylococcus aureus*. *Infect. Immun.* 2007; **75**: 4519-4527.

[47] Korem M, Sheoran AS, Gov Y, Tzipori S, Borovok I, Balaban N. Characterization of RAP, a quorum sensing activator of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 2003; **223**: 167-175.

[48] Cvitkovitch DG, Li Y, Ellen RP. Quorum sensing and biofilm formation in Streptococcal infections. *J. Clin. Invest.* 2003; **112**: 1626-1632.

[49] Lazazzera BA, Solomon JM, Grossman AD. An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*. *Cell* 1997; **89**: 917-925.

[50] Comella N, Grossman AD. Conservation of genes and processes controlled by the quorum response in bacteria: characterization of genes controlled by the quorum-sensing transcription factor ComA in *Bacillus subtilis*. *Mol. Microbiol.* 2005; **57**: 1159-1174.

[51] Perego M, Brannigan JA. Pentapeptide regulation of aspartyl-phosphate phosphatases. *Peptides* 2001; **22**: 1541-1547.

[52] Bischofs IB, Hug JA, Liu AW, Wolf DM, Arkin AP. Complexity in bacterial cell-cell communication: Quorum signal integration and subpopulation signaling in the *Bacillus subtilis* phosphorelay. *Proc. Natl. Acad. Sci. U.S.A.* 2008; **106**: 6459-6464.

[53] Sifri CD, Mylonakis E, Singh KV, Qin X, Garsin DA, Murray BE, Ausubel FM, Calderwood SB. Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus fsr in *Caenorhabditis elegans* and mice. *Infect. Immun.* 2002; **70**: 5647-5650.

[54] Kuipers OP, de Ruyter PGGA, Kleerebezem M, de Vos WM. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* 1998; **64**: 15-21.

[55] Johnsborg O, Dipe DB, Nes IF. Structural analysis of the peptide pheromone receptor PlnB, a histidine protein kinase from *Lactobacillus plantarum*. *J. Bacteriol.* 2003; **185**: 6913-6920.

CHAPTER III

EXPLORING THE CHEMICAL SPACE OF QUORUM SENSING PEPTIDES

"The language of Friendship is not words, but meanings."

Henry David Thoreau

ABSTRACT

Quorum sensing peptides, *i.e.* signalling molecules produced by mainly Gram-positive bacteria, can exert different effects, ranging from inter-species bacterial virulence to bacterial-host interactions. To better comprehend these functional differences, we explored their chemical space, bacterial species distribution and receptor-binding properties using multivariate data analyses, with information obtained from the Quorumpeps database. The quorum sensing peptides can be categorized into three main clusters, which, in turn, can be divided into several subclusters: the classification is based on characteristic chemical properties, including peptide size/compactness, hydrophilicity/lipophilicity, cyclisation, and the presence of (unnatural) S-containing and aromatic amino acids. Most of the bacterial species synthesize peptides located into one cluster. However, some *Streptococcus*, *Stapylococcus*, *Clostridium*, *Bacillus* and *Lactobacillus* species produce peptides that are distributed over more than one cluster, with the quorum sensing peptides of *Bacillus subtilis* even occupying the total peptide space. The AgrC, FsrC and LamC receptors are only activated by cyclic (thio)lacton or lactam quorum sensing peptides, while the lipophilic isoprenyl-modified peptides solely bind the ComP receptor in *Bacillus* species.

CHAPTER III

EXPLORING THE CHEMICAL SPACE OF

QUORUM SENSING PEPTIDES

Main focus in this chapter:

- To indicate chemical differences and similarities between quorum sensing peptides.

- To link the chemical diversity of quorum sensing peptides with bacterial taxonomy and receptor interaction.

1. INTRODUCTION

'Quorum sensing' is the phenomenon of cell-to-cell communication between bacteria, leading to a regulated gene expression in response to fluctuations in cell density. Gram-positive bacteria thereby produce and release oligopeptide molecules as communication tools in order to control *e.g.* virulence, competence, sporulation or biofilm formation [1]. The quorum sensing peptides described in the literature is up-to-date stored in the Quorumpeps database (http://quorumpeps.ugent.be), giving both (physico)chemical information about the peptides and quorum sensing process-related details (*e.g.* receptor, activity, species) [2].

Gram-positive bacteria are commensally present in and on the human body, thereby mainly occupying the gastrointestinal tract, vagina, skin, mouth and oropharynx of healthy individuals. The microbiome composition however differs remarkably between individuals, due to *e.g.* variations in diet, environment, host genetics or early microbial exposure [3]. *Streptococcus* species are highly present in the oral cavity, causing dental caries (predominantly by *Streptococcus mutans*) [4] or forming dental biofilms (*Streptococcus mitis*) after quorum sensing process activation [5]. *Lactobacillus* species (*e.g. Lactobacillus sakei* and *Lactobacillus plantarum*) are mainly found in human vaginal specimens, the oral cavity and the gastrointestinal tract [3,6,7]. *Lactobacillus* species in the oral cavity can be linked to dental plaque (biofilm) formation, next to hyposalivation and dental caries. *Staphylococcus aureus* is a natural inhabitant of the nose and skin, but can cause various infections in human beings as well, ranging from minor skin infections to severe pneumonia.

Many infections caused by *S. aureus* are not caused by free-living cells, but rather by biofilms, which may require quorum sensing mechanisms and the use of signaling peptides [8]. Other Gram-positive cocci can cause nosocomial infections (scleral buckles, contact lenses, vascular grafts, orthopedic devices, penile prostheses), involving biofilm formation, as well [9]. *Escherichia coli*, although its Gram-negative classification, also produces a signaling peptide (*i.e.* Extracellular Death Factor, EDF) to activate the quorum sensing cascade system [10]. This bacterium is commensally present in the human colon but can cause biofilm-involved biliary tract infection, bacterial prostatitis or urinary cystitis [9]. Although not yet demonstrated, the presence of quorum sensing peptides in the human body is very likely, seen the biological incidence of Gram-positive bacteria and the physiological detection of other quorum sensing signaling molecules [11].

The quorum sensing process thus can contribute to host virulence due to *e.g.* biofilm formation after intra-species bacterial communication. This communication phenomenon can be extended to different bacterial species as well (*i.e.* inter-species), thereby promoting bacterial virulence: *e.g.* the lantibiotics nisin and subtilin, produced by *Lactococcus lactis* and *Bacillus subtilis* respectively and originally investigated for their antimicrobial activities, were recently found to act as signaling molecules that regulate their own synthesis (*i.e.* quorum sensing peptides) [12]. The effects of the quorum sensing peptides are however not limited to the bacteria themselves (intra- and interspecies) [13]: recent investigations from our group have revealed a selective crosstalk phenomenon between these peptides (*e.g.* Phr0662, EDF and PhrG) and mammalian cancer cells, thereby influencing tumour cell invasion and angiogenesis [14,15]. Moreover, the *Bacillus subtilis* quorum sensing peptide CSF (Competence and Sporulation Factor) activates p38 mitogen-activated protein kinase and protein kinase B in host intestinal epithelial cells as well and induces cytoprotective heat shock protein synthesis [16].

To better comprehend the effects of the different quorum sensing peptides, as well as to structure this group of molecules, we investigated their chemical space using multivariate data analyses, with data available from the Quorumpeps database.

2. MATERIALS AND METHODS

Chemical descriptors

Before calculating the different chemical properties of the Quorumpeps peptides (n = 231, September 2014), the geometrical structure of these peptides was optimized using HyperChem 8.0 (Hypercube, Gainesville, FL, USA). The geometry optimization *in vacuo* was obtained by the molecular mechanics MM+ force field method using the Polak–Ribière conjugate gradient algorithm

with a root mean square of 0.1 kcal/(Å×mol) as stop criterion. Afterwards, these Cartesian coordinate matrices were used to calculate more than 3000 descriptors, using Dragon 5.5 (Talete, Milan, Italy), HyperChem 8.0 and Marvin Beans 5.3.3 (Chemaxon, Budapest, Hungary) software programs. After removing the non-discriminative descriptors which were constant for all peptides, the remaining descriptors were divided by the peptide's molecular weight as some descriptors were highly correlated with this descriptor. A final 231 x 1468 data-matrix was then obtained. Next, the data were transformed by z-scaling to minimize the effects of differences in measurement units and variance and to render the data dimensionless [17].

Multivariate data-analyses

Multivariate data-analyses were performed using Principal Component Analysis (PCA) and Hierarchical Cluster Analysis with SIMCA-P+ 12.0 (Umetrics AB, Umeå, Sweden) and SPSS 21.0 (IBM, Illinois, USA) software programs, respectively. To obtain a dendrogram of the chemical clustering, average-linkage clustering was performed using the Euclidean distance as the dissimilarity criterion. Peptide numbering used in the Quorumpeps database is retained in this study.

3. RESULTS AND DISCUSSION

Chemical space

The first two principal components (PCs) of the calculated PCA-model explain already more than 60% of the total variability (Table 1). Based on the dendrogram of the Hierarchical Cluster Analysis and the score plot of the PCA, the 231 quorum sensing peptides could be categorized into three main clusters, which again could be subdivided into several other subclusters (Figure 1).

Table 1. PCA summary describing the eigenvalues of the covariance matrix, the total variance explained (R^2, cumulative) and the predictive ability (Q^2, cumulative).

Principal Component	Eigenvalue	Cumulative R^2	Cumulative Q^2
1	127	0.548	0.542
2	20.4	0.636	0.626
3	13.1	0.693	0.678
4	11.9	0.745	0.728
5	6.07	0.771	0.744
6	5.25	0.794	0.763
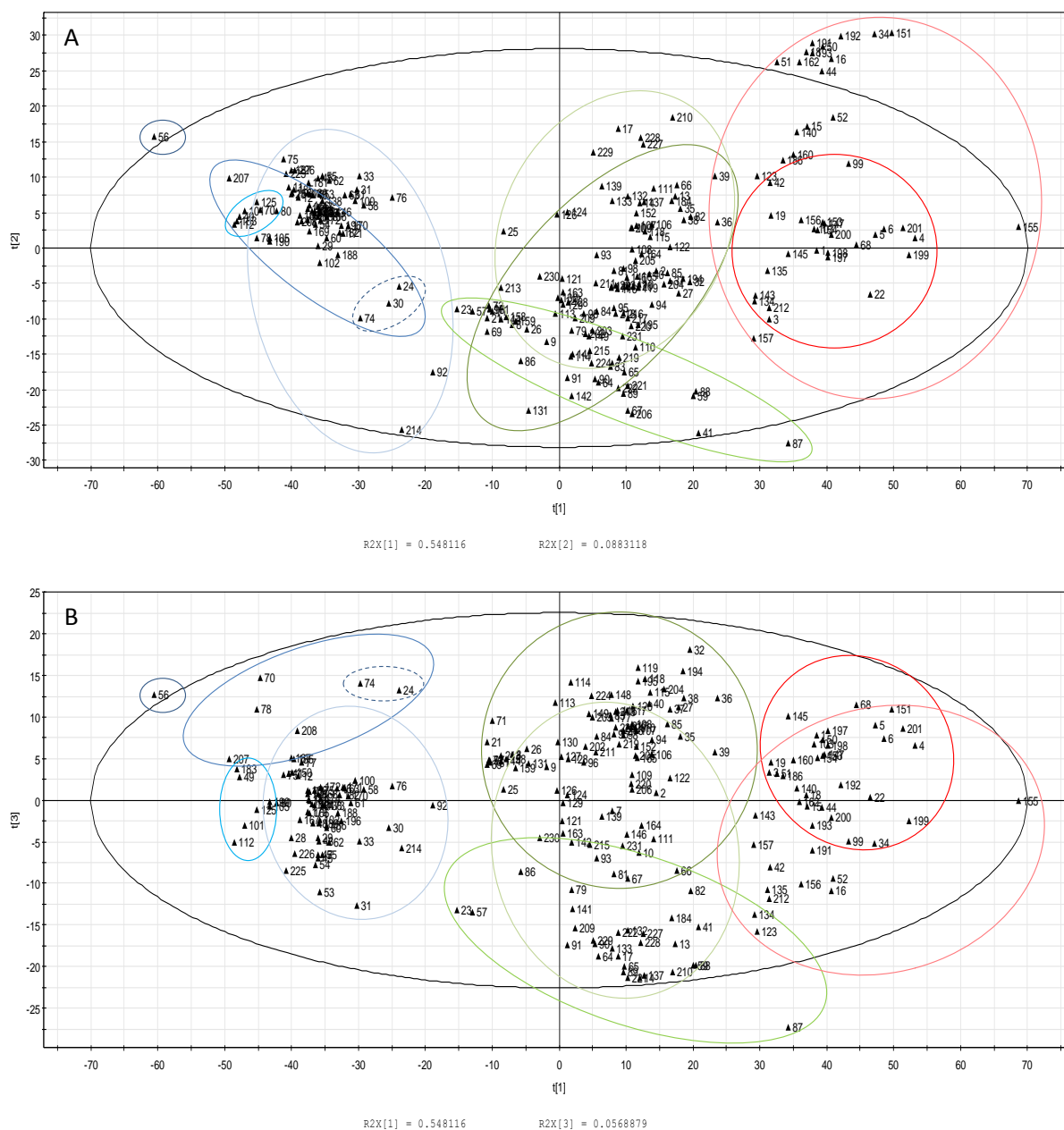
Figure 1. Score plot of the first en second (A) and first and third (B) principal component (PC) from PCA of the quorum sensing signalling peptides (1468 descriptors). Three main clusters can be distinguished (blue, green, red), each subdivided into smaller groups.

The chemical space of the quorum sensing peptides, divided into three main clusters (Figure 1), is mainly described by the peptide size and its compactness (1st principal component), given by the Radial Distribution Function (RDF) descriptors, Burden eigenvalues (BEH, BEL), Randic shape indices, autocorrelation descriptors (ATS, GATS), Weighted Holistic Invariant Molecular (WHIM) descriptors, Balaban indices and the lopping centric index. Second, lipophilicity/hydrophilicity determines the chemical space as well through the logP values, topological polar surface area (TPSA) descriptors and number of H-bond donor (nHDon) and acceptor (nHAcc) atoms, together with connectivity indices describing peptide cyclisation and HOMA, AROM and ARR aromaticity descriptors (2nd principal component). The 3rd principal component is mainly defined by the S-evaluating descriptors, describing *e.g.* thiol groups, thiolactons or disulfides (Figure 2).

The **first cluster** (Figure 1, blue) predominantly contains 4 different subclusters, *i.e.* the lantibiotics, the larger linear quorum sensing peptides, the bacteriocin-related peptides and the very large peptide Carnobacteriocin B2. The lantibiotics nisin A (ID70), subtilin (ID78), pep5 (ID207) and mersacidin (ID24) are classified in one group, together with the anti-HIV peptide Siamycin I (ID74). The lantibiotics are heat-stable, post-translationally modified antimicrobial peptides containing dehydrated amino acids (dehydroalanine and dehydrobutyrine). This peptide group is also characterized by specific polycyclic thioether amino acids (lanthionine and β-methyllanthionine) [18] or disulfide bridges, which are responsible for the separate clustering as observed in the loading plots by the S-evaluating descriptors (Figure 2). Due to the high difference in peptide size/compactness (principal component 1) and lipophilicity (principal component 2), mersacidin and Siamycin I are however separated from the other lantibiotics, forming a different subcluster. The linear bacteriocin-related peptides (IDs 49, 101, 112, 125, 183) form a separate subcluster, despite the identical size as the lantibiotics: these peptides are classified separately due to the absence of the characteristic lanthionine and β-methyllanthionine amino acids. These bacteriocin-related peptides are in turn separated from the medium-sized quorum sensing peptides of the second cluster, again based on peptide size and shape. The fourth subcluster contains the very large bacteriocin Carnobacteriocin B2 (ID56), a representative of the cationic and heat-stable antimicrobial peptides with quorum sensing activity, with a molecular weight of more than 4000 Dalton [19,20].
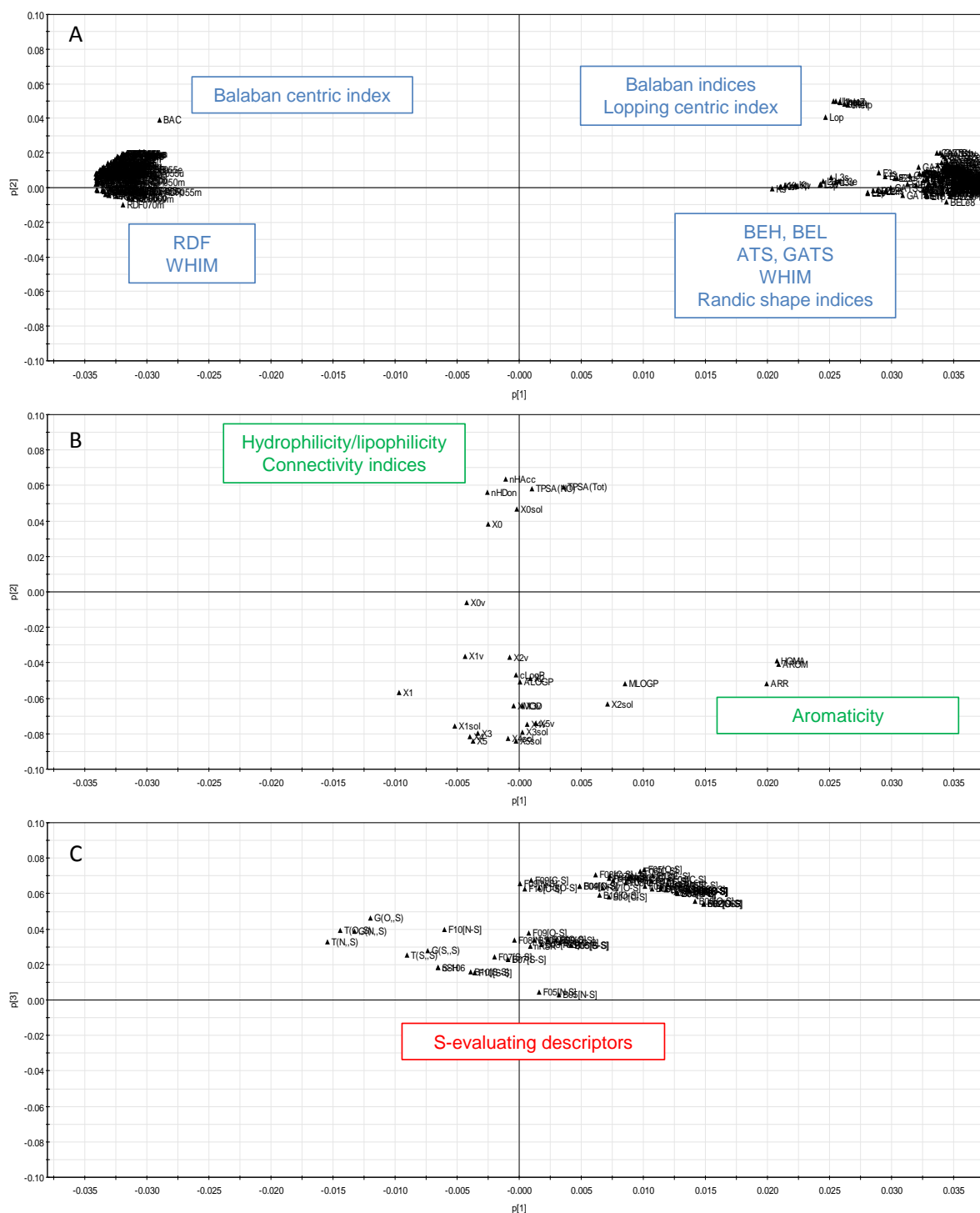
Figure 2. Loading plots of the first, second and third principal components, with visualisation of the most important descriptor groups. (A) PC1 describes the peptide size and compactness (blue), (B) PC2 its hydrophilicity/lipophilicity, cyclisation and aromaticity (green), and (C) PC3 the presence of sulphur atoms or sulphur-containing groups (red).

The **second cluster** (Figure 1, green) contains different subclusters as well: the medium-sized cyclised signalling peptides, including Gelatinase Biosynthesis-Activating Pheromone (GBAP, ID97) and its derivatives, the isoprenyl-modified peptides and the medium-sized linear peptides. The peptides can

be cyclised between the carboxyl end or COOH-side chain (D and E amino acids) of the peptide and the side chain of cysteine or serine, or its amino terminus, resulting in a thiolacton, a lacton or a lactam moiety; the ring is typically composed of 5-9 amino acids. The post-translationally modified quorum sensing peptides, containing a geranyl or farnesyl molecule on a tryptophan amino acid, form a tricyclic structure as well by this modification (*i.e.* a newly formed five-membered ring, next to the two tryptophan rings). Both groups of cyclic peptides are chemically separated from each other based on the difference in lipophilicity (logP values) and (valence and solvation) connectivity indices. RNAIII-inhibiting peptide and its medium-sized derivatives belong to the linear peptides of this cluster. However, not all of the RNAIII-inhibiting peptide-derivatives are grouped into the same (second) cluster: 2 smaller derived peptides with RNAIII-inhibiting biological properties, *i.e.* IDs 156 and 157, are grouped in the third cluster of compact quorum sensing peptides.

This **third cluster** (Figure 1, red) contains the smaller, compact peptides, again subdivided into the small thiolacton or lactam cyclised quorum sensing peptides and the linear peptides, including the Extracellular Death Factor (EDF, ID19) and its derivatives. The cyclised peptides thus clearly occupy a distinct portion of the quorum sensing peptide space as they are spread over both the second and third cluster, according to their size distribution.

Representative quorum sensing peptides from each subcluster are listed in Figure 3, together with some representative key descriptor values.

Peptide sequence	ID	Typical descriptor values (z-score)
VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	56	cLogP = -32.59 (-1.13) RDF095m = 515.51 (1.65)
I(Dhb)AI(Dha)LA(Abu)PGAK(Abu)GALMGANMK(Abu)A(Abu)AHASIHV(Dha)K	70	T(O..S) = 5975 (6.62) X5 = 44.17 (-0.21)
A(Abu)F(Abu)LPGGGGVA(Abu)L(Abu)(Dha)EAI	24	T(O..S) = 1111 (1.89) X5 = 25.51 (0.35) cLogP = -8.44 (-0.53)
GWWEELLHETILSKFKITKALELPIQL	112	T(0..S) = 0 (-0.56) RDF095m = 353.76 (1.85)
DLRNIFLKIKFKKK	31	T(O..S) = 0 (-0.56) cLogP = -2.91 (0.41) X5 = 22.75 (-0.53)
FW(Geranyl)E	87	T(O..S) = 0 (-0.56) cLogP = 4.36 (3.13) X5 = 10.84 (2.88)
DSACVFGA (thiolacton bridge)	36	T(O..S) = 86 (-0.10) cLogP = -3.84 (-0.68) X5 = 9.20 (-0.85)
ALILTLVS	17	T(O..S) = 0 (-0.56) cLogP = 0.26 (1.02) X5 = 9.51 (-1.39) RDF095m = 33.86 (-0.58)
NNWNN	19	T(O..S) = 0 (-0.56) RDF095m = 33.45 (-0.24) X5 = 9.11 (0.22)
CVGIW (thiolacton bridge)	22	T(O..S) = 24 (-0.39) RDF095m = 13.30 (-1.19) X5 = 8.10 (0.73)

↓ Cluster   └→ Principal Component

Figure 3. Overview of representative quorum sensing peptides from different (sub)clusters. The values of some representative key descriptors responsible for the clustering are given on the right. Coloring is used as indicated in Figure 1 (cluster) and Figure 2 (principal component).

Species distribution

Bacillus species, *i.e. B. anthracis*, *B. cereus*, *B. halodurans*, *B. mojavensis*, *B. pumilus*, *B. stearothermophilus*, *B. subtilis* and *B. thuringiensis*, synthesize a diverse collection of peptides, ranging from small and medium-sized linear peptides, over isoprenyl-modified structures, to large complex lantibiotic molecules (Figure 4). The *Bacillus* species are solely responsible for the production of the geranyl- or farnesyl-containing peptides. Next to these lipophilic compounds, (small) hydrophilic peptides can be synthesized as well, as observed in the score plot by the 2nd principal component: both directions of the axis are 'filled' with quorum sensing peptides. The (thio)lacton or lactam cyclised peptides are synthesized by either *Lactobacillus plantarum*, Staphylococci, *Clostridium* species or *Enterococcus faecalis*, of which the latter is the only one responsible for the production of a 9-membered cyclic structure (*i.e.* GBAP). Next to these (small or medium-sized) cyclised structures, *Enterococcus faecalis* and *Clostridium acetobutylicum* synthesize medium-sized linear peptides as well, while this is not observed for *C. sporogenes* or *C. botulinum*. The same is true for *Lactobacillus plantarum*: both cyclised and linear peptides are produced by the

bacteria, with the linear bacteriocin-related peptides (IDs 49 and 125, first cluster) being larger compared to the cyclised peptide (ID22, third cluster). The other *Lactobacillus* species, *i.e. Lactobacillus sakei*, only synthesizes these large linear peptides. Next to the medium-sized cyclised peptides of the second cluster, *Staphyloccus areus* and *Staphylococcus epidermidis* are the only *Staphylococcus* species that make a medium-sized linear peptide for activation of the quorum sensing pathway, namely RNAIII-inhibiting peptide. Moreover, *S. epidermidis* produces a (large) lantibiotic peptide as well (first cluster). The *Carnobacterium* species that are included in the Quorumpeps database only produce large linear peptides, including the largest peptide of the database, Carnobacteriocin B2. The larger linear quorum sensing peptides are mainly synthesized by *Streptococcus* species, with a few medium-sized linear peptides produced by *S. mutans* and *S. thermophilus*.

Figure 4. Distribution of quorum sensing peptides, according to the species origin.
The score plot of the *Bacillus* peptides indicates a wide spread of the peptides over the three clusters
(Derived: chemically modified quorum sensing peptides).

The phylogenetic tree of Firmicutes [21,22], *i.e.* the most abundant phylum in the Quorumpeps database, distinguishes 4 main groups: (1) *Thermoanaerobacterium* and *Clostridium* species; (2) *Phytoplasma*, *Mycoplasma* and *Ureaplasma* species; (3) *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Carnobacterium* and *Streptococcus* species; and (4) *Staphylococcus*, *Listeria*, and *Bacillus* species. In contrast to group 1, 3 and 4, there are no reported Quorumpeps peptides produced by *Phytoplasma*, *Mycoplasma* or *Ureaplasma* species. The second and third chemical clusters of quorum sensing peptides contain peptides that are synthesized by all three Firmicutes groups. The first cluster

however does not contain peptides from *Clostridium* species and is therefore not completely covered by the Firmicutes species.

Moreover, some bacterial (Firmicutes) species are not (yet) reported to synthesize quorum sensing peptides while others do so, despite the phylogenetic relationship between both species (www.patricbrc.org). For example, *Lactobacillus reuteri*, a commensal bacterium of the human gut [23], is closely related to *Lactobacillus plantarum*, which is already listed in the Quorumpeps database. However, no quorum sensing peptides have until now been reported for the former bacterial species. This is the case for *C. sporogenes* and *C. botulinum* as well: while only *C. acetobutylicum* was found to synthesize linear peptides, it is very likely that the other *Clostridium* species synthesize these peptides too, seen the strong phylogenetic relationship between these *Clostridium* species. We therefore believe research concerning the quorum sensing system is far from being completed, with several signaling peptides still to be elucidated which can then strengthen or complete the obtained clustering results.

While the meaning of the term 'Quorum sensing' has evolved over time, with still several interpretations possible, there is consensus that the increased production of the quorum sensing signals by unicellular organisms should occur during specific stages in response to certain conditions. The extracellular accumulation of the quorum sensing signal is recognized by a specific receptor, leading to *i.a.* their own synthesis [12,24]. Although not (yet) considered *sensu stricto* to be quorum sensing peptides, some peptides originating from proteins (cryptic peptides) have similar structures as their quorum sensing counterparts. For example, BLAST [25] searches of ID76, an analogue of the quorum sensing peptide produced by *Enterococcus faecium*, revealed that part of the amino acid sequence is synthesized by *i.a. Streptococcus agalactiae* (located at position 269874 to 269918 of the complete genome). These protein-derived peptides thus can have quorum sensing-related effects and should therefore be further investigated.

Microbial receptor-binding properties

The AgrC receptors are only activated by the (thio)lacton or lactam cyclised peptides, containing maximum 6 amino acids in their ring structure. The size of the peptides is not crucial: both medium-sized and small cyclic peptides are found to bind with these receptor subtypes; yet, no large cyclic peptides were investigated for their AgrC receptor-binding potential. The comparable FsrC receptor is activated by 9-membered (thio)lacton or lactam cyclic peptides, with again no linear binding peptides included in the Quorumpeps database. Here, medium-sized as well as large (ID74) quorum sensing peptides were found to bind the FsrC receptor. Only one cyclic peptide, *i.e.* LamD (ID22),

interacts with the LamC receptor of *Lactobacillus plantarum*. The lipophilic peptides, containing a geranyl or farnesyl moiety on a tryptophan amino acid, bind with the ComP receptor; the interaction with other peptides is apparently not yet reported in the literature. The Rap receptor is mainly activated by short linear peptides of *Bacillus* species, next to the medium-sized linear peptides of *Clostridium acetobutylicum*.

4. CONCLUSIONS

The quorum sensing peptides (n = 231; Quorumpeps database) can chemically be divided into three main clusters, which again can be categorized into several subclusters. The clustering is caused by differences in peptide size and compactness, lipophilicity/hydrophilicity, cyclisation and the presence of sulphur atoms and aromatic amino acids. While most of the Gram-positive bacteria synthesize chemically similar peptides, *i.e.* peptides that are grouped into the same cluster, the quorum sensing peptides produced by *Bacillus subtilis* are chemically very diverse, occupying the total space of quorum sensing peptides.

5. REFERENCES

[1]   Miller MB, Bassler BL. Quorum Sensing in Bacteria. *Annu. Rev. Microbiol.* 2001; **55**: 165-199.

[2]   Wynendaele E, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke F, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides. *Nucleic Acids Res.* 2013; **41**: D655-659.

[3]   The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* 2012; **486**: 207-214.

[4]   Gross EL, Beal CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL. Beyond Streptococcus mutans: Dental Caries Onset Linked to Multiple Species by 16S rRNA Community Analysis. *Plos One* 2012; **7**: e47722.

[5]   Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, Haffajee AD, Socransky SS, Oppenheim FG. Identification of early microbial colonizers in human dental biofilm. *J. Appl. Microbiol.* 2004; **97**: 1311-1318.

[6]   Vaughan EE, de Vries MC, Zoetendal EG, Ben-Amor K, Akkermans ADL, de Vos WM. The Intestinal LABs. *Antonie van Leeuwenhoek* 2002; **82**: 341-352.

[7]   Badet C, Thebaud NB. Ecology of lactobacilli in the oral cavity: a review of literature. *Open Microbiol. J.* 2008; **2**: 38-48.

[8]   Li Y-H, Tian X. Quorum Sensing and Bacterial Social Interactions in Biofilms. *Sensors* 2012; **12**: 2519-2538.

[9]   Costerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* 1999; **284**: 1318-1322.

[10]   Kolodkin-Gal I, Hazan R, Gaathon A, Carmeli S, Engelberg-Kulka H. A Linear Pentapeptide Is a Quorum-Sensing Factor Required for mazEF-Mediated Cell Death in *Escherichia coli*. *Science* 2007; **318**: 652-655.

[11]   Kumari A, Pasini P, Daunert S. Detection of bacterial quorum sensing N-acyl homoserine lactones in clinical samples. *Anal. Bioanal. Chem.* 2008; **391**: 1619-1627.

[12]   Antunes LCM, Ferreira RBR, Buckner MMC, Finlay BB. Quorum sensing in bacterial virulence. *Microbiology* 2010; **156**: 2271-2282.

[13]   Waters CM, Bassler BL. Quorum Sensing: Cell-to-Cell Communication in Bacteria. *Annu. Rev. Cell Dev. Biol.* 2005; **21**: 319-346.

[14]   Wynendaele E, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Crosstalk between the microbiome and cancer cells by quorum sensing peptides. Manuscript submitted 2014.

[15]   De Spiegeleer B, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, Wynendaele E. Quorum Sensing Peptides Influence Breast Cancer Cells. Manuscript submitted 2014.

[16]   Fujiya M, Musch MW, Nakagawa Y, Hu S, Alverdy J, Kohgo Y, Schneewind O, Jabri B, Chang EB. The *Bacillus subtilis* Quorum-Sensing Molecule CSF Contributes to intestinal Homeostasis via OCTN2, a Host Cell Membrane Transporter. *Cell Host Microbe* 2007; **1**: 299-308.

[17]   Eriksson L, Johansson E, Kettaneh-Wold N, Trygg J, Wikstrom C, Wold S. *Multi- and Megavariate Data Analysis: Part I - Basic Principles and Applications*, Umetrics Academy, **2006**.

[18]   Kuipers OP, Rollema HS, Yap WMGJ, Boot HJ, Siezen RJ, de Vos WM. Engineering dehydrated amino acid residues in the antimicrobial peptide nisin. *J. Biol. Chem.* 1992; **267**: 24340-24346.

[19]   Sturme MHJ, Francke C, Siezen RJ, de Vos WM, Kleerebezem M. Making sense of quorum sensing in lactobacilli: a special focus on *Lactobacillus plantarum* WCFS1. *Microbiology* 2007; **153**: 3939-3947.

[20]   Dirix G, Monsieurs P, Dombrecht B, Daniels R, Marchal K, Vanderleyden J, Michiels J. Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. *Peptides* 2004; **25**: 1425-1440.

[21]   Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, Bork P. Toward automatic reconstruction of a highly resolved tree of life. *Science* 2006; **311**: 1283-1287.

[22]   http://www.ncbi.nlm.nih.gov/Taxonomy/ (September 2014).

[23]   Reuter G. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr. Issues Intest. Microbiol*. 2001; **2**: 43-53.

[24]   Winzer K, Hardie KR, Williams P. Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr. Opin. Microbiol.* 2002; **5**: 216-222.

[25]   http://blast.ncbi.nlm.nih.gov/Blast.cgi (October 2014).

CHAPTER IV

QUALITY EVALUATION OF SYNTHETIC QUORUM SENSING PEPTIDES USED IN R&D

"Quality begins on the inside... then works its way out"

Bob Moawad
(Author)

ABSTRACT

Peptides are becoming an important class of molecules in the pharmaceutical field. Closely related peptide-impurities in peptides are inherent to the synthesis approach and have demonstrated to potentially mask biomedical experimental results. Quorum sensing peptides are attracting high interest in R&D and therefore a representative set of quorum sensing peptides, with a requested purity of at least 95.0%, was evaluated for their purity and nature of related impurities. In-house quality control (QC) revealed a large discrepancy between the purity levels as stated on the supplier's certificate of analysis and our QC results. By using our QC analysis flowchart, we demonstrated that only 44.0% of the peptides met the required purity. The main compound of one sample was even found to have a different structure compared to the desired peptide. We also showed that the majority of the related impurities were lacking amino acid(s) in the desired peptide sequence. Relying on the certificates of analysis as provided by the supplier might have serious consequences for peptide research and peptide-researchers should implement and maintain a thorough in-house QC.

CHAPTER **IV**

QUALITY EVALUATION OF SYNTHETIC

QUORUM SENSING PEPTIDES USED IN R&D

Main focus in this chapter:

▪ To demonstrate the need for a thorough quality control of synthetic peptides.

▪ To give an overview of expected related impurities in peptides.

1. INTRODUCTION

Peptides are becoming an important class of molecules in the biomedical and pharmaceutical field owing to their high affinity, strong selectivity for their targets and low toxicity [1]. Despite potential limitations such as overall low oral bio-availability, low metabolic resistance, potential immunogenicity, poor membrane permeability and financial aspects, several peptide drugs have entered the market [2,3]. The promising future of peptide therapeutics is further highlighted by the number of peptides in clinical and preclinical phases [4]. In 2012, approximately 200 peptides entered the clinical phase, while another 400 were at advanced preclinical stages [4-6].

Quorum sensing peptides are a group of peptides currently attracting high interest. Quorum sensing, the process of cell-to-cell communication between bacteria, has been the subject of a great number of scientific research papers [7-9]. Three main groups of quorum sensing molecules can be distinguished: *N*-acylhomoserine lactone derivatives (AHL or auto-inducer-1), quorum sensing peptides and boron-furan derivatives (auto-inducer-2). The quorum sensing peptides, mainly found in Gram-positive bacteria, show a large structural diversity: short, linear fragments as well as cyclic (thiolacton) derivatives, with or without post-translational isoprenyl modifications, are observed [10]. These peptides bind to (i) bacterial membrane associated receptors, or (ii) cytoplasmatic receptors, after which the transcription of the target genes is activated. Interfering with this bacterial quorum sensing pathway might open interesting application perspectives.

Chemical peptide synthesis for medicinal purposes has become economically viable [11]. The possibility to produce small, medium (5 to 20 residues) to large (20 to 50 residues) peptides evolved dramatically, hereby frequently outperforming the biotechnological approaches as they are known to date [6]. In 1965, Merrifield cleared the way for Solid-Phase Peptide Synthesis (SPPS), thus introducing and facilitating a totally new concept in peptide synthesis [12]: a peptidic chain, fixed to a solid support, is created by the consecutive addition of the appropriate amino acids. The reaction can be automated and possible solubilization-issues are avoided due to the fixation of the peptide to the solid matrix [13]. During SPPS, different side-reactions can occur, resulting in several types of peptide impurities [14]: *e.g.* (i) diketopiperazine structures [15,16], (ii) aspartimide residues [17-19], (iii) cysteine racemization [16], (iv) diastereoisomeric products [20,21], (v) dimers [22], (vi) acid precursors and protected sequences [20], (vii) oxidation and reduction of amino acids [19,23], (viii) amino acid deletions [15,23-26], (ix) amino acid insertions [23,24], (x) products of side chain reactivity and (xi) amino acid modifications during cleavage [16,19,23,24,26-30]. Thus, the crude peptides obtained from SPPS mostly will contain many by-products. These impurities, if present even after purification, have to be quantified and characterized to meet pharmaceutical regulatory requirements, but they need to be under control as well during the unregulated biomedical research and discovery phases [31,32]. Due to budgetary constraints, peptides used in research are often purchased at undefined or low purity levels, *e.g.* 70.0% [31]. Generally, the peptide purity used for biomedical research and discovery purposes ranges from as low as 50.0% to more than 95.0% [22,26]. However, closely related impurities of the target peptide may possess stronger binding affinities compared to the native peptide [26], thus potentially causing erroneous conclusions. Investigations by de Beukelaar *et al.* indicated different immune responses of a protein-spanning peptide pool of 70.0% pure peptides due to the impurities [33]. False-positive results in a HIV-vaccine trial were also ascribed to impurities in the peptide-mixtures [34]. Zhang *et al.* were unable to reproduce their initial obestatin results *in vitro*, possibly due to impurities present in the initially examined peptide [35]. Additionally, Verbeken *et al.* observed different biofunctional responses in a set of tissue-organ bath experiments caused by impurities of the examined peptides [31]. Impurities are thus able to potentially mask biomedical experimental outcomes and may cause false negative or positive results.

Quorum sensing peptides are currently being actively investigated, *i.a.* for their possible role in the crosstalk between the microbiome and its host [36,37]. Therefore, a thorough in-house QC analysis of a selected set of quorum sensing peptides will be conducted, followed by identification of the observed impurities. These data not only demonstrate the need for routine QC in peptide research, but also can help in building a global overview of expected related impurities in peptides.

2.   MATERIALS AND METHODS

Chemicals and reagents

A set of 98 representative peptides was selected from the Quorumpeps database [38]. Linear peptide synthesis was conducted by an international supplier, while another supplier synthesized the cyclic peptides, both by means of Fmoc-SPPS. A minimal purity of 95.0% was requested at order. The sequences of the 98 peptides are provided in Table 1. Acetonitrile HPLC-MS and UPLC-MS grade were purchased from Fisher Scientific (Aalst, Belgium). Formic acid (LC-MS grade) and DMSO (p.a. ≥ 99.9%) were obtained from Sigma-Aldrich (Diegem, Belgium). Trifluoroacetic acid (TFA), $NaH_2PO_4 \cdot H_20$ and Na_2HPO_4 were purchased from Merck (Overijse, Belgium). Water was purified using an Arium 611 purification system (Sartorius, Gottingen, Germany) yielding ≥ 18.2 MΩ.cm quality water. Eppendorf Protein LoBind 2.0 and 1.5 mL tubes were purchased from Eppendorf (Nijmegen, the Netherlands). UPLC/HPLC vials and inserts were purchased from Waters (Milford, MA, USA). The Vydac Everest C_{18} (250 mm × 4.6 mm, 5 μm) column and Alltech Prevail Organic Acid column (250 mm × 4.6 mm, 5 μm), both protected with guard columns, were obtained from Grace (Deerfield, IL, USA). The Acquity UPLC BEH 300 C_{18} column (100 mm × 2.1 mm, 1.7 μm) with guard column was purchased from Waters (Milford, MA, USA).

Sample preparation

0.5 – 1 mg of each lyophilized, linear peptide was dissolved in water or a mixture of water and DMSO, depending on the calculated logP values and the solubility in water, to obtain a concentration of approximately 1.0 mg/mL (Table 1). The quantity of DMSO used was determined experimentally, by adding DMSO stepwise to the peptide-water suspension until no more precipitate was observed. The dissolved peptides were stored at -35°C. The cyclic peptides (*i.e.* ID5, 22, 32, 40, 71, 97, 107, 148 and 218) were dissolved in DMSO-water (50/50, V/V) to obtain a concentration of approximately 0.5 mg/mL.

HPLC and UPLC

QC purity profiling

Three LC systems were consecutively applied in the analysis. To avoid carry-over peaks, 50% acetonitrile in water was always used as needle wash.

Each peptide was first analysed with the Acquity UPLC BEH300 C_{18} column. Sample compartment was kept at 5°C (± 2°C) and column maintained at 30°C (± 2°C). Injection volume was set at 2 μL for the linear and 5 μL for the cyclic peptides. Mobile phases consisted of 0.1% (m/V) formic acid in water (A)

and 0.1% (m/V) formic acid in acetonitrile (B). A general linear gradient was applied running from 95% A to 20% A during 22 min followed by returning to initial condition and re-equilibration with a flow rate set at 0.5 mL/min. Analysis was conducted using a Waters Acquity UPLC Class BioQuaternary Solvent Manager, a Waters Acquity Bio-sample Manager, combined with a Flow Through Needle and a Waters Acquity UPLC PDA (500 nL – 10 mm path length analytical flow cell) detector (Waters, Milford, MA, USA). Empower 2 software was employed for data acquisition and analysis. Peptides and related impurities were quantified by area-normalization at 210 nm, using a sampling rate of 20 points/s and a detector time constant of 0.1 s. A reporting threshold (*i.e.* 0.1% of main peak area under the curve in the UV chromatogram) and identification threshold (*i.e.* 0.5% of main peak area under the curve in the UV chromatogram) as stated in the European Pharmacopoeia were applied [39]. Solvent and system peaks, observed in the blank (*i.e.* the solvent used to dissolve a certain peptide, Table 1), were excluded.

If no retention of the peptides was observed using the Acquity UPLC BEH300 C_{18} column, peptides were analysed with the Vydac Everest C_{18} column on a Waters Alliance 2695 HPLC apparatus equipped with a Waters 2695 Separations Module, combined with a Flow Through Needle, and a Waters 2996 Photodiode Array Detector with Empower 2 software for data acquisition. Mobile phases consisted of 0.1% (m/V) TFA in water (A) and 0.1% (m/V) TFA in acetonitrile (B). A general linear gradient was applied running from 95% A to 20% A during 30 min followed by returning to initial condition and re-equilibration with a flow rate set at 1.0 mL/min. For peptide ID191, a slightly modified linear gradient was applied, *i.e.* running from 98% A to 80% A during 10 min followed by returning to initial conditions and re-equilibration with a flow rate set at 1.0 mL/min. Sample compartment was kept at 5°C (± 2°C) and column maintained at 30°C (± 2°C); 20 µL of the samples dissolved in suitable solvent composition were injected. UV detection was performed at 215 nm, using a sample rate of 1.0 point/s combined with a detector time constant of 0.2 s. The flow rate was set to 1.0 mL/min. The peptide purity was quantified through normalization, by calculating the area (UV) of the most abundant peak as a percentage of total peak areas.

Finally, peptides ID18, 51, and 192 were analysed using a Grace Alltech Prevail Organic Acid column (250 mm × 4.6 mm, 5 µm), using the same Waters Alliance 2695 HPLC equipment as used with the Vydac column. Mobile phases consisted of 10 mM phosphate buffer (pH 7) (A) and acetonitrile (B). A general linear gradient was applied running from 95% A to 60% A during 20 min followed by returning to initial condition and re-equilibration with a flow rate set at 1.0 mL/min. Sample compartment was kept at 5°C (± 2°C) and column maintained at 30°C (± 2°C). 20 µL of the samples dissolved in suitable solvent composition were injected. UV detection was performed at 215 nm, using a sample rate of 1.0 point/s combined with a detector time constant of 0.2 s. The peptide

purity was quantified through normalization, by calculating the area (UV) of the most abundant peak as a percentage of total peak areas.

Identification of main LC peak and related impurities

Mass spectroscopic characterization was performed with a Thermo HPLC system consisting of a Waters 2487 dual λ absorbance UV/VIS-detector (UV at 215 nm), a Finnigan LCQ ion trap mass spectrometer with electrospray ionization (ESI) and Xcalibur software version 2.0 for data acquisition. The LC-MS equipment is yearly qualified using a tune solution and qualification solution according to the EDQM guideline [40]. The tune solution consists of 100 μL 1.0 mg/mL caffeine (Sigma-Aldrich, Diegem, Belgium) in methanol, 15 μL 5 nmol/μL MRFA (Met-Arg-Phe-Ala) (Research Plus inc., Atlantic City, NJ, USA) in 50/50 methanol/water (V/V), 2.5 mL 0.1% (V/V) Ultramark 1621 (Alfa Aesar, Karlsruhe, Germany) in acetonitrile, 50 μL glacial acetic acid (Riedel de Haen, Diegem, Belgium) and 2.34 mL of 50/50 methanol/water (V/V). The EDQM qualification solution consists of a 10.0 μg/mL reserpine (Flandria, Ghent, Belgium) solution in acidified methanol (*i.e.* 1% acetic acid in methanol (V/V)). The reserpine solution is infused in the mass spectrometer with a flow rate of 3 μL/min with a collision energy of 35.00 eV. The *m/z* value of 609.4 is selected as parent ion and the *m/z* range for the daughter ions is set to *m/z* 165.0-800.0. The obtained *m/z* values are compared to the acceptance criteria as set by the EDQM.

For the analysis of the quorum sensing peptides, column temperature was set at 30°C (± 2°C) and samples were kept at room temperature. 20 μL of aqueous peptide solution was injected into the HPLC system and a flow rate of 1.0 mL/min was applied. All 15 selected quorum sensing peptides were analysed using the Vydac Everest C_{18} column (250 mm × 4.6 mm, 5 μm). Mobile phases consisted of 0.1% (m/V) formic acid in water (A) and 0.1% (m/V) formic acid in acetonitrile (B) with a flow rate of 1.00 mL/min. An isocratic period consisting of 95% A during 5 min was maintained, followed by a linear gradient running from 95% A to 40% A in 60 min and returning to the initial conditions and re-equilibration afterwards. The ion transfer capillary was operated at 250°C, nitrogen was used as sheath gas (80 arbitrary units corresponding to 1.2 L/min) and helium as auxiliary gas (20 arbitrary units corresponding to 0.2 L/min). The spray voltage was 4.50 kV and the capillary voltage was 46 V. MS[1] (100 – 2000 *m/z*) and MS[2] (data dependent, *i.e.* highest abundant MS[1] *m/z* value for MS[2]) data were obtained. All *m/z* values possessing an abundance higher than 25% in the according MS[1] spectrum were subject to identification via their respective MS[2] fragmentation pattern. Theoretical MS[2] fragmentation patterns of the peptides were calculated *in silico* with ProteinProspector [41] (University of California, San Francisco, CA, USA). The proposed sequences of the related impurities was confirmed using the by ProteinProspector generated MS[2] spectra. The

nomenclature originally proposed by Roepstorff and Fohlman is used to annotate MS² fragments [42].

3. RESULTS AND DISCUSSION

Quorum sensing peptide analysis flow chart

To determine the purity of the QS peptides, we developed a UPLC/HPLC-analysis flow chart (Figure 1). Each peptide was analysed first with the Acquity UPLC BEH300 C_{18} column, using the method described above. A C_{18} column with a 0.1% (m/V) formic acid in water/acetonitrile gradient is widely used for peptide analysis and was therefore selected as the initial method of analysis. If no retention of the peptides was observed, the peptides were analysed with the Vydac Everest C_{18} column with TFA as ion-pairing reagent, which is known to have a narrowing effect on peak width and an increased retention. Finally, if no retention was observed with the aforementioned columns, the Prevail Organic Acid column (mobile phase of pH 7) with a 10 mM phosphate buffered water/acetonitrile gradient was used for peptide quality control. Previous experience indicated that peptides which had no retention using standard C_{18} peptide columns might have appropriate retention using a polar-embedded column for highly hydrophilic compounds, like the Prevail Organic Acid column.

Baseline separation with solvent peaks?

1. Acquity UPLC BEH300 C_{18} 0.1% formic acid in water/acetonitrile gradient

2. Vydac Everest C_{18} column 0.1% trifluoroacetic acid in water/acetonitrile gradient

3. Grace Alltech Prevail Organic Acid column 10 mM phosphate buffer/acetonitrile gradient

Quality control of peptide
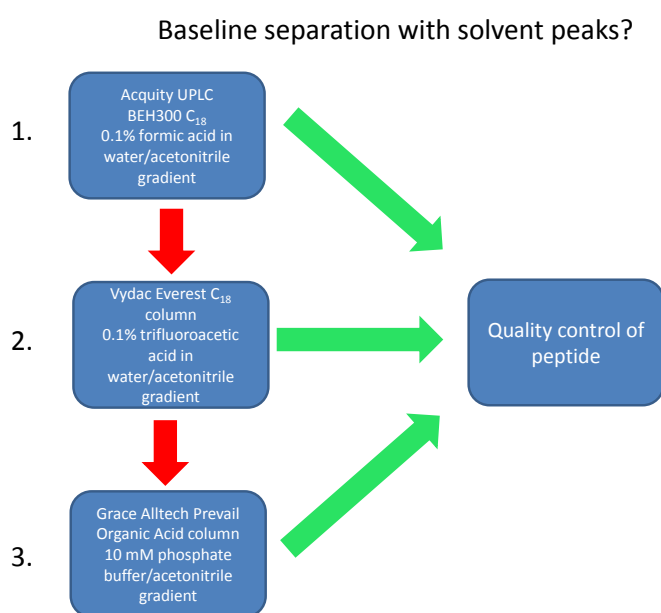
Figure 1. (U)HPLC column flow chart for quorum sensing peptide analysis.

QC purity assay

Despite a requested purity of at least 95.0%, only 43 out of 98 peptides met these purity specifications (Figure 2). However, all 98 peptides were accompanied by a certificate of analysis stating at least 95.0% purity. Our QC analysis upon arrival showed purity levels sometimes far below the demanded purity level (Table 1).

Figure 2. Purity levels of quorum sensing peptides determined by in-house QC.

Table 1. Peptide information.

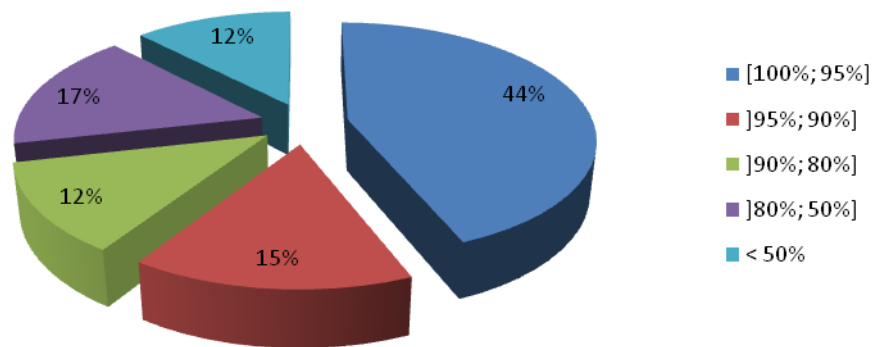| Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[1]	Purity[2] (%)
2	FNTIPSY	839.95	Water	C_{18}-FA	83.31
5	Ac-CGSLF, thiolacton linkage between C1 and F5	549.72	Water + 50% DMSO	C_{18}-FA	88.30
7	FNTWPSY	913.00	Water	C_{18}-FA	98.46
10	ADLPFEF	837.93	Water	C_{18}-FA	99.20
11	AGTKPQGKPASNLVECVFSLFKKCN	2667.14	Water	C_{18}-FA	72.93
13	AIFILAS	733.91	Water + 58% DMSO	C_{18}-FA	97.82
14	AITLIFI	790.01	Water + 60% DMSO	C_{18}-FA	72.48
15	AKDEH	598.61	Water	C_{18}-TFA	69.26
16	AKTVQ	545.64	Water	C_{18}-TFA	96.20
17	ALILTLVS	829.05	Water + 60% DMSO	C_{18}-FA	94.43
18	ARNQT	588.62	Water	Organic Acid	92.33
19	NNWNN	660.64	Water	C_{18}-TFA	92.66
22	CVGIW, thiolacton linkage between C1 and W5	558.78	Water + 50% DMSO	C_{18}-FA	60.39
24	CTFTLPGGGGVCTLTSECIC	1962.3	Water + 1% DMSO	C_{18}-FA	12.21
25	CVFSLFKKCN	1188.47	Water	C_{18}-FA	47.92
28	DIRHRINNSIWRDIFLKRK	2480.91	Water	C_{18}-FA	93.68
30	DLRGVPNPWGWIFGR	1770.03	Water	C_{18}-FA	91.62
31	DLRNIFLKIKFKKK	1791.26	Water	C_{18}-FA	76.74
32	DMCNGYF, thiolacton linkage between C3 and F7	831.04	Water + 50% DMSO	C_{18}-FA	58.05
34	DRVGA	516.55	Water	C_{18}-TFA	98.30
40	DSVCASYF, thiolacton linkage between C4 and F8	873.06	Water + 50% DMSO	C_{18}-FA	31.61
42	DWRFLNSIRDLIFPKRK	2204.61	Water	C_{18}-FA	97.86
44	EKMIG	576.71	Water	C_{18}-FA	99.41
45	EMRISRIILDFLFLRKK	2178.71	Water	C_{18}-FA	81.91
46	EMRKSNNNFFHFLRRI	2109.44	Water	C_{18}-FA	96.43
47	EMRLPKILRDFIFPRKK	2187.72	Water	C_{18}-FA	98.46
49	EQLSFTSIGILQLLTIGTRSCWFFYCRY	3346.92	Water + 17% DMSO	C_{18}-FA	52.58

Table 1. Peptide information (continued).

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[1]	Purity[2] (%)
50	ERGMT	592.67	Water	C_{18}-TFA	98.76
51	ERNNT	632.63	Water	Organic Acid	98.24
52	ERPVG	556.62	Water	C_{18}-TFA	99.75
53	ESRLPKILLDFLFLRKK	2116.62	Water	C_{18}-FA	65.11
54	ESRLPKIRFDFIFPRKK	2177.62	Water	C_{18}-FA	99.30
55	ESRVSRIILDFLFQRKK	2135.54	Water	C_{18}-FA	96.90
56	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	4969.46	Water	C_{18}-FA	33.57
58	DSRIRMGFDFSKLFGK	1904.22	Water	C_{18}-FA	95.72
62	ESRISDILLDFLFQRKK	2108.47	Water	C_{18}-FA	99.27
71	QNCPNIFGQWM, lacton linkage between S3 and M11	1319.70	Water + 50% DMSO	C_{18}-FA	77.64
75	SINSQIGKATSNLVECVFSLFKKCN	2731.2	Water	C_{18}-FA	30.03
76	SNLVECVFSLFKKCN	1731.06	Water	C_{18}-FA	80.63
81	FNTIPKY	881.04	Water	C_{18}-FA	99.29
82	NTIPKY	733.87	Water	C_{18}-FA	69.22
84	FFNTCPSY	978.09	Water	C_{18}-FA	96.54
85	FNTCPSY	830.91	Water	C_{18}-FA	92.98
92	FHWWQTSPAHFS	1530.66	Water	C_{18}-FA	99.54
93	FLVMFLSG	913.14	Water + 33% DMSO	C_{18}-FA	91.31
97	QNSPNIFGQWM, lacton linkage between S3 and M11	1303.63	Water + 50% DMSO	C_{18}-FA	43.95
99	GKAEF	550.61	Water	C_{18}-FA	99.33
100	GKATSSISKCVFSFFKKC	1968.36	Water + 33% DMSO	C_{18}-FA	28.65
101	GLWEDILYSLNIIKHNNTKGLHHPIQL	3167.67	Water	C_{18}-FA	96.92
102	GLWEDLLYNINRYAHYIT	2254.53	Water + 33% DMSO	C_{18}-FA	97.98
103	GNWNN	603.59	Water	C_{18}-FA	97.70
105	GSQKGVYASQRSFVPSWFRKIFRN	2846.25	Water	C_{18}-FA	99.31
107	GVNACSSLF, thiolacton linkage between C5 and F9	879.13	Water + 50% DMSO	C_{18}-FA	39.15
111	GWWEDFLYRFNIIEQKNTKGFYQPIQL	3434.91	Water + 50% DMSO	C_{18}-FA	89.72
121	ILSGAPCIPW	1056.29	Water	C_{18}-FA	77.04

Table 1. Peptide information (continued).

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[1]	Purity[2] (%)
123	IRFVT	634.78	Water	C$_{18}$-FA	99.53
125	KSSAYSLQMGATAIKQVKKLFKKWGW	2985.59	Water	C$_{18}$-FA	66.39
132	LFSLVLAG	819.01	Water + 33% DMSO	C$_{18}$-FA	96.92
133	LFVVTLVG	847.06	Water + 60% DMSO	C$_{18}$-FA	97.74
134	LPFEF	651.76	Water + 50% DMSO	C$_{18}$-FA	17.17
135	LPFEH	641.72	Water	C$_{18}$-FA	94.23
137	LVTLVFV	790.01	Water + 50% DMSO	C$_{18}$-FA	84.02
138	MAGNSSNFIHKIKQIFTHR	2229.59	Water + 17% DMSO	C$_{18}$-FA	81.90
140	MKAEH	614.72	Water	C$_{18}$-FA	66.91
143	MPFEF	669.79	Water	C$_{18}$-FA	86.24
146	NEVPFEF	880.95	Water	C$_{18}$-FA	99.90
147	NGWNN	603.59	Water	C$_{18}$-FA	94.53
148	YSTCDFIM, thiolacton linkage between C4 and M8	961.25	Water + 50% DMSO	C$_{18}$-FA	44.40
151	NNGNN	531.48	Water	C$_{18}$-TFA	96.79
152	NNNWNNN	888.85	Water	C$_{18}$-FA	98.42
153	NNWGN	603.59	Water	C$_{18}$-FA	99.10
154	NNWNG	603.59	Water	C$_{18}$-FA	99.33
155	NWN	432.44	Water	C$_{18}$-FA	98.40
156	FNTIP	589.69	Water	C$_{18}$-FA	99.27
157	FNTWP	662.75	Water	C$_{18}$-FA	99.23
160	QKGMY	625.74	Water	C$_{18}$-FA	99.04
162	QRGMI	603.74	Water	C$_{18}$-FA	94.36
164	SDLPFEH	843.89	Water	C$_{18}$-FA	91.37
165	SDMPFEF	871.96	Water	C$_{18}$-FA	99.44
166	SGSLSTFFLLFNRSFTQALGK	2321.66	Water	C$_{18}$-FA	92.99
174	SGSLSTFFRLFNRSFTQALG	2236.52	Water	C$_{18}$-FA	97.41
176	SGSLSTFFRLFNRSFTQALGK	2364.69	Water	C$_{18}$-FA	98.96
177	SGSLSTFFRLFNRSFTQALGKIR	2634.04	Water	C$_{18}$-FA	55.68
180	SGSLSTFFRLFNRSQTQALGK	2345.65	Water	C$_{18}$-FA	99.06

Table 1. Peptide information (continued).

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[1]	Purity[2] (%)
184	SIFTLVA	749.90	Water + 33% DMSO	C_{18}-FA	93.17
186	SKDYN	625.64	Water	C_{18}-TFA	92.88
188	SLSTFFRLFNFSFTQALG	2083.37	Water + 33% DMSO	C_{18}-FA	85.61
191	SRKAT	561.64	Water	C_{18}-TFA	78.31
192	SRNAT	547.57	Water	Organic Acid	81.74
193	SRNVT	575.62	Water	C_{18}-TFA	81.49
206	SYPGWSW	881.94	Water	C_{18}-FA	99.45
207	TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK	3595.31	Water	C_{18}-FA	32.42
208	TNRNYGKPNKDIGTCIWSGFRHC	2668.01	Water	C_{18}-FA	89.19
210	VAVLVLGA	740.94	Water + 33% DMSO	C_{18}-FA	92.98
212	VPFEF	637.73	Water	C_{18}-FA	97.65
214	WPFAHWPWQYPR	1670.89	Water	C_{18}-FA	58.94
215	FNTWPKY	954.10	Water	C_{18}-FA	95.52
218	YNPCSNYL, thiolacton linkage between C4 and L8	955.18	Water + 50% DMSO	C_{18}-FA	16.87

[1]: C_{18}-FA: Acquity UPLC BEH 300 C_{18} column (100 mm × 2.1 mm, 1.7 μm).

 C_{18}-TFA: Vydac Everest C_{18} column (250 mm × 4.6 mm, 5 μm).

 Organic Acid: Grace Alltech Prevail Organic Acid column (250 mm × 4.6 mm, 5 μm).

[2]: Purity assigned based on UV chromatogram with internal normalization to the most abundant peak.

In Figure 3, the chromatogram of ID76, as provided by the supplier on the certificate of analysis versus our QC analysis is given. The mobile phases of the supplier consisted of 0.1% (m/V) TFA in acetonitrile (A) and 0.1% (m/V) TFA in water (B). A general linear gradient was applied running from 22% A to 47% A during 22 min with a Kromasil C_{18}-5 (250 mm × 4.6 mm, 5 µm) column with quantification at 220 nm. The peptide was analysed in our laboratory with the Acquity UPLC BEH300 C_{18} column, using the operational details as described above.
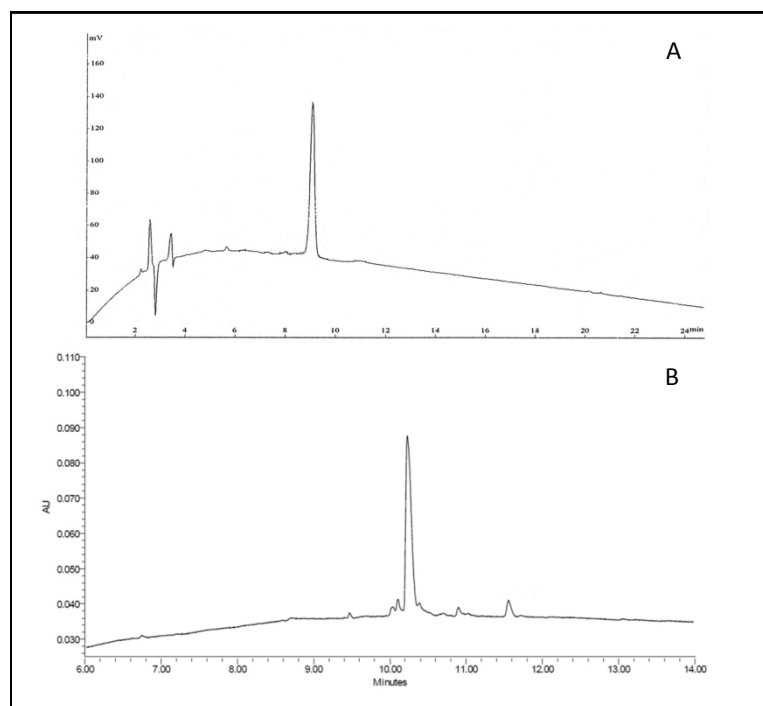

Figure 3. The in-house QC control shows a distinct difference in peptide purity. (A) Chromatogram of supplier; Kromasil C_{18}-5 (250 mm × 4.6 mm, 5 µm), quantification at 220 nm; (B) chromatogram of in-house QC; Vydac Everest C_{18} column (250 mm × 4.6 mm, 5 µm), quantification at 210 nm.

The method applied by the supplier clearly lacked selectivity to determine the purity level of the peptide. We demonstrated the necessity of a reliable QC method, *i.e.* the need of a suitable chromatographic system to analyse peptide purity. The supplier claimed ≥ 95.0% purity using his system, where we determined a purity level of 80.6%.

Especially the cyclic peptides (n = 9) are characterized by low purity levels: none of the peptides had a purity exceeding 90.0%. Of all cyclic peptides, 1 peptide had a purity between 80.0 and 90.0%, whereas 8 others had purity levels below 80.0%. Nevertheless, each of these peptides was accompanied by a certificate of analysis stating a purity of at least 95.0%. After communication with the supplier, they revealed that they were unable to produce these cyclic peptides at the requested purity level.

Identification of related impurities

A set of 15 peptides with varying purities was selected to determine the identity of the impurities (Table 1, depicted in red). The main peak was characterized as the desired peptide for 14 out of 15 peptides. The main peak from peptide ID25 was not the requested amino acid sequence. Instead of CVFSLFKKCN, a peptide with a mass difference of -2.1 Da compared to the requested peptide sequence was noted. Therefore, the formation of a disulfide bond (and thus cyclisation) between C_1 and C_9 is suggested. Because sulfur-containing amino acids are prone to oxidation [43], a spontaneous cyclisation is suspected for this peptide (Figure 4).

The MS^1 spectrum of a related peptide impurity of peptide ID134 (retention time of 29.75 min) showed an impurity with a monoisotopic mass of 666.22 Da (Figure 5). The difference with the monoisotopic mass of the requested peptide sequence (LPFEF) amounts 14.04 Da, a mass difference that could be associated with the addition of a methyl group. The MS^2 fragmentation pattern of the related peptide impurity, found in Figure 5C, showed a difference of +14 Da with the requested peptide sequence fragmentation pattern at b_4, y_2-H_2O, y_4, MH-H_2O, while the b_3 fragment of requested peptide and related impurity are the same, suggesting thus the addition of a methyl group to Glu4.

A related impurity of peptide ID82 (retention time 9.34 min) possessed a *m/z* value of 621.14 (Figure 6). Compared to the desired peptide, *i.e.* NTIPKY, there was a mass difference of 114.02 Da, a mass difference which could be bridged by the deletion of Asn1. The suggested sequence TIPKY is confirmed by the MS^2 spectrum in Figure 6C. On the other hand, we also demonstrated a related impurity characterized by the incorporation of an additional amino acid at the C-terminus of peptide ID121 (retention time 35.70 min) (Figure 7). The MS^2 spectrum (Figure 7C) confirmed the addition of an extra Trp residue at the C-terminus of peptide ID121, thus resulting in the related impurity ILSGAPCIPWW. Another example of a related peptide impurity was the combination of both deleted and additionally inserted amino acids in the desired peptide sequence. In Figure 8, such an example is given: ILSGAPCIPPPP is a related impurity of peptide ID121 and lacks the C-terminal Trp residue, but is characterized by 3 additional Pro residues at the C-terminus.
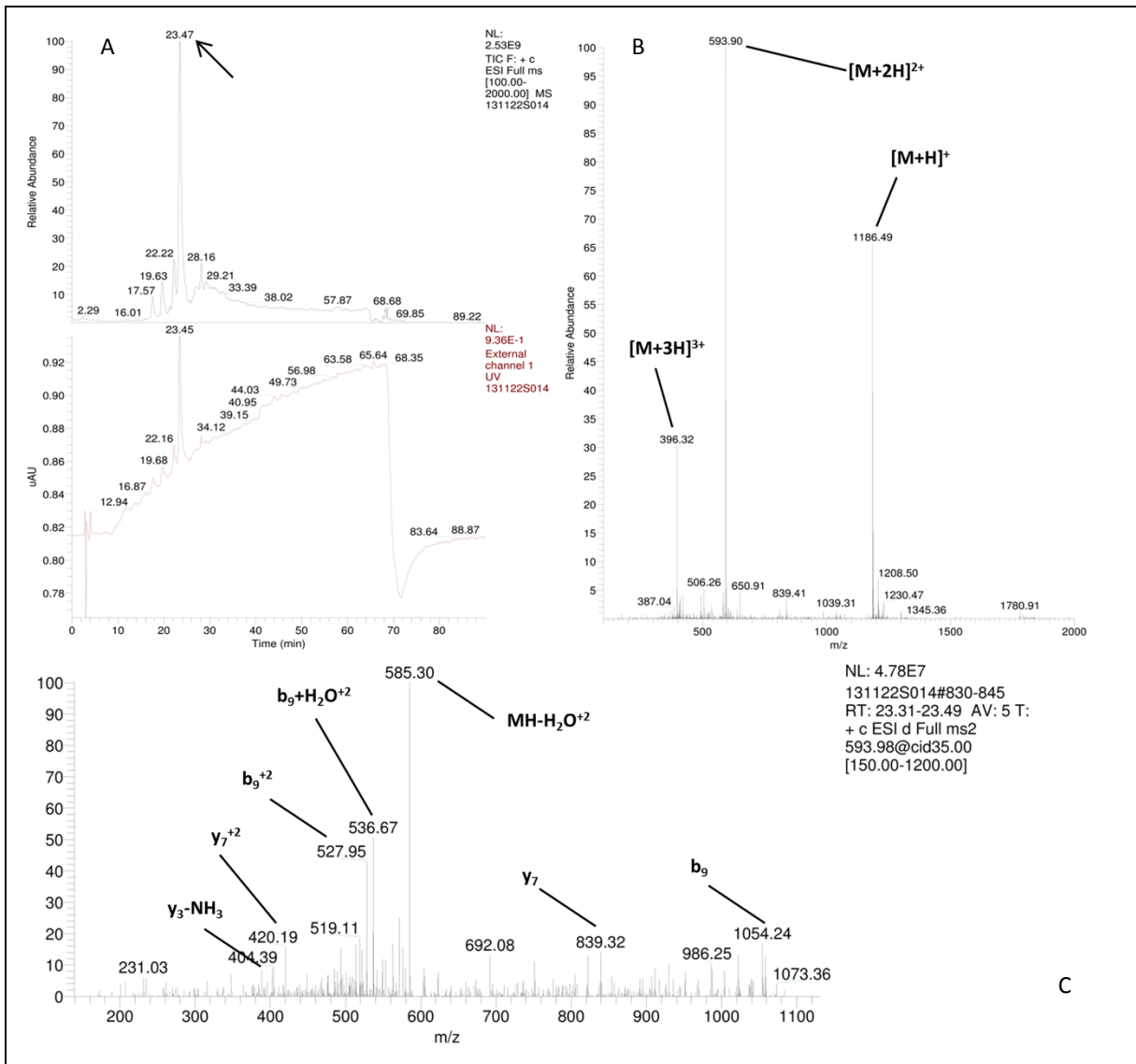
Figure 4. TIC and UV chromatogram (A), MS[1] (B) and MS² (C) spectra of the related impurity $C_1VFSLFKKC_9N$ (disulfide bond between C_1 and C_9) of peptide ID25 at a retention time of 23.47 minutes with appointed isotopic mass patterns and b- and y-fragmentation.
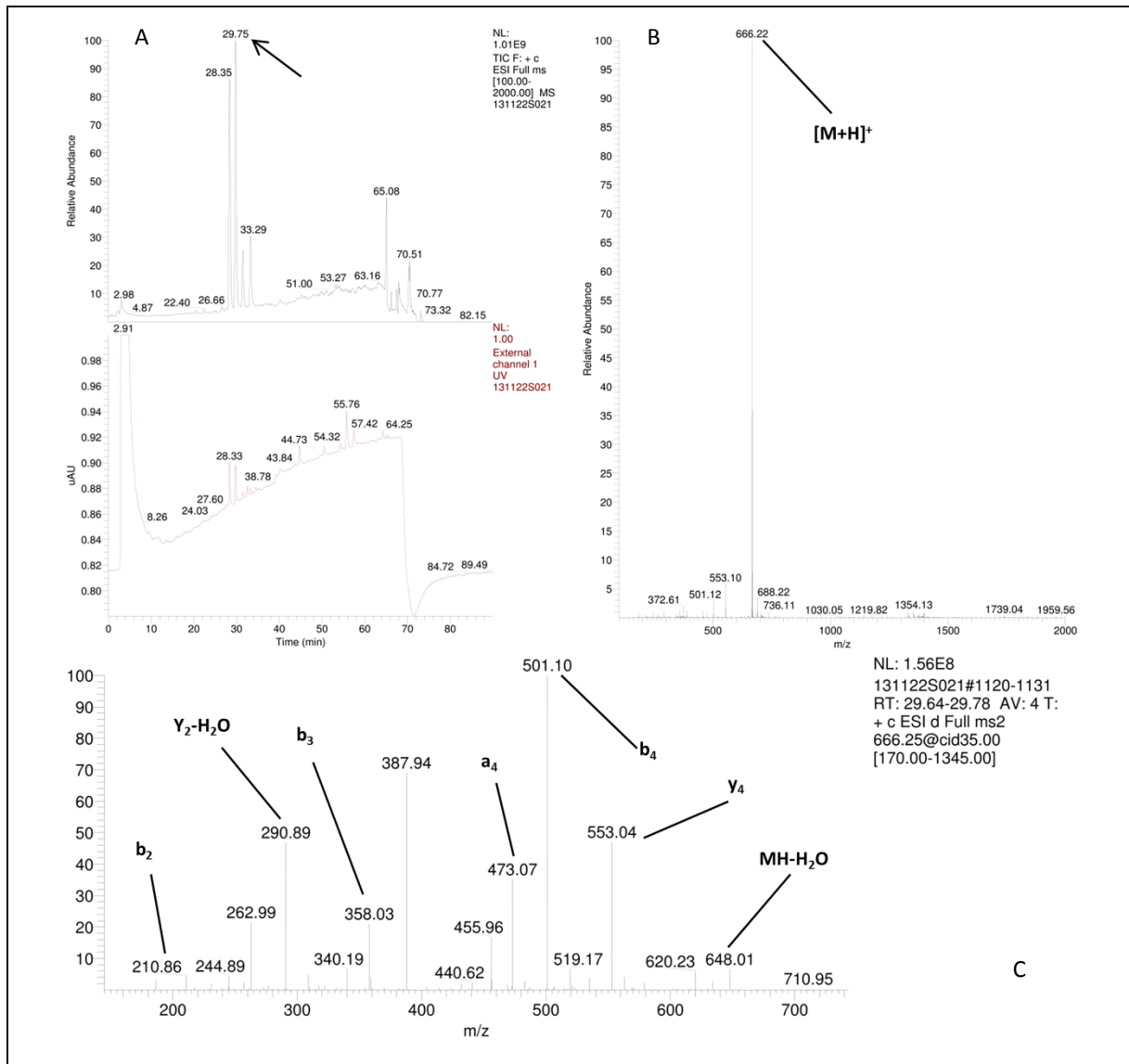
Figure 5. TIC and UV chromatogram (A), MS[1] (B) and MS² (C) spectra of the related impurity LPFE(methyl)F of peptide ID134 at a retention time of 29.75 minutes with appointed isotopic mass patterns and b- and y-fragment allocation.
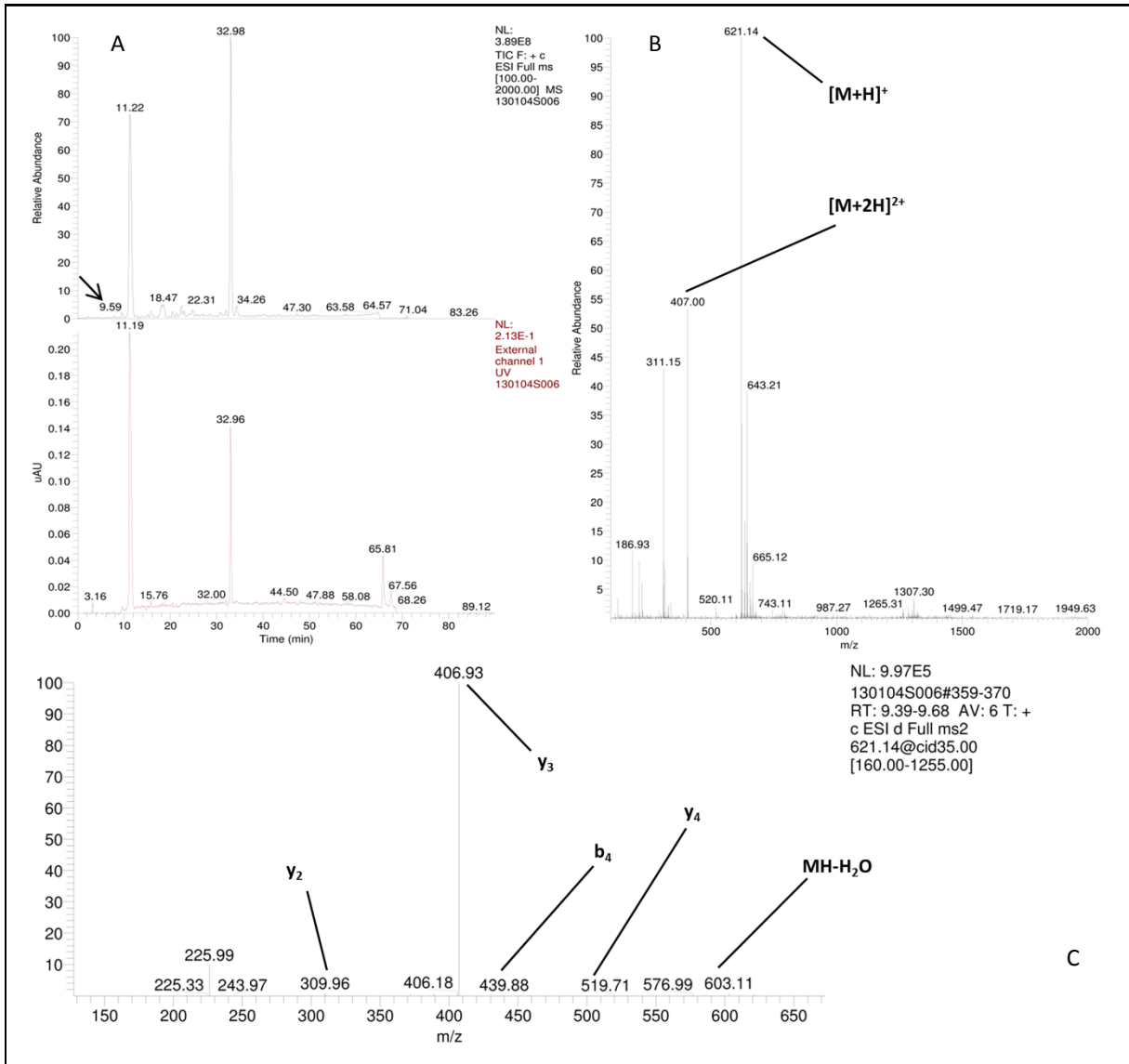
Figure 6. TIC and UV chromatogram (A), MS[1] (B) and MS² (C) spectra of the related impurity TIPKY of peptide ID82 at a retention time of 9.59 minutes with appointed isotopic mass patterns and b- and y-fragment allocation.

Figure 7. TIC and UV chromatogram (A), MS^1 (B) and MS² (C) spectra of the related impurity ILSGAPCIPWW of peptide ID121 at a retention time of 35.70 minutes with appointed isotopic mass patterns and b- and y-fragment allocation.

Figure 8. TIC and UV chromatogram (A), MS^1 (B) and MS² (C) spectra of the related impurity ILSGAPCIPPPP of peptide ID121 at a retention time of 33.55 minutes with appointed isotopic mass patterns and b- and y-fragment allocation.
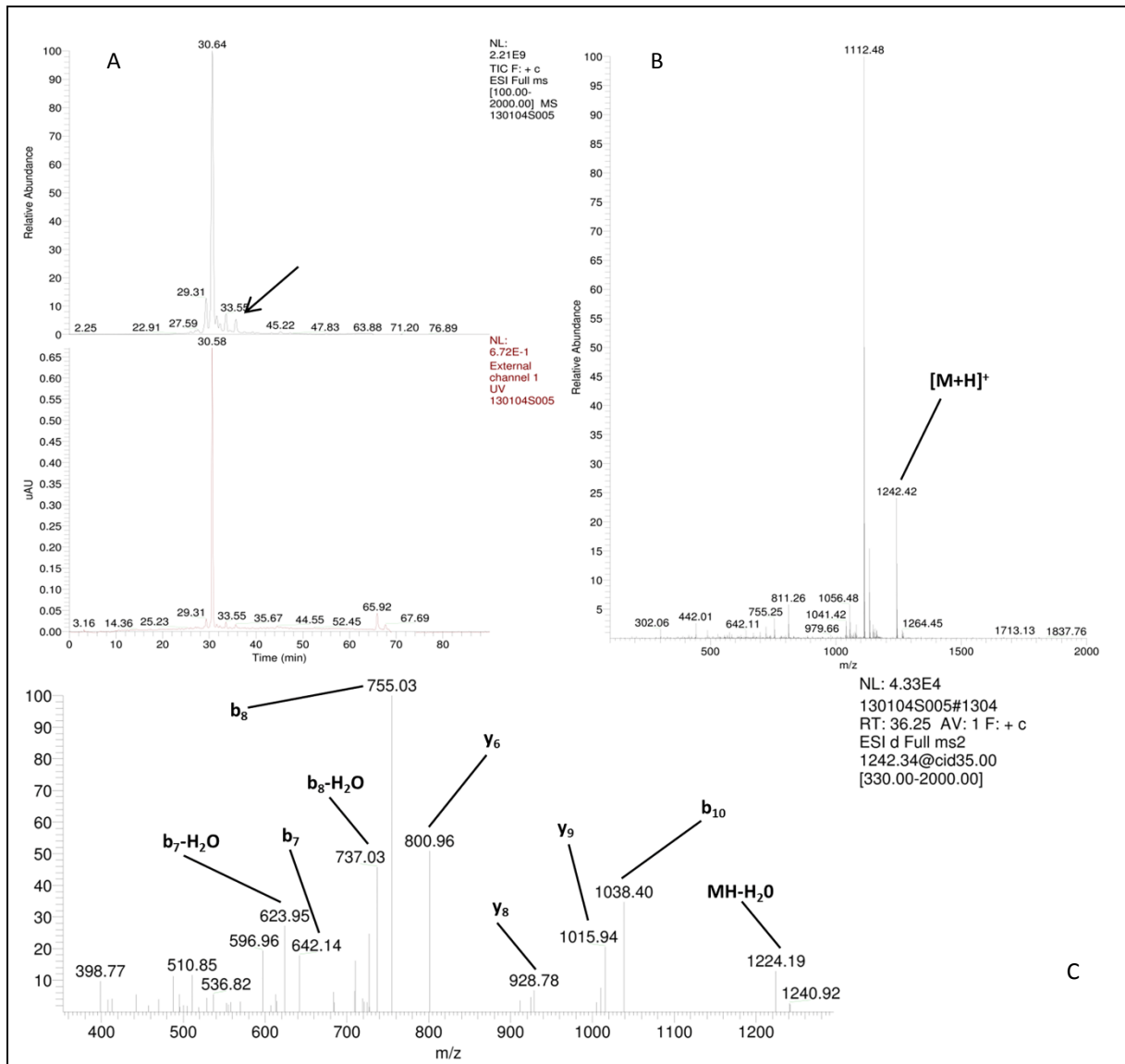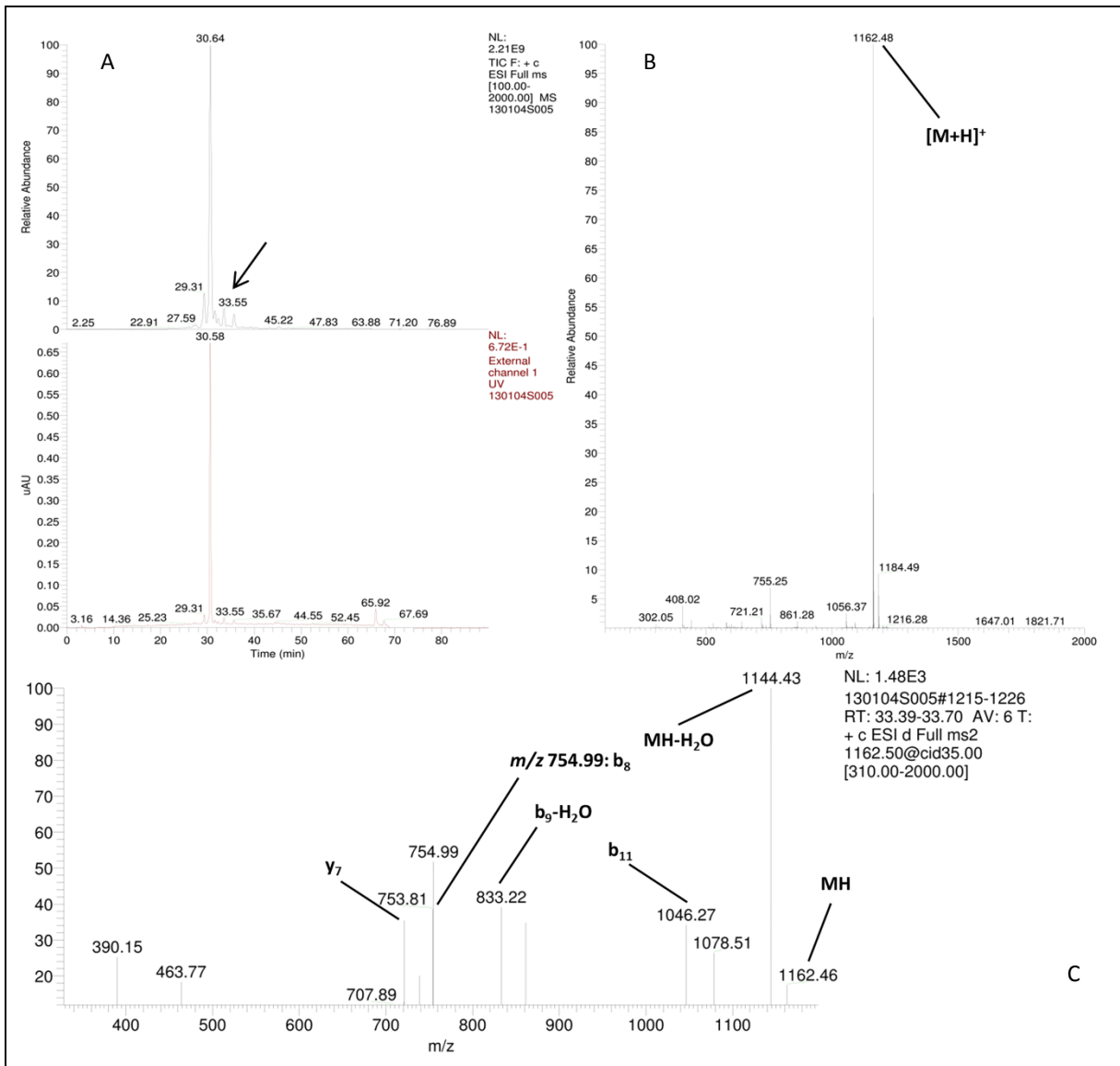
In total, 84 impurities (Table 2) were observed and 73% could be assigned a structure related to the requested peptide by means of MS². Analysis of these identified impurities revealed a high abundance of impurities with at least one deletion (*i.e.* in 74% of all identified impurities is involved) (Figure 9). Inappropriate deletion of blocking groups during synthesis results in such sequences, thereby lacking at least 1 amino acid [20,44]. A minority of the observed peptide impurities were characterized by the incorporation of additional amino acids. During synthesis, an excess of amino acid equivalents used to assure a maximum coupling efficiency might result in the incorporation of additional amino acids [20,45]. Impurities can also be a combination of alteration types, *e.g* one or more desired amino acid(s) might be missing at the N-terminus whilst at the C-terminus additional amino acid(s) are incorporated.
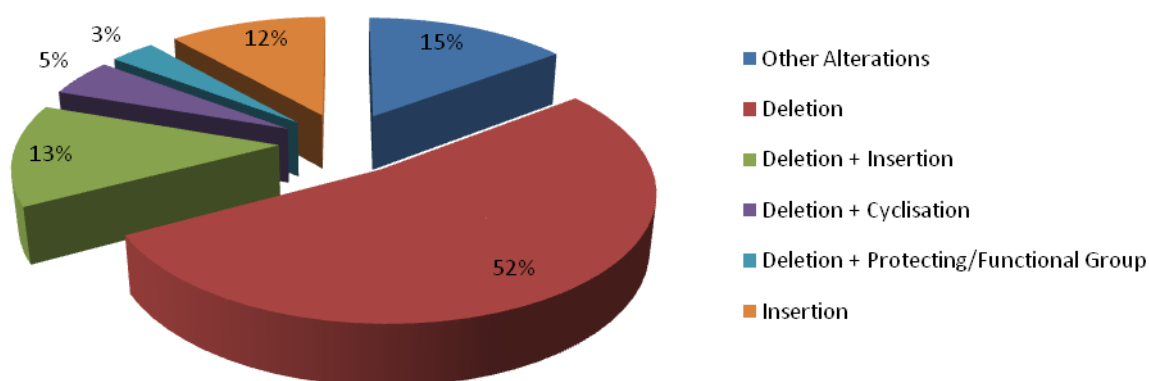
Figure 9. Types of related peptide impurities found in the 61 experimentally determined sequences.

4. CONCLUSIONS

In conclusion, "purified" synthetic peptides still contain a considerable amount and wide variety of related impurities, with peptides missing amino acid(s) being the most prominent. As a consequence, a thorough in-house quality characterization of peptides, already used in basic biomedical research, is mandatory. Remarkably, there is a large discrepancy between the certificates of analysis provided by the suppliers and the in-house quality control, which can at least partly be explained by the methods used. Finally, researchers should also be aware that the main peak observed in the chromatogram might be an impurity instead of the requested peptide; hence MS is requested in this QC.

Table 2. Overview of the observed peptides (blue) and impurities (purple).

Peptide ID	Purity (%)[1]	M_r (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
19 NNWNN	92.66	660.12	NNWNN	4.59	-
		661.10	Deamidated	5.73	Not yet identified
44 EKMIG	99.41	576.19	EKMIG	3.83	-
76 SNLVECVFSLFKKCN	80.63	1778.20	**X**FSLFKKCN	35.18	Not yet identified
		1761.98	Not yet identified	38.31	Not yet identified
		1728.69	SNLVECVFSLFKKCN	40.59	-
		1641.10	*NLVEC5VFSLFKKC13N, disulfide bond between C_5 and C_{13}	45.59	Deletion + cyclisation
134 LPFEF	17.17	518.10	LPE(methyl)F	22.40	Deletion + functional group
		651.18	LPFEF	28.35	-
		665.22	LPFE(methyl)F	29.75	Functional group
		709.28	LPFEF**X**	31.49	Not yet identified
		679.21	Not yet identified	33.29	Not yet identified
162 QRGMI	94.36	-	ND[4]	4.15	-
		546.07	QRMI	4.94	Deletion
		603.27	QRGMI		-
		474.98	RGMI		Deletion
		-	ND[4]	5.80	-
		586.17	QR(ammonia-loss)GMI	14.82	Ammonia loss
		2143.56	QRGMRR**X**GMRGMRGMGMI	17.74	Not yet identified
153 NNWGN	99.10	546.05	NNWN	4.56	Deletion
		603.06	NNWGN	5.05	-
		604.05	NNWGGG	6.90	Deletion + insertion

Table 2. Overview of the observed peptides (blue) and impurities (purple) (continued).

Peptide ID	Purity (%)[1]	M_r (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
25 CVFSLFKKCN	47.92[2]	985.42	FSLFKKCN	17.57	Deletion
		1084.45	VFSLFKKCN	19.53	Deletion
		1187.46	CVFSLFKKCN	21.22	-
		1086.42	C_1FSLFKKC$_8$N, disulfide bond between C_1 and C_8		Deletion + cyclisation
		1185.50	C_1VFSLFKKC$_9$N, disulfide bond between C_1 and C_9	23.47	Cyclisation
		1027.39	FKLFKKCN or acetyl-FSLFKKCN	25.26	Deletion + insertion or deletion + acetylation
		1219.36	C_1VFSFFKKC$_9$N, disulfide bond between C_1 and C_9	25.74	Deletion + insertion + cyclisation
		1235.41	CFFSLFKKCN	28.16	Deletion + insertion
		1057.35	C_1VFSLFC$_8$N, disulfide bond between C_1 and C_8		Deletion + cyclisation
		1126.56	KVFLFKKCN	29.21	Deletion + insertion
		1213.38	KVFSLFKKCN	31.52	Deletion + insertion
164 SDLPFEH	91.37	696.16	SDLPEH	4.12	Deletion
		746.10	SDLFEH	14.72	Deletion
		570.06	acetyl-PFEH	16.31	Acid precursor + deletion
		714.27	SDLPFH		Deletion
		728.95	SLPFEH		Deletion
		785.22	SD**X**LPL	16.87	Not yet identified
		470.00	SDLH		Deletions
		529.05	PFEH	17.36	Deletions
		843.24	SDLPFEH		-
		641.17	LPFEH		Deletions
		756.15	DLPFEH	18.33	Deletion
		939.30	SDLPPFEH or PSDLPFEH	26.76	Insertion
		1308.75	**X**LPFEH or LPFEH**X**	31.06	Not yet identified

Table 2. Overview of the observed peptides (blue) and impurities (purple) (continued).

Peptide ID	Purity (%)[1]	M_r (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
53 ESRLPKILLDFLFLRKK	65.11	2115.99	ESRLPKILLDFLFLRKK	29.87	-
		1620.81	SKILLDFLFLRKK	31.11	Deletion
		1959.46	ESRLPKILLDFLFLKK	35.89	Deletion
		1430.67	**X**DFLFLRKK	38.98	Not yet identified
		2131.14	Not yet identified	39.73	Not yet identified
		1627.78	**X**LDFLFLRKK	41.53	Not yet identified
		1740.71	**X**LLDFLFLRKK	43.52	Not yet identified
138 MAGNSSNFIHKIKQIFTHR	81.90	-	ND[4]	4.11	-
		2027.64	GNSSNFIHKIKQIFTHR	21.09	Deletion
		2229.01	MAGNSSNFIHKIKQIFTHR		-
		-	ND[4]	23.01	-
		2091.64	MAGNSSNFIHKIKQIFTR	23.90	Deletion
		1834.12	MAGNSSNFIHKIKQIF		Deletion
		1307.38	HKIKQIFTHR		Deletion
		2159.99	MAGNS**X**IHKIKQIFTHR	24.53	Not yet identified
		2123.85	RNSSNFIHKIKQIFTHR	25.34	Deletion + insertion
		1995.71	MANNFIHKIKQIFTHR		Deletion
121 ILSGAPCIPW	77.04	-	Not yet identified	24.73	Not yet identified
		-	Not yet identified	25.85	Not yet identified
		614.10	ISIPW or LSIPW or PCIPW	26.06	Deletion
		830.13	SGAPCIPW	27.25	Deletion
		-	GAPCIP**X**PCIPW		Not yet identified
		754.22	ILSGAPCI(dehydrated)	27.59	Deletion + loss of water
		1027.41	**X**PW		Not yet identified
		942.27	ILSGAPCPW	29.31	Deletion
		952.41	ILSGAPIPW		Deletion

Table 2. Overview of the observed peptides (blue) and impurities (purple) (continued).

Peptide ID	Purity (%)[1]	M$_r$ (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
121 ILSGAPCIPW (continued)	77.04	969.88	ILSGAPCIPT	30.64	Insertion
		1041.37	Deamination product		Not yet identified
		1055.46	ILSGAPCIPW		-
		927.34	ILSPCIPW	31.71	Deletion
		998.40	ILSAPCIPW		Deletion
		968.35	ILGAPCIPW	32.56	Deletion
		1161.48	ILSGAPCIPPPP	33.55	Deletion + insertion
		-	Not yet identified	35.70	Not yet identified
		1241.42	ILSGAPCIPWW	36.25	Insertion
125 KSSAYSLQMGATAIKQV KKLFKKWGW	66.39	2912.62	Not yet identified	29.63	Not yet identified
		2984.46	KSSAYSLQMGATAIKQVKKLFKKWGW	29.73	-
		3089.70	KSSAYYSLQMATAIKQVKKLFKKWGW	32.36	Deletion + insertion
31 DLRNIFLKIKFKKK	76.74	1675.62	LRNIFLKIKFKKK	19.42	Deletion
		1790.03	DLRNIFLKIKFKKK	20.19	-
		1633.78	DLNIFLKIKFKKK	21.96	Deletion
82 NTIPKY	69.22	644.18	**X**KY	4.12	Not yet identified
		620.22	TIPKY	9.59	Deletion
		406.07	PKY	11.22	Deletion
		734.24	NTIPKY		
		897.30	NTIPKYY	15.78	Insertion
		569.09	YPKY or PKYY		Deletion + insertion
		605.87	NTIPY(hydroxylated)	16.14	Deletion + hydroxylation
		662.18	acetyl-TIPKY	18.12	Protection group + deletion

Table 2. Overview of the observed peptides (blue) and impurities (purple) (continued).

Peptide ID	Purity (%)[1]	M_r (exp.) (Da)	Impurity sequence[3]	Retention time (min)	Alteration type
82 NTIPKY (continued)	69.22	1275.50	NTIPKTTIPKY or TIPKYTIPKNT or TIPKTNTIPKY	18.52	Insertion
		517.05	K(ammonia-loss)PKY	24.73	Deletion + insertion + modification
		976.34	NNK NKN ⎤=TIPKY KNN ⎦ Or NNQ NQN ⎤=TIPKY QNN ⎦	32.98	Insertion
75 SINSQIGKATSNLVECVF SLFKKCN	30.03	2421.00	Not yet identified	28.40	Not yet identified
		2743.23	Not yet identified	33.63	Not yet identified
		2256.32	SINSQIGKATSNLVECVFSLF	35.20	Deletion
		2729.40	SINSQIGKATSNLVECVFSLFKKCN	40.99	-
		2786.42	SINSQIGGKATSNLVECVFSLFKKCN	46.27	Insertion

[1]: Purity assigned based on UV chromatogram with internal normalization to the most abundant peak.

[2]: The peptide C_1VFSLFKKC$_9$N (disulfide bond between C_1 and C_9) was the most abundant peak and was not the desired sequence.

[3]: Structural deviations were defined as follows:

- Chemical modifications of an amino acid are depicted in between brackets following the modified amino acid.

- **X** signals a "semi-identified" impurity with already part of the peptide sequence allocated in the MS² fragmentation pattern.

[4]: Solely observed in UV spectrum, no signal in TIC.

5.   REFERENCES

[1]   Craik D, Fairlie DP, Liras S, Price D. The future of peptide-based drugs, *Chem. Biol. Drug Des.* 2013; **81**: 136–147.

[2]   Berggren R, Moller M, Moss R, Poda P, Smietana K. Outlook for the next 5 years in drug innovation. *Nat. Rev. Drug Discov.* 2012*;* **11**: 435–436.

[3]   Van Arnum P. Peptides gain traction in drug development. *Pharm. Technol.* 2012; **36**: 42–43.

[4]   Albericio F, Kruger H. Therapeutic peptides. *Future Med. Chem.* 2012; **4**: 1527–1531.

[5]   Danho W, Swistok J, Khan W, Chu X-J, Cheung A, Fry D, Sun H, Kurylko G, Rumennik L, Cefalu J, Defalu G, Nunn P. Oppertunities and challenges of developing peptide drugs in the pharmaceutical industry, In *Peptides for Youth* (Eds: Del Valle S, Escher E, Lubell W), Springer Sciences and Business Media, NewYork, **2009**, pp. 467–468.

[6]   Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. *Drug Discov. Today* 2010; **15**: 40–56.

[7]   Williams P. Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology* 2007; **153**: 3923–3938.

[8]   Hughes DT, Sperandio V. Inter-kingdom signaling: communication between bacteria and their hosts. *Nature Rev. Microbiol.* 2008; **6**: 111–120.

[9]   LaSarre B, Federle MJ. Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol. Mol. Biol. Rev.* 2013; **77**: 73–111.

[10]   Wynendaele E, Gevaert B, Stalmans S, Verbeke F, De Spiegeleer B. Exploring the chemical space of quorum sensing peptides. *Submitted for publication* **2014**.

[11]   Bruckdorfer T, Marder O, Albericio. From production of peptides in milligram amounts for research to multi-tons quantities for drugs of the future. *Curr. Pharm. Biotechnol.* 2004; **5**: 29–43.

[12]   Merrifield R. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 1963; **85**: 2149–2154.

[13]   Guzman F, Barberis S, Illanes A. Peptide synthesis: chemical or enzymatic. *J. Biotechnol.* 2007; **10**: 279–314.

[14]   D'Hondt M, Bracke N, Tavernier L, Gevaert B, Verbeke F, Wynendaele E, De Spiegeleer B. Related impurities in peptide medicines. *J. Pharm. Biomed. Anal.* **2014**; doi: 10.1016/j.jpba.2014.06.012.

[15]   Takahashi D, Yamamoto T. Development of an efficient liquid-phase peptide synthesis protocol using a novel fluorine-derived anchor support compound with Fmoc chemistry; AJIPHASE®. *Tetrahedron Lett.* 2012; **53**: 1936–1939.

[16]   Stawikowski M, Fields GB. Introduction to peptide synthesis. *Curr. Protoc. Protein Sci.* 2012; **18**: 1–17.

[17]   Dolling R, Beyermann M, Haenel J, Kernchen F, Krause E, Franke P, Brudel M, Bienert M. *3rd International Symposium on Innovation and Perspectives in SPPS* (Ed: Epton R), Mayflower Scientific Ltd, Birmingham, 1994, pp. 489.

[18]   Yang Y, Sweeney WV, Schneider K, Thörnqvist S, Chait BT, Tam JP. Aspartimide formation in base-driven 9-fluorenylmethoxycarbonyl chemistry. *Tetrahedron Lett.* 1994; **35**: 9689–9692.

[19]   Hoitink M, Beijnen J, Boschma M, Bult A, Hop E, Nijholt J, Versluis C, Wiese G, Underberg WJM. Identification of the degradation products of gonadorelin and three analogues in aqueous solution. *Anal. Chem.* 1997; **69**: 4972–4978.

[20]   Sanz-Nebot V, Benavente F, Castillo A, Barbosa J. Liquid chromatography-electrospray mass spectrometry of multicomponent peptide mixtures. Characterization of a mixture from the synthesis of the hormone goserelin. *J. Chromatogr. A* 2000; **889**: 119–133.

[21]   Riester D, Wiesmüller K, Stoll D, Kuhn R. Racemization of amino acids in solid-phase synthesis investigated by capillary electrophoresis. *Anal. Chem.* 1996; **68**: 2361–2365.

[22]   De Spiegeleer B, Vergote V, Pezeski A, Peremans K, Burvenich C. Impurity profiling quality control testing of synthetic peptides using liquid chromatography-photodiode array-fluorescence and liquid chromatography-electrospray ionization-mass spectrometry: the obestatin case. *Anal. Biochem.* 2008; **376**: 229–234.

[23]   Sanz-Nebot V, Fernando B, Toro I, Barbosa J. Separation and characterization of complex crude mixtures produced in the synthesis of therapeutic peptide hormones by liquid chromatography coupled to electrospray mass spectrometry (LC-ESI-MS). *Anal. Chim. Acta.* 2004; **521**: 25–36.

[24]   Eggen I, Bakelaar F, Petersen A, Ten Kortenaar PB, Ankone NH, Bijsterveld HE, Bours GH, El Bellaj F, Hartsuiker MJ, Kuiper GJ, Ter Voert EJ. A novel method for repetitive peptide synthesis in solution without isolation of intermediates. *J. Peptide Sci.* 2005; **11**: 633–641.

[25]   Ball H, Mascagni P. Chemical synthesis and purification of proteins: a methodology. *Int. J. Peptide Protein Res.* 1996; 48: 31–47.

[26]   Metzger JW, Kempter C, Wiemuller KH, Jung G. Electrospray mass spectrometry and tandem mass spectrometry of synthetic multicomponent peptide mixtures: determination of composition and purity. *Anal. Biochem.* 1994; **219**: 261–277.

[27]   King DS, Fields CG, Fields GB. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Pept. Protein Res.* 1990; 36: 255–66.

[28]   Albericio F, Kneib-Cordonier N, Biancalana S, Gera L, Masada RI, Hudson D, Barany G. Preparation and application of the 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) handle for the solid-phase synthesis of C-terminal peptide amides under mild conditions. *J. Org. Chem.* 1990; **155**: 3730–3743.

[29]   Solé NA, Barany G. Optimization of solid-phase synthesis of [Ala8]-dynorphin A. *J. Org. Chem.* 1992; **57**: 5399–5403.

[30]   Leon J, Reubsaet E, Beijnen H, van Bennekom WP, Bult A, Hoekstra AJ, Hop E, van Os PJ, Teeuwsen J, Underberg WJ. Reduction of Cys^{36}–Cys^{42} and Cys^{64}–Cys^{74}disulfide bonds in recombinant human granulocyte colony stimulating factor. *J. Pharm. Biomed. Anal.* 1999; **19**: 837–845.

[31]   Verbeken M, Wynendaele E, Lefebre R, Goossens E, De Spiegeleer B. The influence of peptide impurity profiles on functional tissue-organ bath response: the 11-mer peptide INSL6[151 – 161] case. *Anal. Biochem.* 2012; **421**: 547–555.

[32]   WHO, Handbook: Quality practices in basic biomedical research, http://www.who.int/tdr/publications/documents/quality_practices.pdf, 2006.

[33]   De Beukelaar J, Gratama J, Smitt P, Verjans GM, Kraan J, Luider TM, Burgers PC. The impact of impurities in synthetic peptides on the outcome of T-cell stimulation assays. *Rapid Commun. Mass Spectrom.* 2007; **21**: 1282–1288.

[34]   Currier J, Galley L, Wenschuh H, Morafo V, Ratto-Kim S, Gray CM, Maboko L, Hoelscher M, Marovich MA, Cox JH. Peptide impurities in commercial synthetic peptides and their implications for vaccine trial assessment. *Clin. Vaccine. Immunol.* 2008; **15**: 267–276.

[35]   Zhang J, Klein C, Ren P-G, Kass S, Ver Donck L, Moechars D, Hsueh AJW. Response to comment on "obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effect on food intake". *Science* 2007; **315**: 766.

[36]   Wynendaele E, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Crosstalk between the microbiome and cancer cells by quorum sensing peptides. *Submitted for publication* **2014**.

[37]   De Spiegeleer B, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, Wynendaele E. Quorum sensing peptides promote angiogenesis and invasion of breast cancer cells. *Submitted for publication* **2014**.

[38]   Wynendaele E, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke F, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides. *Nucleic Acids Res.* 2013; 41: D655–D659.

[39]   European Pharmacopoeia 7.0. European Directorate for the quality of Medicines and Healthcare, Strassbourg, France. 07/2009:2034. Substances for Pharmaceutical Use.

[40]   OMCL network of the Council of Europe, Qualification of equipment, Annex 1: Qualification of HPLC equipment. PA/PH/OMCL(11)04, 2011.

[41]   Baker PR, Clauser KR. http://prospector.ucsf.edu. (September 2014).

[42]   Roepstorff P, Fohlman J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* 1984; **11**: 601.

[43]   Lai MC, Topp EM. Solid-state chemical stability of proteins and peptides. *J. Pharm. Sci.* 1999; **88**: 489–500.

[44]   Sanz-Nebot V, Garcés A, Barbosa J. Investigation of crudes of synthesis of carbetocin by liquid chromatography coupled to electrospray ionization mass spectrometry. *J. Chromatogr. A.* 1999; 883: 267–275.

[45]   Taicrib A, Scriba G, Neusüß C. Identification and characterization of impurities of tetracosactide by capillary electrophoresis and liquid chromatography coupled to time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* 2011; **401**: 1365–1375.

CHAPTER V

CROSSTALK BETWEEN THE MICROBIOME AND COLON CANCER CELLS BY QUORUM SENSING PEPTIDES

'Look deep into nature, and then you will understand everything better."

Albert Einstein

Parts of this chapter were published:

ABSTRACT

To date, the precise role of the human microbiome in health and disease states remains largely undefined. Complex and selective crosstalk systems between the microbiome and mammalian cells are also not yet reported. Research up till now mainly focused on bacterial synthesis of virulence factors, reactive oxygen/nitrogen species (ROS/RNS) and hydrogen sulphide, as well as on the activation of exogenous mutagen precursors by intestinal bacteria. We discovered that quorum sensing peptides, produced by bacteria, interact with mammalian cells, *in casu* cancer cells: Phr0662 (*Bacillus* sp.), EntF-metabolite (*Enterococcus faecium*) and EDF-derived (*Escherichia coli*) peptides initiate colon cancer cell invasion as well as promote angiogenesis. Our findings thus indicate that the human microbiome, through their quorum sensing peptides, is one of the factors responsible for cancer metastasis.

CHAPTER **V**

CROSSTALK BETWEEN THE MICROBIOME

AND COLON CANCER CELLS

BY QUORUM SENSING PEPTIDES

Main focus in this chapter:

- To explore the biological role of quorum sensing peptides on colon cancer cells, as part of the microbiome-host interaction.

- To initially identify the target of quorum sensing peptides involved in tumour progression.

1. INTRODUCTION

Studies of the human microbiome revealed an individual and age-related diversity of microbes, occupying different habitats like skin, mouth, mammary gland, vagina and gut [1-5]. The beneficial effects of the gastrointestinal microbiota are elaborated using probiotics. These products, most often positioned as functional foods, claim to restore the gut microbiota composition, possibly preventing gut inflammation or other intestinal or systemic disease phenotypes [6]. The most commonly studied organisms for probiotic therapies in the treatment of gastrointestinal diseases include organisms of the genera *Bacillus*, *Enterococcus*, *Escherichia*, *Faecalibacterium* and *Propionibacterium*. *Enterococcus faecium*, together with *Bacillus subtilis* and *Lactobacillus* spp., were investigated as probiotics for acute gastroenteritis, while *Streptococcus* spp., *B. subtilis* and *E. coli* were explored for their use as probiotics in irritable bowel syndrome; a reduced duration or decreased abdominal pain of both gastrointestinal diseases was established. Manipulation of the microbiota with rationally selected pre- or probiotics can inhibit pathogens, strengthen epithelial barrier functions and supply the host with key nutrients (*e.g.* vitamins) [7]. It has to be noted that certain safety aspects should be taken into account when using probiotics: taxonomic identification of the probiotic strain is necessary to avoid pathogenicity, as well as inhibiting the risk of infection and antimicrobial

resistance [8]. The use of probiotics was already associated with diverse side effects, *e.g.* bacteremia, fungemia and gastrointestinal ischemia; critically ill patients and immune-compromised individuals are the most-at-risk populations [9].

The interactions of the bacteria with the host can affect metabolic, neurological, inflammatory and immunological functions as well, and also the development of cancer can directly or indirectly be promoted [10]. For example, the progress of colorectal cancer can directly be initiated by DNA-damaging superoxide radicals or genotoxins, both produced by gut (mucosa-associated) bacteria. Indirectly, bacteria can induce cell proliferation or pro-carcinogenic pathways by T-helper cells or Toll-like receptors, respectively [11]. Human microbiome studies have revealed significant differences between cancer patients and healthy controls regarding the relative abundance of certain microbes. In colon cancer patients, an increased population of *e.g. Escherichia coli* was observed in feces, inducing colitis and colibactin synthesis and thereby promoting inflammation and cancer [12].

Although the quorum sensing process within Gram-positive bacterial colonies is already extensively described in literature, the direct link between quorum sensing peptides and tumour development remains unexplored. Recent investigations revealed that the quorum sensing process is activated in the human gut: a set of acylhomoserine lactone (AHL) molecules, *i.e.* signaling molecules produced by Gram-negative bacteria, were identified in human feces of gastrointestinal disease patients as well as healthy subjects [13]. Moreover, bacterial quorum sensing molecules are likely to play a role in bacterial colonization of mucosa, thus requiring quorum sensing-mediated biofilm formation [14]. Finally, Casula and Cutting showed the germination of *Bacillus subtilis* spores in the murine gastrointestinal tract, thereby probably requiring signaling peptides for quorum sensing pathway activation [15]. Although thus not yet investigated, it is very likely that also quorum sensing peptides are found in the human intestinal tract.

In this study, we utilize a collagen invasion assay, transcriptome assay, Chick Chorioallantoic Membrane (CAM) assay, cytokine profiling and phospho-receptor tyrosine kinase array to investigate the influence of quorum sensing peptides on mammalian cancer cells. Our preliminary observations unexpectedly reveal that quorum sensing peptides stimulate metastasis behavior of human colon cancer cells, thereby opening new perspectives on the role and applications of the microbiome on the guest's health, with the possibility of translating these findings into other biological and applied medical fields as well.

2.   MATERIALS AND METHODS

Cell culture

An epithelial subclone from human ileocecal colorectal adenocarcinoma cells (ATCC$^{®}$ CCL-224), *i.e.* HCT-8/E11, was grown in high-glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% (V/V) fetal bovine serum (FBS), 1% (w/V) L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen/GIBCO, Gent, Belgium) in a humidified atmosphere of 5% CO_2.

Collagen type I invasion assay

Morphology changes of HCT-8/E11 cells were investigated using previously described methods [16]. In brief, 10 000 cells were seeded per well in a 48-well plate, containing 150 µl of collagen type I gel per well, thereby investigating cell morphology 24 hours post-treatment (Leica DMI3000B phase contrast microscope). Peptide solutions (> 80% purity; all from GL Biochem, Shanghai, China) were prepared using ultrapure water, obtaining final peptide concentrations of 1 µM, 100 nM and 10 nM after 1:10 dilution using growth medium; the placebo sample solely contained ultrapure water. Two independent morphology 'scorings' were obtained for each of the 3 replicates; peptides were found positive if collagen-invasion or cell-stretching at minimum 2 consecutive concentrations was established. To quantify these visual results, the number of cells containing invasive extensions were counted and compared to the total number of cells in the field.

Human Transcriptome Array

Peptide treated cells were analysed in duplicate (independent treatment and analysis) for whole transcriptome expression using Affymetrix GeneChip Human Transcriptome Array 2.0. by AROS Applied Biotechnology A/S (Aarhus, Denmark); RNA expression was compared with placebo (ultrapure water) treated samples. Data analysis was performed using Transcriptome Analysis Console (Affymetrix) and MetaCore (Thompson Reuters) software programs.

Chick Chorioallantoic Membrane (CAM) assay

The Chick Chorioallantoic Membrane (CAM) assay was performed as described by Sys *et al.*: 6 days after (pre-treated, 100 nM) tumour cell transfer to the fertilized eggs, CAM was microscopically scored and histologically examined after H&E staining [17,18]. For quantification of microscopically observed neovascularisation, an average CAM score was calculated: the number of blood vessels in

the 1 mm diameter ring around the 2 mm radius tumour centre was determined. Significant differences were evaluated using the Mann–Whitney U test.

Cytokine profiling

Cytokine expression was investigated using the Human XL Cytokine Array kit (R&D Systems, Abingdon, United Kingdom), following the instructions of the supplier. In brief, cell supernatant was obtained after 24 hours of incubation with Phr0662 (100 nM) and 102 different cytokines analysed in duplo using spotted capture antibodies, followed by incubation with detection antibodies and chemiluminescent visualization. Membranes are finally exposed to X-ray films for 1-10 minutes. The mean (blank corrected, n = 2) pixel density is calculated using ImageJ software and compared with placebo treatment. Significant differences were evaluated using the independent samples t-test.

Phospho-receptor tyrosine-kinase array

A human phospho-receptor tyrosine kinase (RTK) array kit (R&D Systems, Abingdon, United Kingdom) was used to detect phosphorylation levels of 49 different RTK after Phr0662 (100 nM) treatment of HCT-8/E11 cells, following the manufacturer's instructions. Cell lysates were prepared after 5 minutes of peptide incubation and incubated with capture antibodies (in duplo), followed by incubation with detection antibodies and Horseradish Peroxidase for chemiluminsescent visualization. X-ray films were again analysed for mean (blank corrected, n = 2) pixel density and compared with placebo results using the ImageJ software program. Significant differences were evaluated using the independent samples t-test.

3. RESULTS

Epithelial to mesenchymal (EMT)-like transition and related cell migration activities

To investigate the potential role of chemically diverse quorum sensing peptides in oncology [19], we explored their morphologic effect on a human epithelial colon cancer cell line, *i.e.* HCT-8/E11. Three quorum sensing peptides, or metabolites thereof, were found to significantly induce tumour cell invasion through a collagen type I extracellular matrix: Phr0662 (ERNNT), an EntF-metabolite (SNLVECVFSLFKKCN) and an EDF-analogue (NWN) (Figure 1), while others, *e.g.* cAM373 (AIFILAS) and iAD1 (LFVVTLVG), both originating from *Enterococcus faecalis*, do not induce invasive characteristics. This epithelial to mesenchymal (EMT)-like process (*i.e.* observation of invasive cellular extensions) is

one of the main mechanisms involved in colorectal cancer metastasis, establishing metastatic disseminations with potential life-threatening consequences [20].

Peptide Phr0662 is synthesized by *Bacillus* species [21]. The quorum sensing peptide designated Extracellular Death Factor (EDF), originally described by Kolodkin-Gal *et al.*, is responsible for the *mazEF* and *chpBIK*-mediated cell death in *Escherichia coli*. This intestinal commensal bacterium synthesizes this EDF peptide during logarithmic growth stages when stress-situations are induced [22,23]. Our study now enlarges the role of EDF to mammalian cells. EntF is synthesized by *Enterococcus faecium*, a commensal bacterium in healthy humans and animals, which is responsible for a number of nosocomial infections [24]. *Enterococcus faecium*, together with *Enterococcus faecalis*, is the primary source of reactive oxygen species (ROS), causing genomic alterations which are correlated to colorectal cancer [25]. However, based on the results of this study, its quorum sensing peptide (or its metabolite) can contribute to metastatic tumour behavior as well.
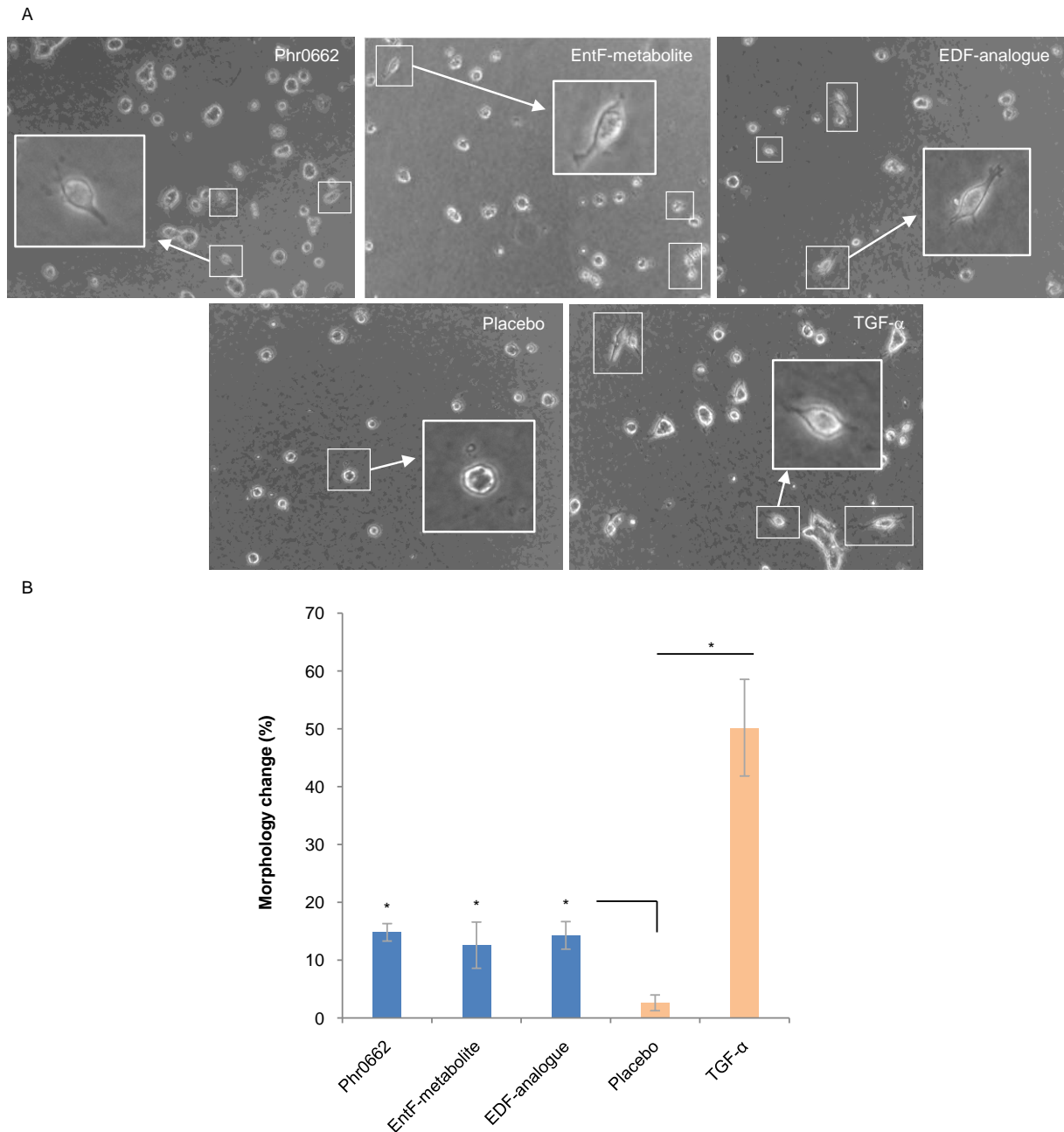
Figure 1. Collagen invasion of 3 quorum sensing peptides at 10 nM. (A) Morphologic changes of HCT-8/E11 cells, observed 24 hours post-treatment with Phr0662 (ERNNT), EntF-metabolite (SNLVECVFSLFKKCN) and EDF-analogue (NWN). Placebo sample serves as negative control and TGF-α (0.1 µg/ml) as the very strong positive control. (B) Mean (n = 3) percentage of cancer cells with induced morphology changes: a significant difference is observed between the peptide and placebo treatments (* $p < 0.05$ (Mann-Whitney U test)). Error bars represent SEM values.

The microscopically observed invasive characteristics were further corroborated by whole transcriptome analyses on both placebo and peptide-treated tumour cells (Table 1). RNA extraction was performed on a heterogenous mixture (*i.e.* cells with and without induced invasive cellular extensions) instead of on isolated HCT-8/E11 cells, and only two duplicates were performed in this

exploratory study, so the standard (parametric) ANOVA p-values are only given for the sake of completeness but are not meant to conclude any statistical significance (requiring more data and non-parametric evaluation). This exploratory study was meant to observe trends based on the mean fold-change. The up- or downregulation of certain genes indeed supported the phenotypic observations of cellular invasiveness: *e.g.* the upregulation of histone cluster 1 H4 (*HIST1H4A-F/H-L*) and *CXorf61* (Cancer/Testis Antigen 83 (*CT83*) or Kita-Kyushu Lung Cancer Antigen (*KKLC1*)) is associated with cell cytoskeleton remodelling. Histone H4 is involved in different biological pathways through its link with β-arrestin 1, activin A and Notch1. Via β-arrestin 1, Histone H4 is linked to cell migration and metastasis in colon cancer *in vivo* [26]. The Histone H4-activin A link is again associated with cell migration and cytoskeleton remodeling (via Smad3) and cell proliferation (via Smad4) in colon cancer [27,28]; in normal human epithelial cells, activin A can induce EMT as well. Finally, via the link with Notch1, in colon cancer, it causes metastasis and cell invasion; a deregulation of the Notch signaling pathway thus affects EMT and tumour aggressiveness [29,30]. Through its link with micro-RNA 520h (*miR-520h*), *CXorf61* upregulation is associated with increased tumour cell mobility and enhanced *in vitro* cell invasion activity [31]. Moreover, downregulation of micro-RNA 558 (*miR-558*) is associated with a bad prognosis of colon cancer outcome, indicating tumour metastases and thus EMT-like behaviour [32]. Micro-RNA 644a (*miR-644a*) functions by directly binding to its target site in the 3' untranslated region of glyceraldehyde-3-phosphate dehydrogenase and β-actin, thereby significantly repressing their expression [33]. Downregulation of *miR-664a* thus upregulates β-actin levels, leading to increased cell motility and cell migration [30].

Table 1. Transcriptome alterations after quorum sensing peptide addition to HCT-8/E11 cells. Gene expression 24 hours post-treatment, compared to placebo samples. Mean fold change is calculated from duplicate samples (cut off: > 1.5 or < -1.5).

Phr0662				
Gene symbol		Fold change	ANOVA p-value	Description
UP-REGULATED	SCARNA10	1.99	0.024	Small Cajal body-specific RNA 10
	RN5S348	1.69	0.272	RNA, 5S ribosomal 348
	MIR4521	1.60	0.335	MicroRNA 4521
	HIST1H4A-F/H-L	2.02	0.647	Histone cluster 1, H4d,…
	CXorf61	1.54	0.054	Chromosome X open reading frame 61
	SNORA26	1.63	0.095	Small nucleolar RNA, H/ACA box 26
DOWN-REGULATED	RPL36AP33	-1.75	0.576	Ribosomal protein L36a pseudogene 33
	RNU6-51	-1.99	0.565	RNA, U6 small nuclear 51
	MIR222	-1.60	0.593	MicroRNA 222
	SNORD121B	-1.88	0.242	Small nucleolar RNA, C/D box 121B
	SNORD85	-1.84	0.323	Small nucleolar RNA, C/D box 85
	ARL17A	-1.74	0.543	ADP-ribosylation factor-like 17A
	RNU7-47P	-1.72	0.463	RNA, U7 small nuclear 47 pseudogene

EntF-metabolite				
Gene symbol		Fold change	ANOVA p-value	Description
UP-REGULATED	RN5S62	1.79	0.534	RNA, 5S ribosomal 62
	OR52E6	1.58	0.479	Olfactory receptor, family 52, subfamily E, member 6
DOWN-REGULATED	RNU7-47P	-1.83	0.340	RNA, U7 small nuclear 47 pseudogene
	MIR597	-1.65	0.599	MicroRNA 597
	MIR548T	-1.58	0.597	MicroRNA 548t
	RNY4	-1.56	0.007	RNA, Ro-associated Y4
	MIR558	-1.68	0.090	MicroRNA 558
	LOC286437	-1.64	0.390	Uncharacterized LOC286437
	RNY4P2	-1.61	0.231	RNA, Ro-associated Y4 pseudogene 2
	MIR644A	-1.98	0.443	MicroRNA 644a
	MIR3975	-1.63	0.088	MicroRNA 3975
	MIR553	-1.95	0.328	MicroRNA 553

EDF-analogue				
Gene symbol		Fold change	ANOVA p-value	Description
UP-REGULATED	OR52E6	1.70	0.384	Olfactory receptor, family 52, subfamily E, member 6
DOWN-REGULATED	MIR222	-1.51	0.641	MicroRNA 222
	RNU7-19P	-1.68	0.008	RNA, U7 small nuclear 19 pseudogene

Angiogenesis confirms metastatic potential of quorum sensing peptides

Downregulation of microRNA 222 (*miR-222*), as observed 24 hours after Phr0662 and EDF-analogue peptide addition, is associated with tumour growth reduction (through PUMA) and apoptosis induction [34], next to a bad cancer prognosis [32]. MicroRNA 222 is also highly expressed in endothelial cells, possessing anti-angiogenic properties through its targets c-kit (mast/stem cell growth factor receptor kit, CD117), p27Kip1, p57Kip2 (cyclin-dependent kinase inhibitor 1C) and cyclin G1 [35]. The downregulation after quorum sensing peptide addition thus initiates angiogenesis. This angiogenic effect was analysed for Phr0662, seen the highly changed transcriptomic profile of the cancer cells after 24 hours of treatment. Our Chick Chorioallantoic Membrane (CAM) assay results confirmed these pro-angiogenic properties (Figures 2a and 2b): its addition clearly promoted neovascularisation, thereby facilitating metastasis of the tumour. Without tumour cells, the addition of the peptide alone did not promote neovascularisation, confirming the crosstalk between the peptide and the cancer cells (*i.e.* indirect angiogenic effect) (Figure 2c). Histological evaluation of the membrane confirmed the invasive tumour cell properties through the chorion into the mesoderm, induced by the quorum sensing peptide (Figure 2d).
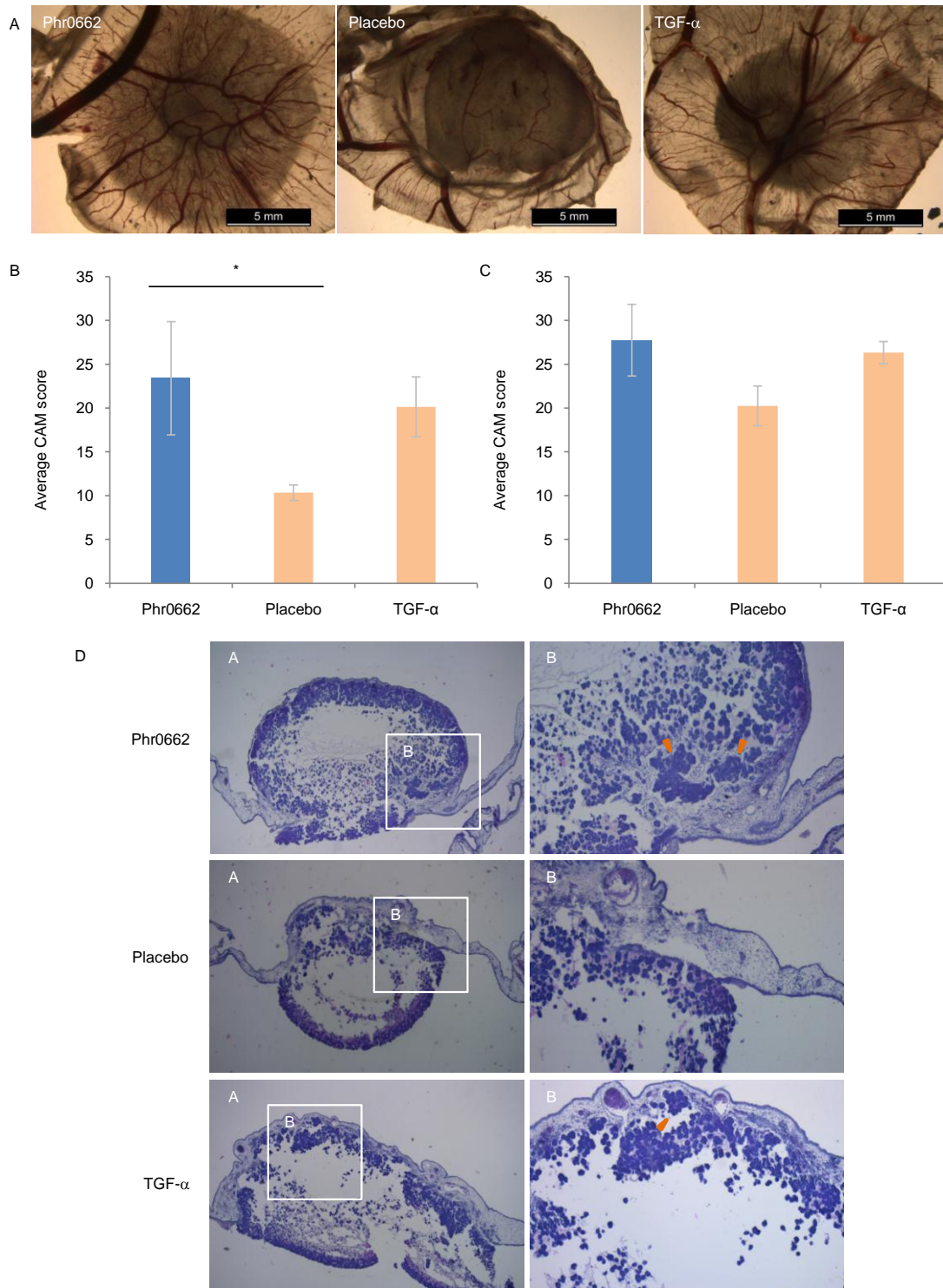
Figure 2. Neovascularisation and cell invasion after Phr0662 (100 nM) addition to HCT-8/E11 cells on CAM. Placebo sample serves as negative control and TGF-α (0.1 μg/ml) as positive control. (A) Macroscopic images, observed 6 days after (pre-treated) tumour cell transfer to the eggs. (B) Average CAM Score (*i.e.* number of blood vessels in the 1 mm diameter ring around the 2 mm radius centre) in the presence of tumour cells (mean ± SEM, n = 5 (Phr0662), n = 7 (TGF-α) or n = 3 (placebo)). (C) Average CAM Score when no tumour cells are present (mean ± SEM, n = 8 (Phr0662 and placebo) or n = 6 (TGF-α)). (D) Histological H&E evaluation of the CAM, with orange arrowheads indicating invasion of the tumour cells into the mesoderm. * p < 0.05 (Mann-Whitney U test)

Cytokine screening after Phr0662 treatment of the cancer cells revealed pro-angiogenic effects as well (Figure 3): the quantitative increase of the vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), interleukin 6 (IL-6) and stromal cell-derived factor 1 (SDF-1α) all indicated an induction of angiogenesis by the investigated peptide [36]. IL-6 was also found to drive tumour initiation and subsequent growth and metastasis, with a poor clinical outcome observed in colorectal cancer patients. Through its link with STAT3, it promotes the expression of VEGF and Fibroblast Growth Factor (FGF), supporting the rapid vascularization required for tumour growth and metastasis [37,38].
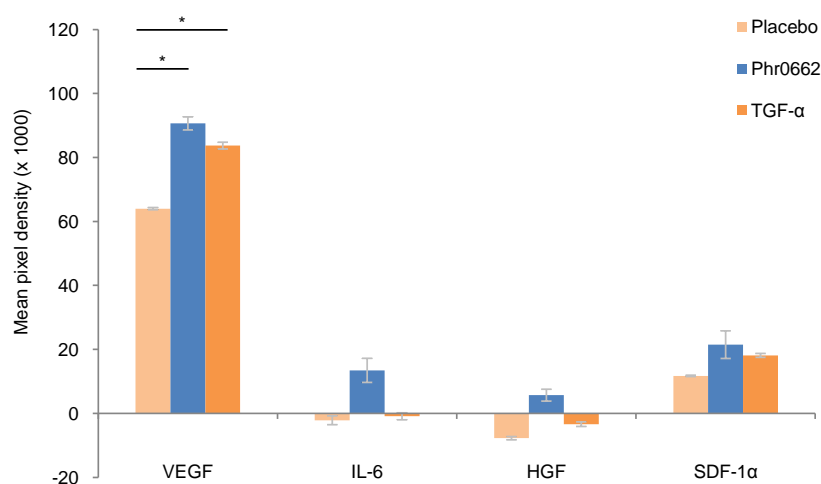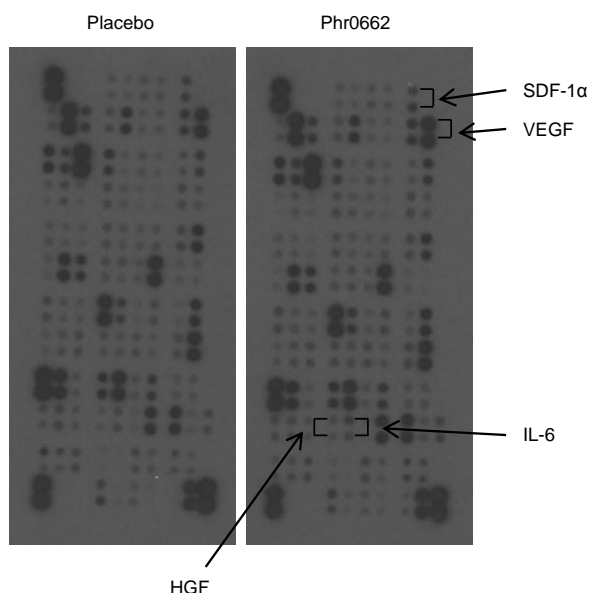

Figure 3. Effect of quorum sensing peptide Phr0662 (100 nM) on cytokine expression in HCT-8/E11 colon cancer cells. Placebo sample serves as the negative control and TGF-α (0.1 µg/ml) as the positive control. (A) Mean (n = 2, ± SEM) pixel density of some pro-angiogenic cytokines, demonstrating increasing cytokine concentrations after Phr0662 addition (24 hours of peptide incubation); * p < 0.05 (independent samples t-test). (B) Human XL Cytokine Array visually demonstrating increased cytokine levels after peptide treatment of HCT-8/E11 cells, compared to placebo samples. The selected pro-angiogenic cytokines (in duplo) are indicated.

Phr0662 targets the epidermal growth factor receptors EGFR and ErbB2

Receptor Tyrosine Kinase (RTK) screening of Phr0662-treated HCT-8/E11 cells revealed a 2 times higher ErbB2 (HER2/neu) and a significant increase in epidermal growth factor receptor (EGFR, HER1 or ErbB1) phosphorylation compared to the placebo cell samples (Figure 4). A comparable activity is observed for the Phr0662 quorum sensing peptide and the autocrine ligand TGF-α, 5 minutes after HCT-8/E11 treatment.

The EGFR is overexpressed in many types of cancers, especially colorectal cancer, and is significantly associated with tumour-node-metastasis and a more aggressive clinical tumour progression [39]. This regulation of cellular functions is achieved by activating different intracellular signaling cascades: phosphatidylinositol 3-kinase (PI3K/Akt), the ras/raf/MEK/mitogen-activated protein kinase (MAPK) and the signal transducer and activator of transcription (STAT) pathways. EGFR signaling regulates the synthesis and secretion of different angiogenic growth factors as well, including VEGF; overexpression of ErbB2 also leads to increased angiogenesis [40].
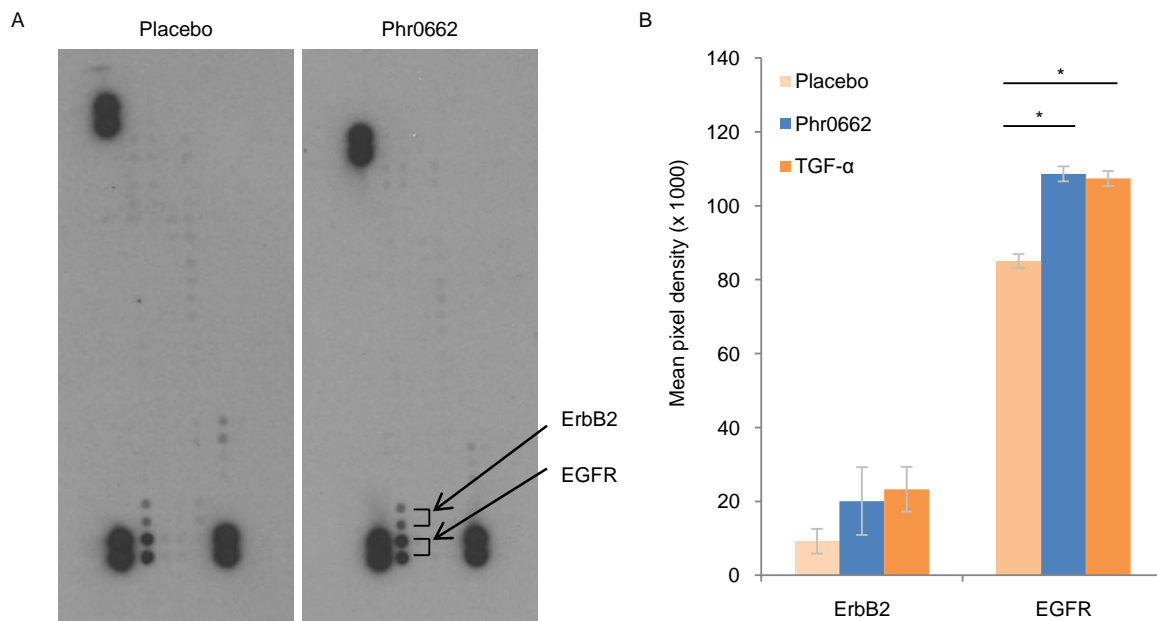

Figure 4. Effect of quorum sensing peptide Phr0662 (100 nM) on receptor tyrosine kinase activation in colon cancer cells. Placebo sample serves as the negative control and TGF-α (0.1 µg/ml) as the positive control. (A) Receptor tyrosine kinase (RTK) array demonstrating the tyrosine phosphorylation level of 42 RTK after Phr0662 treatment of HCT-8/E11. Each RTK is spotted in duplicate and the phosphorylated EGFR and ErbB2 are indicated. (B) Mean (n = 2, ± SEM) pixel density of ErbB2 and EGFR spots after Phr0662 addition (5 minutes of peptide incubation); * p < 0.05 (independent samples t-test). TGF-α serves as the cognate ligand for EGFR, demonstrating thus EGFR binding properties for the quorum sensing peptide.

4. DISCUSSION

Our results suggest that quorum sensing peptides exert (part of) their effects through the epidermal growth factor receptor (EGFR), thereby activating the Ras/raf/MEK/MAPK, PI3K/Akt and STAT intracellular signaling cascades [40], leading to an altered gene transcription and finally tumour metastasis (Figure 5). β-arrestin 1, which is linked to HIST1H4 and thus possibly upregulated after peptide treatment, can activate the EGFR pathway as well, thereby playing a pivotal role in colon cancer metastasis [26]. Next, Smad proteins (*i.e.* Smad 2, 3 and 4) can be stimulated through Activin A binding with its ActRII receptor, linking both cell cytoskeleton remodeling and cell migration to quorum sensing peptide presence [27,41]. The upregulation of *HIST1H4* can be associated with increased Notch1 stimulation, again connected to cancer metastasis, together with angiogenesis and NF-κB production [29,42]; NF-κB in turn contributes to the progression of colorectal cancer by regulating cell proliferation, angiogenesis and tumour metastasis [43]. Micro-RNA's block mRNA translation and thus impede the synthesis of specific proteins by recruiting the micro-RNA-induced silencing complex (miRISC) to target mRNAs [44]; downregulation of micro-RNA 222 and 644a thus increases KIT and β-actin protein expression, respectively, thereby promoting endothelial cell proliferation and migration (angiogenesis) [35,45] and tumour cell invasiveness and motility [33]. β-actin can be upregulated through the IL-6 receptor pathway as well: the increased IL-6 expression after quorum sensing peptide treatment, as observed with our cytokine array, activates β-actin phosphorylation, again promoting tumour cell migration [46]. Both the transcriptome outcomes and cytokine results thus correspond well and are visually confirmed by the collagen invasion and CAM assays. A good correlation is observed for VEGF as well: an increased IL-6 pathway activation leads to VEGF upregulation [47], which is again observed in our cytokine array; VEGF can then trigger the VEGF receptor (VEGFR) pathway in endothelial cells, leading to altered gene transcription and subsequently angiogenesis. The pro-angiogenic cytokines SDF-1α and HGF both activate the Ras/raf/MEK/MAPK, PI3K/Akt and STAT intracellular signaling cascades through CXCR4 and MET receptor binding, respectively. These cytokine-receptor axes are involved in tumour progression, angiogenesis and metastasis [48,49].
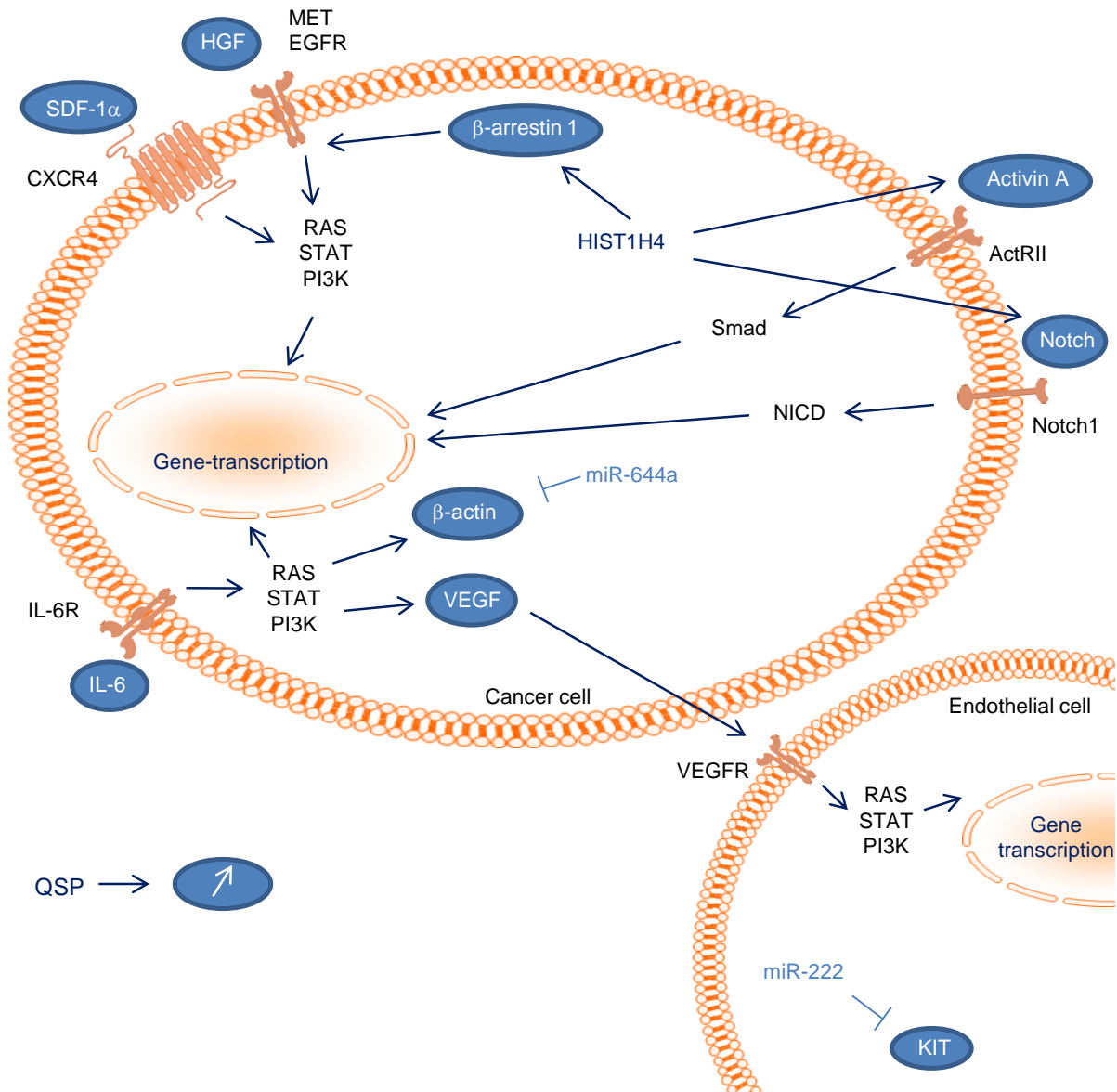
Figure 5. Possible pathway-map for quorum sensing peptides in tumour progression. Quorum sensing peptides promote cancer metastasis and tumour progression by activating different receptor pathways.

These exploratory results thus indicate metastasis-promoting characteristics for some quorum sensing peptides. Assuming the *in vivo* presence of these molecules influenced by the individual microbiota composition, our findings may potentially have a great impact on the patient's health, with effects on cancer metastasis. Exploiting our findings, it would be interesting to consider how we can prevent these pro-metastatic effects? First, it may be important to adjust the patient's life style, including diet and hygiene measures. The influence of food patterns cannot be neglected, *e.g.* switching from a low-fat, plant (non- or limited digestible) polysaccharide-rich diet to a high-fat, high-sugar diet can shift the structure of the gut microbiota within a single day [50,51]. Appropriate hygiene measures will diminish the risk of unwanted infections and thus an alteration in the

microbiota and quorum sensing peptide composition. Second, blocking the tumour receptor targets with designed peptide antagonist, directly or through probiotics, can help in the prevention of cancer metastasis as well.

5. CONCLUSIONS

Our results clearly indicate a new way of crosstalk between the microbiome and mammalian cancer cells, *i.e.* through certain quorum sensing peptides excreted by mainly Gram-positive bacteria. By activating one of the suggested pathways, the quorum sensing peptides can promote cancer metastasis and thus possibly alter cancer outcome. These *in vitro* findings are not only important from a fundamental biological and evolutionary point of view, but may also have health consequences, clearly awaiting further *in vivo* studies.

6. REFERENCES

[1]   Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan TJ, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota. *Science* 2012; **338**: 120-123.

[2]   Schwabe RF, Wang TC. Bacteria Deliver a Genotoxic Hit. *Science* 2012; **338**: 52-53.

[3]   Garagnani P, Pirazzini C, Franceschi C. Colorectal Cancer Microenvironment: Among Nutrition, Gut Microbiota, Inflammation and Epigenetics. *Curr. Pharm. Des.* 2013; **19**: 765-778.

[4]   The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* 2012; **486**: 207-214.

[5]   Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW. Gut microbiota composition correlates with diet and health in the elderly. *Nature* 2012; **488**: 178-184.

[6]   Hemarajata P, Versalovic J. Effects of probiotics on gut Microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therap. Adv. Gastroenterol.* 2013; **6**: 39-51.

[7]   Preidis GA, Versalovic J. Targeting the Human Microbiome With Antibiotics, Probiotics, and Prebiotics: Gastroenterology Enters the Metagenomics Era. *Gastroenterology* 2009; **136**: 2015-2031.

[8]   Ishibashi N, Yamazaki S. Probiotics and safety. *Am. J. Clin. Nutr.* 2001; **73**: 465s-470s.

[9]   Didari T, Solki S, Mozaffari S, Nikfar S, Abdollahi M. A systematic review of the safety of probiotics. *Expert Opin. Drug Saf.* 2014; **13**: 227-239.

[10]   Plottel CS, Blaser MJ. Microbiome and Malignancy. *Cell Host Microbe* 2011; **10**: 324-335.

[11]   Marchesi JR, Dutilh BE, Hall N, Peters WHM, Roelofs R, Boleij A, Tjalsma H. Towards the Human Colorectal Cancer Microbiome. *PLOS ONE* 2011; **6**: e20447.

[12]   Bultman SJ. Emerging roles of the microbiome in cancer. *Carcinogenesis* 2014; **35**: 249-255.

[13]   Kumari A, Pasini P, Deo SK, Flomenhoft D, Shashidhar H, Daunert S. Biosensing Systems for the Detection of Bacterial Quorum Signaling Molecules. *Anal. Chem.* 2006; **78**: 7603-7609.

[14]   Macfarlane S, Woodmansey EJ, Macfarlane GT. Colonization of Mucin by Human Intestinal Bacteria and Establishment of Biofilm Communities in a Two-Stage Continuous Culture System. *Appl. Environ. Microbiol.* 2005; **71**: 7483-7492.

[15]   Casula G, Cutting SM. Bacillus probiotics: Spore germination in the gastrointestinal tract. *Appl. Environ. Microbiol.* 2002; **68**: 2344-2352.

[16]   De Wever O, Hendrix A, De Boeck A, Westbroek W, Braems G, Emami S, Sabbah M, Gespach C, Bracke M. Modeling and quantification of cancer cell invasion through collagen type I matrices. *Int. J. Dev. Biol.* 2010; **54**: 887-896.

[17]   Sys G, Van Bockstal M, Forsyth R, Balke M, Poffyn B, Uyttendaele D, Bracke M, De Wever O. Tumor grafts derived from sarcoma patients retain tumor morphology, viability, and invasion potential and indicate disease outcomes in the chick chorioallantoic membrane model. *Cancer Lett.* 2012; **326**: 69-78.

[18]   Ribatti D, Nico B, Vacca A, Presta M. The gelatin-sponge-chorioallantoic membrane assay. *Nat. Protoc.* 2006; **1**: 85-91.

[19]   Wynendaele E, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke F, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides. *Nucleic Acids Res.* 2013; **41**: D655-D659.

[20] Loboda A, Nebozhyn MV, Watters JW, Buser CA, Shaw PM, Huang PS, Van't Veer L, Tollenaar RAEM, Jackson DB, Agrawal D, Dai H, Yeatman TJ. EMT is the dominant program in human colon cancer. *BMC Med. Genomics* **2011**; doi: 10.1186/1755-8794-4-9.

[21] Perego M, Brannigan JA. Pentapeptide regulation of aspartyl-phosphate phosphatases. *Peptides* 2001; **22**: 1541-1547.

[22] Kolodkin-Gal I, Hazan R, Gaathon A, Carmeli S, Engelberg-Kulka HA. Linear Pentapeptide Is a Quorum-Sensing Factor Required for mazEF-Mediated Cell Death in Escherichia coli. *Science* 2007; **318**: 652-655.

[23] Belitsky M, Avshalom H, Erental A, Yelin I, Kumar S, London N, Sperber M, Schueler-Furman O, Engelberg-Kulka H. The *Escherichia coli* Extracellular Death Factor EDF Induces the Endoribonucleolytic Activities of the Toxins MazF and ChpBK. *Mol. Cell* 2011; **41**: 625-635.

[24] Willems RJL, van Schaik W. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. *Future Microbiol.* 2009; **4**: 1125-1135.

[25] Huycke MM, Joyce W, Wack MF. Augmented Production of Extracellular Superoxide by Blood Isolates of *Enterococcus faecalis. J. Infect. Dis.* 1996; **173**: 743-746.

[26] Buchanan FG, Gorden DL, Matta P, Shi Q, Matrisian LM, DuBois RN. Role of β-arrestin 1 in the metastatic progression of colorectal cancer. *Proc. Natl. Acad. Sci. U.S.A.* 2006; **103**: 1492-1497.

[27] Roberts AB, Tian F, DaCosta Byfield S, Stuelten C, Ooshima A, Saika S, Flanders KC. Smad3 is key to TGF-β-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine Growth Factor Rev.* 2006; **17**: 19-27.

[28] Wildi S, Kleeff J, Maruyama H, Maurer CA, Büchler MW, Korc M. Overexpression of activin A in stage IV colorectal cancer. *Gut* 2001; **49**: 409-417.

[29] Wang Z, Li Y, Kong D, Sarkar FH. The role of Notch Signaling Pathway in Epithelial-Mesenchymal Transition (EMT) During Development and Tumor Aggressiveness. *Curr. Drug Targets* 2010; **11**: 745-751.

[30] Bunnell TM, Burbach BJ, Shimizu Y, Ervasti JM. β-actin specifically controls cell growth, migration, and the G-actin pool. *Mol. Biol. Cell* 2011; **22**: 4047-4058.

[31] Su JL, Chen PB, Chen YH, Chen SC, Chang YW, Jan YH, Cheng X, Hsiao M, Hung MC. Downregulation of microRNA miR-520h by E1A Contributes to Anti-cancer Activity. *Cancer Res.* 2010; **70**: 5096-5108.

[32] Galon J, Mlecnik B, Pages F, Fridman H. Method for predicting the outcome of colon cancer by analyzing mirna expression. 2013, US Patent application number: 20130331291.

[33] Sikand K, Singh J, Ebron JS, Shukla GC. Housekeeping Gene Selection Advisory: Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and β-Actin Are Targets of miR-644a. *PLOS ONE* 2012; **7**: doi: 10.1371/journal.pone.0047510.

[34] Yu L, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA Induces the Rapid Apoptosis of Colorectal Cancer Cells. *Mol. Cell* 2001; **7**: 673-682.

[35] Le X, Merchant O, Bast Jr RC, Calin GA. The Roles of MicroRNAs in the Cancer Invasion-Metastasis Cascade. *Cancer Microenviron.* 2010; **3**: 137-147.

[36] Neufeld G, Kessler O. Pro-angiogenic cytokines and their role in tumor angiogenesis. *Cancer Metastasis Rev.* 2006; **25**: 373-385.

[37] Fisher DT, Appenheimer MM, Evans SS. The two faces of IL-6 in the tumor microenvironment. *Semin. Immunol.* 2014; **26**: 38-47.

[38] Seruga B, Zhang H, Bernstein LJ, Tannock IF. Cytokines and their relationship to the symptoms and outcome of cancer. *Nat. Rev. Cancer* 2008; **8**: 887-899.

[39] Spano J-P, Lagorce C, Atlan D, Milano G, Domont J, Benamouzig R, Attar A, Benichou J, Martin A, Morere J-F, Raphael M, Penault-Llorca F, Breau J-L, Fagard R, Khayat D, Wind P. Impact of EGFR expression on colorectal cancer patient prognosis and survival. *Ann. Oncol.* 2005; **16**: 102-108.

[40] De Luca A, Carotenuto A, Rachiglio A, Gallo M, Maiello MR, Aldinucci D, Pinto A, Normanno N. The role of the EGFR signaling in tumor microenvironment. *J. Cell Physiol.* 2007; **214**: 559-567.

[41] Meulmeester E, ten Dijke P. The dynamic roles of TGF-β in cancer. *J. Pathol.* 2011; **223**: 205-218.

[42] Hu Y, Zheng M, Zhang R, Liang Y, Han H. Notch signaling pathway and cancer metastasis, In *Notch signaling in embryology and cancer* (Eds: Reichrath J, Reichrath S), Landes Bioscience, Austin, **2011**, pp. 186-198.

[43] Wang S, Liu Z, Wang L, Zhang X. NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell Mol. Immunol.* 2009; **6**: 327-334.

[44] Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat. Struct. Mol. Biol.* 2012; **19**: 586-593.

[45] Nicoloso MS, Spizzo R, Shimizu M, Rossi S, Calin GA. MicroRNAs – the micro steering wheel of tumour metastases. *Nat. Rev. Cancer* 2009; **9**: 293-302.

[46] Ho YP, Kuo CW, Hsu YT, Huang YS, Yew LP, Huang WF, Lin KC, Hsu JH. β-Actin is a downstream effector of the PI3K/AKT signaling pathway in myeloma cells. *Mol. Cell Biochem.* 2011; **348**: 129-139.

[47] Wei L, Kuo ML, Chen CA, Chou CH, Lai KB, Lee CN, Hsieh CY. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene* 2003; **22**: 1517-1527.

[48] Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin. Cancer Res.* 2010; **16**: 2927-2931.

[49] Cecchi F, Rabe DC, Bottaro DP. Targeting the HGF/Met signaling pathway in cancer therapy. *Expert Opin. Ther. Targets* 2012; **16**: 553-572.

[50] Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Sci. Transl. Med.* 2009; **1**: doi: 10.1126/scitranslmed.3000322.

[51] Zarrinpar A, Chaix A, Yooseph S, Panda S. Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. *Cell metab.* 2014; **20**: 1006-1017.

CHAPTER VI

QUORUM SENSING PEPTIDES PROMOTE ANGIOGENESIS AND INVASION OF BREAST CANCER CELLS

*"Two roads diverged in a wood and I, I took the one less travelled by,
and that has made all the difference."*

*Robert Frost
(°1874 - †1963, American poet)*

Parts of this chapter were published:

ABSTRACT

The role of the human microbiome on cancer progression remains unclear. Therefore, in this study, we investigated the influence of some quorum sensing peptides, produced by the guest's microbiome, on breast cancer cell invasion and thus cancer outcome. Based on microscopy, transcriptome and Chick Chorioallantoic Membrane (CAM) analyses, four peptides (*i.e.* PhrG from *B. subtilis*, CSP from *S. mitis* and EDF from *E. coli*, together with its tripeptide analogue) were found to promote tumour cell invasion and angiogenesis, thereby potentially influencing tumour metastasis. Our results offer not only new insights on the possible role of the microbiome, but also further opportunities in cancer prevention and therapy by competing with these endogenous molecules and/or by modifying people's life style.

CHAPTER **VI**

QUORUM SENSING PEPTIDES PROMOTE ANGIOGENESIS AND INVASION OF BREAST CANCER CELLS

Main focus in this chapter:

- To investigate the possible interactions between quorum sensing peptides and breast cancer cells.
- To study the pro-angiogenic and invasion effects of selected quorum sensing peptides.

1. INTRODUCTION

To date, cancer figures among the leading causes of death in human societies worldwide. Next to a person's genetic factors and carcinogens exposure, the human microbiome recently gathered great attention in tumour development as well [1]. *Helicobacter pylori*, a commensal of the human stomach in almost half of the world's population, was found to cause gastric adenocarcinoma through the CagA and VacA toxins [2]. Colon cancer on the other hand can be influenced by several gut bacteria, producing reactive oxygen/nitrogen species or bacterial toxins (*e.g.* Colibactin from *Escherichia coli*) [3, 4].

The vast majority of bacteria inhabit the human intestine, with as many as 10^{12} cells per gram of the average human faeces. Besides, other habitats like skin, mouth, vagina and mammary gland are occupied as well, next to the occasional presence in the blood (bacteremia or septicemia). The breast milk in the mammary gland is a continuous source of commensal, mutualistic and potentially probiotic bacteria to the infant gut, containing *i.a.* lactic acid bacteria, *Staphylococcus* and *Streptococcus* species [5]. In contrast to the breast milk, the human breast tissue was originally thought to be sterile. However, given the nutrient rich fatty composition of the female breast, together with the blood and lymphatic vasculature, and the diffuse location of the lobules and ducts, bacteria can spread within the mammary glands, resulting in a breast tissue microbiome. Urbaniak *et*

al. indeed found a higher abundance of *Proteobacteria*, *Firmicutes* (specifically the class Bacilli) and *Actinobacteria* in the breast tissue, while no signs or symptoms of infection were assigned [6]. These results, serving as a first indication, are eagerly waiting to be confirmed and further investigated towards its pathophysiological role.

Bacteria communicate with each other by the use of signalling molecules in a cell-density dependent manner, a process called 'quorum sensing'. The signalling molecules differ between the groups of bacteria: while Gram-negative bacteria predominantly use *N*-acyl homoserine lacton (AHL) molecules, the Gram-positive bacteria communicate by the use of oligopeptides; both Gram-positive and Gram-negative bacteria synthesize autoinducer-2 (AI-2) signalling molecules. The quorum sensing process was found to be activated *in vivo* as both the quorum sensing end products (*e.g.* biofilms or toxins) and signalling molecules were detected in different areas of the human body. Bacterial biofilms that can be produced after quorum sensing pathway activation can be detected at diverse physiological sites: *e.g.* dental plaques produced by *Streptococcus mitis* or *Lactobacillus* species, as well as nosocomial infections with vascular grafts or urinary catheters after biofilm formation by Staphylococci or *Escherichia coli*, were observed [7]. Moreover, *N*-acyl homoserine lacton signalling molecules were detected in human biological samples as well, including sputum, faeces and saliva [8,9]. Although thus not yet investigated, it is very likely that also quorum sensing peptides are found in the human body, at their site of origin or distributed throughout the human body via the blood circulation.

The very recent identification of different Gram-positive bacteria in the breast tissue [6] together with the increasing incidence of breast cancer in women in both developed and developing countries [10], excited the research in the microbiome's influence on breast cancer cells. To this end, we investigated the effect of several quorum sensing peptides, synthesized by some commensal bacteria, on a human epithelial breast cancer cell line, *i.e.* MCF-7/AZ.

2. MATERIALS AND METHODS

Cell culture

The epithelial breast adenocarcinoma cell line MCF-7/AZ (ATCC® CCL-224) was grown in high-glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% (V/V) foetal bovine serum (FBS), 1% (w/V) L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen/GIBCO, Gent, Belgium) in a humidified atmosphere of 10% CO_2 at 37°C.

Collagen type I invasion assay

Morphology changes of MCF-7/AZ cells were investigated using previously described methods [11]. In brief, 10 000 cells were seeded per well in a 48-well plate, containing 150 µl of collagen type I gel per well, thereby investigating cell morphology 24 hours post-treatment (Leica DMI3000B phase contrast microscope). Peptide solutions (> 80% purity; all from GL Biochem, Shanghai, China) were prepared using ultrapure water, obtaining final peptide concentrations of 1 µM, 100 nM and 10 nM after 1:10 dilution using growth medium. The placebo sample solely contained ultrapure water. Two independent morphology 'scorings' were obtained for each of the 3 replicates; peptides were found positive if collagen-invasion or cell-stretching at minimum 2 consecutive concentrations was established. To quantify these visual results, the number of cells containing invasive extensions were counted and compared to the total number of cells in the field.

Human Transcriptome Array

Cells treated with either EDF, PhrG or CSP were analysed in duplicate (independent treatment and analysis) for whole transcriptome expression using Affymetrix GeneChip Human Transcriptome Array 2.0. by AROS Applied Biotechnology A/S (Aarhus, Denmark); RNA expression was compared with placebo treated samples. Data analysis was performed using Transcriptome Analysis Console (Affymetrix) and MetaCore (Thompson Reuters) software programs.

Chick Chorioallantoic Membrane (CAM) assay

The Chick Chorioallantoic Membrane (CAM) assay was performed as described by Sys *et al.*: 6 days after (pre-treated, 100 nM) tumour cell transfer to the fertilized eggs, CAM was microscopically scored and histologically examined after H&E staining [12]. For quantification of microscopically observed neovascularisation, an average CAM score was calculated: the number of blood vessels in the 1 mm diameter ring around the 2 mm radius tumour centre was determined [13]. Significant differences were evaluated using the Mann–Whitney U test.

Stability in cell medium and human plasma

In vitro chemical and metabolic stability was determined in cell medium and human plasma, respectively, using previously described procedures [14]. In brief, 100 µg of peptide was incubated in Krebs-Henseleit buffer pH 7.4 with cell medium/plasma (500 µl) at 37°C while shaking. At predetermined time intervals (*i.e.* 0, 6 and 24 hours for cell medium; 0, 30 and 120 minutes for plasma), aliquots were immediately transferred into microtubes containing 1:1 volume of 1% (V/V) trifluoroacetic acid solution in water. The enzyme reaction was further stopped by heating the

solution at 95°C for 5 minutes. Next, the samples were centrifuged to precipitate the denatured proteins and the supernatant analysed using UPLC-PDA. Appropriate placebo solutions were similarly prepared. Assuming first-order kinetics, the rate constant k was obtained from $\ln(P_t/P_{t0}) = -kt$, from which the half-life was determined as $t_{1/2} = \ln(2)/k$.

3. RESULTS

To investigate the crosstalk between the quorum sensing peptides produced by the host microbiome and human breast cancer cells, we selected different signalling peptides from the Quorumpeps® database [15]. Four quorum sensing peptides or analogues thereof were consistently (and concentration-dependently) found to induce tumour cell invasion through a type I collagen extracellular matrix: PhrG from *Bacillus subtilis* (EKMIG), a Competence Stimulating Peptide (CSP) from *Streptococcus mitis* (EMRKSNNNFFHFLRRI) and Extracellular Death Factor (EDF) from *Escherichia coli* (NNWNN), together with its analogue NWN (Figure 1). Other peptides (*e.g.* PhrA (ARNQT) from *Bacillus subtilis* or CSP (ESRLPKIRFDFIFPRKK) from *Streptococcus mitis*) did not induce invasive characteristics. These biologically active peptides were found to be sufficiently stable in cell medium (Table 1), meaning that the effects, observed after 24 hours of treatment, can be associated with the original peptide structures. These observations of invasive cellular extensions are linked to an induced epithelial to mesenchymal transition (EMT), an important process in breast cancer progression and metastasis and the primary cause of breast cancer-related deaths [16, 17].
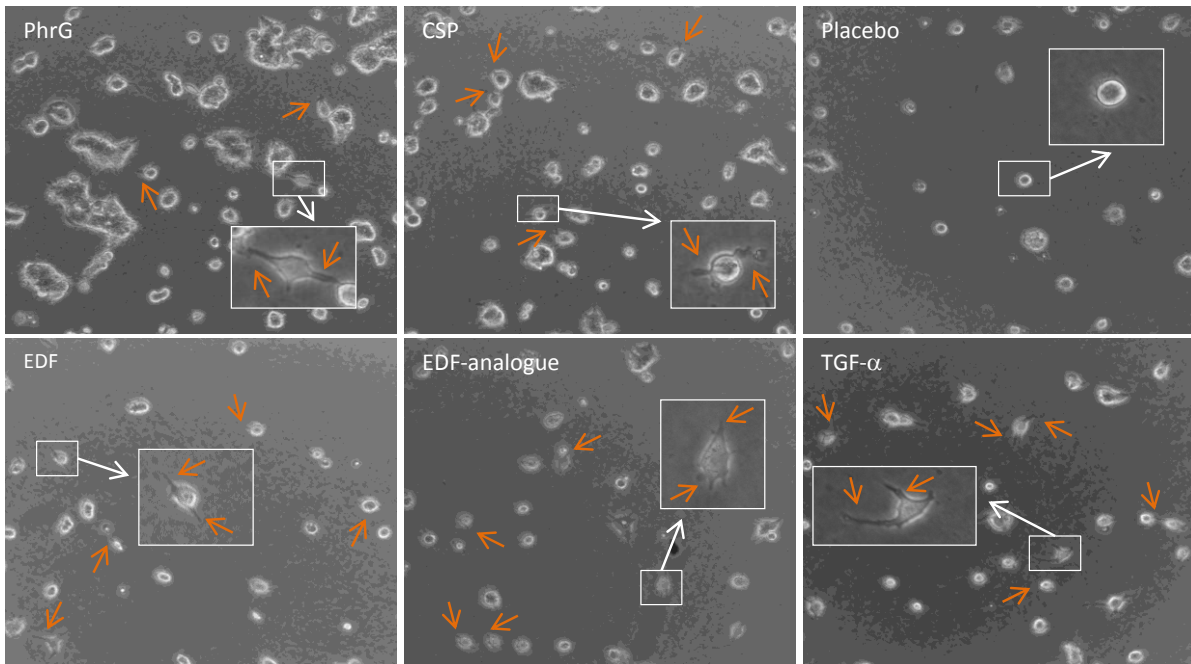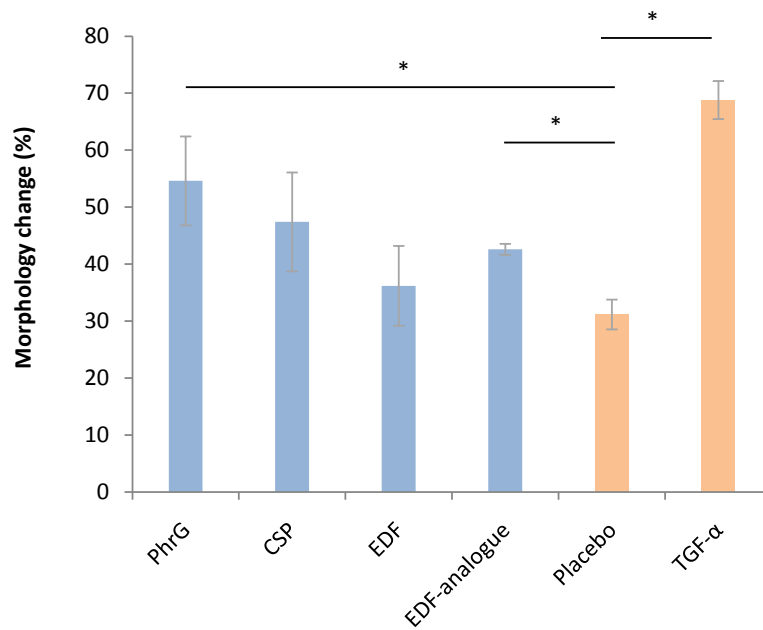
Figure 1. Morphologic changes of non-invasive epithelial MCF-7/AZ cells, induced by quorum sensing peptides at 10 nM. (A) The invasive cellular extensions, observed 24 hours post-treatment with PhrG (EKMIG), CSP (EMRKSNNNFFHFLRRI), EDF (NNWNN) and the EDF-analogue (NWN), indicate tumour-promoting properties of these quorum sensing peptides. The placebo sample serves as the negative control and TGF-α (0.1 μg/ml) as the positive control. (B) Mean (n = 3) number of cancer cells with induced morphology changes: a significant difference is observed between the PhrG peptide or EDF-analogue and the placebo treatment (* $p < 0.05$, Mann-Whitney U test). Error bars represent SEM values.

Table 1. *In vitro* cell medium half-life values of quorum sensing peptides.

Peptide	Sequence	Plasma half-life (hours)
PhrG	EKMIG	160[a]
CSP	EMRKSNNNFFHFLRRI	45.8
EDF	NNWNN	16.2
EDF-analogue	NWN	42.0

[a]The calculated half-life is 157.89 hours

RNA extraction from the MCF-7/AZ cells was performed only in duplicate on a heterogenous mixture (*i.e.* cells with and without induced invasive cellular extensions) instead of on isolated cells, so statistically significant results (ANOVA p-values) are again not yet expected or looked for in this exploratory study; they are rather given for the sake of completeness (similar to the previous chapter). Trends were researched based on the mean fold change. The up-regulation of the Histone cluster 1 H4 gene (*HIST1H4A-F/H-L*) after EDF treatment, which was observed by our transcriptome expression results (Table 2), assigns tumour progressive characteristics to this quorum sensing peptide. Through its link with β-arrestin 1, EDF induces angiogenesis, thereby promoting the survival of breast cancer cells. Moreover, it promotes cytoskeleton reorganization of breast cancer cells as well through the cofilin pathway, which is crucial for tumour migration [18-20]; these morphologic alterations were already observed in our type I collagen invasion assay. With Notch1 over-expression, a poor clinical outcome of breast cancer is correlated, again promoting angiogenesis and thus tumour progression [21]. Reproducible over-expression of the *MTRNR2L2* and *MTRNR2L6* genes, leading to increased synthesis of the Humanin-like proteins 2 and 6 respectively, can be linked to an anti-apoptotic function of the EDF peptide [22]. Finally, up-regulation of *EYA3-IT1* can be associated with increased tumour size and metastasis, due to the tyrosine phosphatase activity of the transcribed proteins, promoting the motility and invasiveness of cancer cells [23].

Table 2. Transcriptome alterations after EDF, PhrG or CSP quorum sensing peptide addition to MCF-7/AZ cells. Gene expression 24 hours post-treatment, compared to placebo samples. Mean fold change is calculated from duplicate samples (cut off: > 1.5 or < -1.5).

Gene symbol	Fold change	ANOVA p-value	Description
Upregulated			
MIR548W	1.92	0.566	MicroRNA 548w
HIST1H4A-F/H-L	1.98	0.330	Histone cluster 1, H4(a-f/h-l)
MTRNR2L2	1.69	0.010	MT-RNR2-like 2
MTRNR2L6	1.58	0.243	MT-RNR2-like 6
RNU2-4P	1.59	0.011	RNA, U2 small nuclear 4, pseudogene
RNU4-4P	1.54	0.065	RNA, U4 small nuclear 4, pseudogene
MIR320C2	1.67	0.076	MicroRNA 320c-2
EYA3-IT1	1.75	0.087	EYA3 intronic transcript 1
IGLJ4	1.58	0.011	Immunoglobulin Lambda Joining 4
SNORD92	1.58	0.261	Small Nucleolar RNA, C/D Box 92
FOXP1-IT1	1.63	0.390	FOXP1 Intronic Transcript 1
MIR635	1.65	0.024	MicroRNA 635
Downregulated			
RN5S217	-1.88	0.127	RNA, 5S ribosomal 217
FOXQ1	-1.72	0.338	Forkhead Box Q1
MAGEB5	-1.75	0.199	Melanoma Antigen Family B, 5
MIR4718	-1.64	0.401	MicroRNA 4718
LOC100128593	-1.56	0.084	Uncategorized LOC100128593

The angiogenic properties of the quorum sensing peptides, as postulated by the transcriptome expression results, were confirmed using the Chick Chorioallantoic Membrane (CAM) assay. From Figure 2, it is clear that all investigated peptides significantly promoted neovascularisation after peptide treatment of the tumour cells, compared to the placebo sample. This recruitment of new blood vessels thereby contributes to the metastasis of tumour cells. Histological evaluation of the membrane confirmed the invasive tumour cell properties through the chorionic layer into the mesoderm, induced by the quorum sensing peptides (Figure 3). Groups of chorionic epithelial cells thereby indicated an aggressive invasion of MCF-7/AZ cells: the chorion is massively disrupted by the tumour cells, resulting in a collapse of the membrane (black arrowheads in Figure 3).
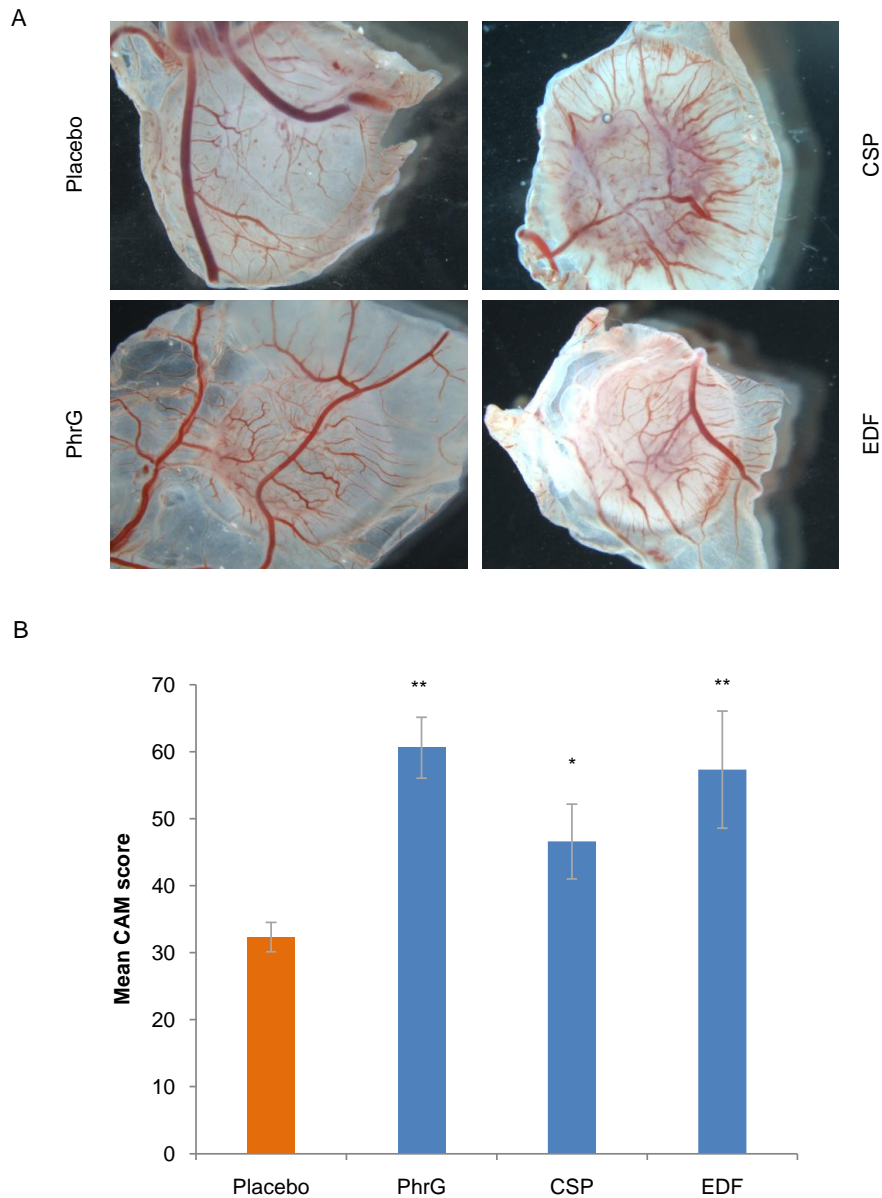
Figure 2. Neovascularisation after quorum sensing peptide treatment (100 nM) of MCF-7/AZ cells on CAM. Placebo sample serves as negative control. (A) Macroscopic images, observed 6 days after (pre-treated, 24 hours) tumour cell transfer to the eggs. (B) Mean CAM score (*i.e.* number of blood vessels in the 1 mm diameter ring around the 2 mm radius centre) in the presence of tumour cells (mean ± SEM, n = 5 (PhrG and CSP), n = 3 (EDF) or n = 9 (placebo)). ** $p < 0.05$, * $p < 0.1$ (Mann-Whitney U test)

Figure 3. Histological H&E evaluation of the chorioallantoic membrane (CAM). Placebo sample serves as negative control. Tumour cells, when treated with the quorum sensing peptide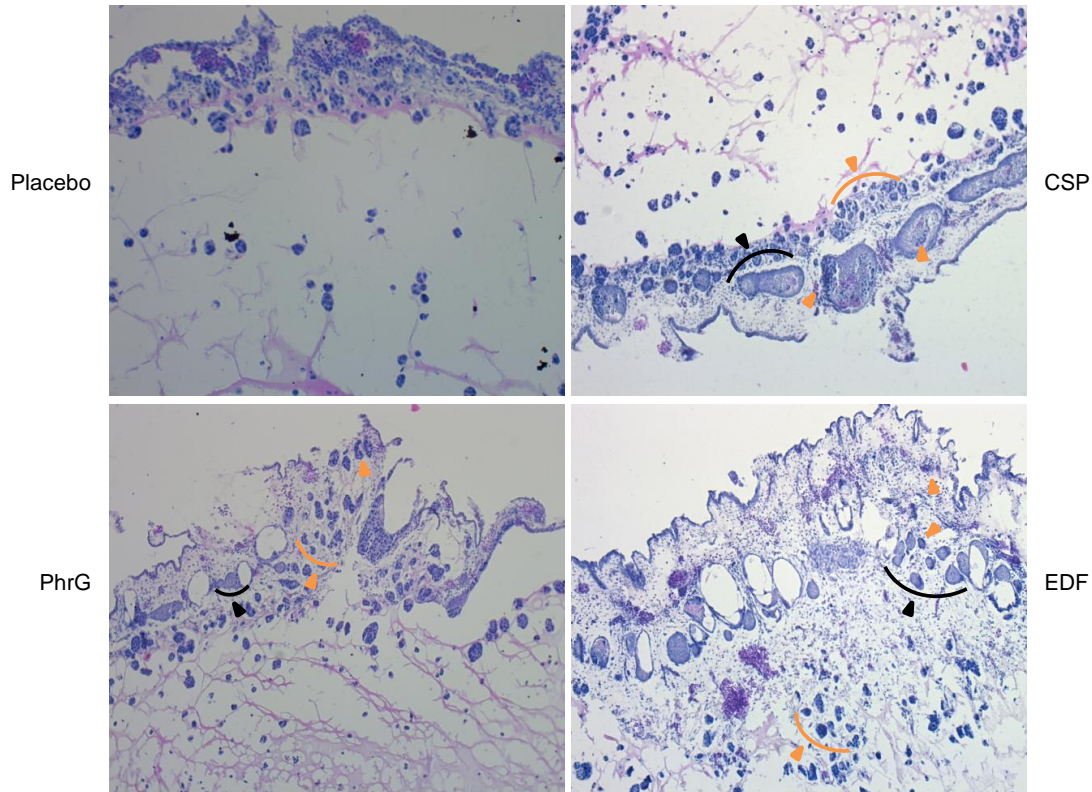s, moved through the chorionic layer into the mesoderm (orange arrowheads). Clusters of chorionic epithelial cells are indicated by black arrowheads, indicating aggressive tumour cell invasion through the membrane.

4.   DISCUSSION

Our *in vitro* results thus clearly indicate that some quorum sensing peptides promote angiogenesis and induce invasion of human breast cancer cells. Based on these results, different questions related to their biological relevance now arise: (1) Do these quorum sensing peptides reach the breast cancer tissue, (2) Can we inhibit these negative microbiome-related effects, and (3) Does this have consequences for the patient's life style?

First, very recent investigations have shown that microbial DNA and viable bacterial cells are present in healthy and cancerous breast tissue, thereby indicating that bacteria or their components may influence the local microenvironment. The predominant bacteria found in breast tumours were *Escherichia* and *Bacillus* [24]. These findings elicit contradictions with previous insights, where it was assumed that the breast tissue was a sterile location, except for the nipple region; nipple duct contamination with skin flora is generally accepted and plays an important role in breast infection [25]. Clearly, future studies are needed to confirm the presence of a breast tissue microbiome under different personalised conditions and describe the different bacterial species that are present. Next,

a thorough exploration of the quorum sensing peptides in the breast tissue should be performed as well; if found, they can negatively influence the nearby breast cancer tissue.

Quorum sensing peptides, produced by commensal or pathogenic bacteria at distant locations, can reach the breast tissue as well via the blood circulation. *N*-acylhomoserine lacton (AHL) molecules, another group of quorum sensing molecules, are already found in human sputum, faeces and saliva, whereby the last biological sample reflects the systemic AHL concentration [8, 26]. Besides, peptides that are synthesized in the gastrointestinal tract can pass the intestinal barrier as well and consequently reach the blood circulation [27]. The same is true for peptides that are synthesized by skin or mouth commensal bacteria: peptides can permeate the human skin or oral mucosa, thereby again reaching the circulation [28,29]. Occasionally, bacteria themselves can also be found in the blood circulation: *Bacillus subtilis* and *Streptococcus mitis*, commensals of the gastrointestinal tract or skin and oropharynx, respectively, were found to be present in the blood of cancer patients [6,30,31]. PhrG and CSP, when present in the blood circulation, thus can affect the clinical outcome of breast cancer, seen its pro-invasive characteristics on breast cancer cells and angiogenesis-promoting properties. Although thus not yet confirmed, quorum sensing peptides might be available from the blood and influence breast cancer progression. One major limitation of this *in vivo* availability, is however the limited stability inherent to peptides, due to the presence of peptidases in the human plasma. As observed in Table 3, the investigated peptides are sufficiently stable in an *in vitro* plasma metabolization study, so this pharmacokinetic property will not be the critical parameter for the biological activity observed with these peptides.

Table 3. *In vitro* plasma half-life values of quorum sensing peptides.

Peptide	Sequence	Plasma half-life (hours)
PhrG	EKMIG	>13[a]
CSP	EMRKSNNNFFHFLRRI	8.7
EDF	NNWNN	>13[a]
EDF-analogue	NWN	4.5

[a]The calculated half-life is 13.16 hours

To answer the second and third question, *i.e.* 'Can we inhibit these microbiome-related effects and does this have consequences for the patient?', the *in vivo* functionality of the quorum sensing peptides should be investigated to confirm our obtained *in vitro* results. If the quorum sensing peptides are found to be present *in vivo*, our preliminary *in vitro* findings of pro-metastatic effects of quorum sensing peptides can open up new perspectives. First, after identification of the tumour target, peptide antagonist can be developed to compete with these endogenous molecules for receptor binding. Second, a person's life style may be adapted to possibly influence the observed

bacteria-related effects. As a change in diet can drastically adapt the microbiota composition, and thus the quorum sensing peptide profile, this may possibly have consequences on cancer outcome [32]. Last, inadequate hygienic measures during *e.g.* surgery can induce breast infection as well. Moreover, during lactation, mastitis should be treated carefully to avoid unwanted bacterial infection of the breast tissue as well [33].

5. CONCLUSIONS

The results of this *in vitro* study indicate that some quorum sensing peptides can stimulate breast cancer metastasis, which may possibly explain the existence of a crosstalk system between the human microbiome and breast cancer cells through quorum sensing peptides. More specifically, we showed that these quorum sensing peptides promote breast cancer cell invasion and angiogenesis.

6. REFERENCES

[1]   World Health Organization. Cancer health topic, fact sheet number 297. http://www.who.int/mediacentre/factsheets/fs297/en/ (Februari 2014).

[2]   Niyaz A, Shivendra T, Nishant N. Helicobacter pylori – a seasoned pathogen by any other name. *Gut Pathogens* **2009**; doi: 10.1186/1757-4749-1-24.

[3]   Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* 2012; **338**: 120-123.

[4]   Schwabe RF, Wang TC. Bacteria Deliver a Genotoxic Hit. *Science* 2012; **338**: 52-53.

[5]   Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am. J. Clin. Nutr.* 2012; **96**: 544-551.

[6]   Urbaniak C, Cummins J, Brackstone M, Macklaim JM, Gloor GB, Baban CK, Scott L, O'Hanlon DM, Burton JP, Francis KP, Tangney M, Reig G. Microbiota of human breast tissue. *Appl. Environ. Microbiol.* 2014; **80**: 3007-3014.

[7]   Costerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* 1999; **284**: 1318-1322.

[8]   Kumari A, Pasini P, Daunert S. Detection of bacterial quorum sensing N-acyl homoserine lactones in clinical samples. *Anal. Bioanal. Chem.* 2008; **391**: 1619-1627.

[9]   Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG. *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. *Infect. Immun.* 2002; **70**: 1783-1790.

[10]   World Health Organization. Cancer program, breast cancer: prevention and control. http://www.who.int/cancer/detection/breastcancer/en/index1.html (February 2014).

[11]   De Wever O, Hendrix A, De Boeck A, Westbroek W, Braems G, Emami S, Sabbah M, Gespach C, Bracke M. Modeling and quantification of cancer cell invasion through collagen type I matrices. *Int. J. Dev. Biol.* 2010; **54**: 887-896.

[12]   Sys G, Van Bockstal M, Forsyth R, Balke M, Poffyn B, Uyttendaele D, Bracke M, De Wever O. Tumor grafts derived from sarcoma patients retain tumor morphology, viability, and invasion potential and indicate disease outcomes in the chick chorioallantoic membrane model. *Cancer Lett.* 2012; **326**: 69-78.

[13]   Ribatti D, Nico B, Vacca A, Presta M. The gelatin-sponge-chorioallantoic membrane assay. *Nat. Protoc.* 2006; **1**: 85-91.

[14]   Vergote V, Van Dorpe S, Peremans K, Burvenich C, De Spiegeleer B. In vitro metabolic stability of obestatin: kinetics and identification of cleavage products. *Peptides* 2008; **29**: 1740-1748.

[15]   Wynendaele E, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke M, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides. *Nucleic Acids Res.* 2013; **41**: D655-D659.

[16]   Burgess DJ. Breast cancer: circulating and dynamic EMT. *Nat. Rev. Cancer* 2013; **13**: 148-149.

[17]   Tomaskovic-Crook E, Thompson EW, Thiery JP. Epithelial to mesenchymal transition and breast cancer. *Breast Cancer Res.* **2009**; doi: 10.1186/bcr2416.

[18]   Shenoy SK, Han S, Zhao YL, Hara MR, Oliver T, Cao Y, Dewhirst MW. β-arrestin1 mediates metastatic growth of breast cancer cells by facilitating HIF-1-dependent VEGF expression. *Oncogene* 2012; **31**: 282-292.

[19]   Zoudilova M, Kumar P, Ge L, Wang P, Bokoch GM, DeFea KA. Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. *J. Biol. Chem.* 2007; **282**: 20634-20646.

[20]   Ge L, Shenoy SK, Lefkowitz RJ, DeFea K. Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2. *J. Biol. Chem.* 2004; **279**: 55419-55424.

[21]   Al-Hussaini H, Subramanayam D, Reedijk M, Sridhar SS. Notch signaling pathway as a therapeutic target in breast cancer. *Mol. Cancer Ther.* 2011; **10**: 9-15.

[22]   Bodzioch M, Lapicka-Bodzioch K, Zapala B, Kamysz W, Kiec-Wilk B, Dembinska-Kiec A. Evidence for potential functionality of nuclearly-encoded humanin isoforms. *Genomics* 2009; **94**: 247-256.

[23]   Pandey RN, Rani R, Yeo EJ, Spencer M, Hu S, Lang RA, Hegde RS. The Eyes Absent phosphatase-transactivator proteins promotes proliferation, transformation, migration and invasion of tumor cells. *Oncogene* 2010; **29**: 3715-3722.

[24]   Xuan C, Shamonki JM, Chung A, DiNome ML, Chung M, Sieling PA, Lee DJ. Microbial dysbiosis is associated with human breast cancer. *Plos One* **2014**; doi: 10.1371/journal.pone.0083744.

[25]   Streinu-Cercel A, Jianu DM, Streinu-Cercel O. Breast Ecology Assessment in the Study of Local Microflora. *Aesthet. Surg. J.* 2013; **33**: 747-748.

[26]   Kaufman E, Lamster IB. The Diagnostic Applications of Saliva – A Review. *Crit. Rev. Oral Biol. Med.* 2002; **13**: 197-212.

[27]   Daniel H. Molecular and Integrative Physiology of Intestinal Peptide Transport. *Annu. Rev. Physiol.* 2004; **66**: 361-384.

[28]   Taevernier L, Veryser L, Roche N, De Spiegeleer B. Human skin kinetics of cyclic depsipeptide mycotoxins. *J. Pept. Sci.* 2014; **20**: S299-S300.

[29]   Veuillez F, Kalia YN, Jacques Y, Deshusses J, Buri P. Factors and strategies for improving buccal absorption of peptides. *Eur. J. Pharm. Biopharm.* 2001; **51**: 93-109.

[30]   Logan NA. *Bacillus* species of medical and veterinary importance. *J. Med. Microbiol.* 1988; **25**: 157-165.

[31]   Han XY, Kamana M, Rolston KVI. Viridans *Streptococci* Isolated by Culture from Blood of Cancer Patients: Clinical and Microbiologic Analysis of 50 Cases. *J. Clin. Microbiol.* 2006; **44**: 160-165.

[32]   Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. *Sci. Transl. Med.* **2009**; doi: 10.1126/scitranslmed.3000322.

[33]   Jahanfar S, Ng C-J, Teng CL. Antibiotics for mastitis in breastfeeding women. *Cochrane Database Syst. Rev.* **2009**; doi: 10.1002/14651858.CD005458.pub2.

CHAPTER VII

QUORUM SENSING PEPTIDES SELECTIVELY PENETRATE THE BLOOD-BRAIN BARRIER

"Biology gives you a brain. Life turns it into a mind."

Jeffrey Eugenides
(° 1960, American writer)

ABSTRACT

Bacteria communicate with each other by the use of signaling molecules, a process called 'quorum sensing'. One group of quorum sensing molecules includes the oligopeptides, which are mainly synthesized by Gram-positive bacteria. Recently, these quorum sensing peptides were found to biologically influence mammalian cells, promoting *i.a.* metastasis of cancer cells. In this study, three quorum sensing peptides were investigated for their brain influx and efflux properties in an *in vivo* mouse model: PhrCACET1 demonstrated a very high influx into the mouse brain (K_{in} = 1.33 µl/(g×min)), while brain permeability of BIP-2 and PhrANTH2 was found to be low (K_{in} = 0.35 µl/(g×min)) and very low (K_{in} = 0.18 µl/(g×min)), respectively. All three quorum sensing peptides were found to be metabolically stable in plasma during the experimental time frame. No significant efflux was observed for the three quorum sensing peptides. Initial tissue distribution data showed remarkably high liver accumulation of BIP-2.

CHAPTER VII

QUORUM SENSING PEPTIDES SELECTIVELY

PENETRATE THE BLOOD-BRAIN BARRIER

Main focus in this chapter:

- To indicate the permeability of quorum sensing peptides through the blood-brain barrier.

- To excite the research on the microbiome's influence on diverse central nervous system disorders.

1.   INTRODUCTION

Bacteria communicate with each other by the use of chemicals (*i.e.* pheromones), produced and released by the bacteria and recognized by others. Once a threshold concentration of these molecules is reached, a coordinated change in bacterial behavior is initiated, *e.g.* biofilm formation, virulence factor production or competence induction. This process of cell-to-cell communication is called 'quorum sensing'. Gram-negative and Gram-positive bacteria have different quorum sensing systems, each activated by specific quorum sensing molecules: *N*-acyl homoserine lactones (AHLs) trigger LuxI/LuxR circuits in Gram-negative bacteria, while Gram-positive bacteria mostly use peptides as signal molecules; both Gram-negative and Gram-positive bacteria produce autoinducer-2 (AI-2) family molecules [1,2]. Although not yet investigated, the presence of quorum sensing peptides in the human body is very likely, seen the biological incidence of Gram-positive bacteria and the *in vivo* detection of biofilms and AHL molecules in human feces, sputum and saliva [3-5]. The quorum sensing signaling molecules were originally found as intra-species communication tools in bacteria, but recent evidence indicates interspecies and host signaling as well [6-8]. Some AHLs exhibit immunomodulating activities by influencing the Th1-Th2 balance in the infected host [9]. In addition, the *Bacillus subtilis* quorum sensing peptide CSF (Competence and Sporulation Factor) activates p38 mitogen-activated protein kinase and protein kinase B (Akt) in host intestinal epithelial cells and induces cytoprotective heat shock protein synthesis [10]. Moreover, investigations from our group have indicated a selective crosstalk phenomenon between these quorum sensing peptides and

mammalian cells: some quorum sensing peptides enhance breast or colon cancer cell invasion and promote angiogenesis, thereby potentially influencing cancer metastasis [11,12].

The brain is protected by a physiological barrier between the bloodstream and the central nervous system, *i.e.* the blood-brain barrier (BBB). This barrier is formed by the endothelium lining the brain capillaries, possessing intercellular tight junctions, pericytes within the capillary basement membrane and astrocyte endfeet [13,14]. Research towards peptide-based therapeutics has been fuelled since the determination of the role of different neuropeptides in several neurological disorders. However, due to the presence of the BBB, inadequate delivery of these medicinally promising peptides to the brain is frequently observed. Currently, a number of peptides are used or investigated for their therapeutic purposes in *e.g.* epilepsy, depression, pain or brain cancer [15,16]. An increased success rate of peptide-based therapies is often observed due to a disruption of the BBB, which occurs in many neurological disorders, including brain cancer. However, in early stages of brain cancer, the BBB is generally intact, so early diagnosis and treatment are limited, though indispensable [17,18]. Some of the neurological diseases were associated with an altered microbiota composition as well. However, no clear explanation was given for these observations [19], with the role of the quorum sensing peptides not yet being investigated. Next to the peptides, a limited number of bacteria is capable of crossing the tight barrier between the bloodstream and the central nervous system as well, leading to bacterial meningitis. Here, the bacteria reach the blood circulation and then traverse the BBB, leading to inflammatory responses from the host and pathophysiological alterations (*e.g.* BBB disruption) [20].

Based on our previous results of metastasis-promoting effects of quorum sensing peptides, together with the presence of an intact BBB in the early stages of brain cancer and the possible link with some other neurological disorders, we investigated the permeability of three chemically diverse quorum sensing peptides through the BBB: if BBB-transport is observed, this may have an impact on the development of several brain pathologies, including cancer.

2. MATERIALS AND METHODS

Reagents

Calcium dichloride dihydrate, **m**agnesium sulphate, potassium chloride, sodium chloride, sodium dihydrogen phosphate hydrate, sodium lactate and urethane were purchased from Sigma-Aldrich (Diegem, Belgium), while Bovine Serum Albumin (BSA), disodium hydrogen phosphate dihydrate, sodium iodide, sodium dihydrogen phosphate monohydrate, sodium metabisulphite and Chloramine-T were obtained from Merck KGaA (Darmstadt, Germany). Calcium dichloride, D-glucose, formic acid

(FA) and HEPES were purchased from Fluka (Diegem, Belgium) and dextran from AppliChem GmbH (Darmstadt, Germany). For the mobile phases, acetonitrile was obtained from Fisher Scientific (Erembodegem, Belgium) and water was purified using an Arium 611 Pro VF purification system (Sartorius, Göttingen, Germany) to laboratory-graded water (18.2 MΩ × cm). For the radiolabeling of the peptides, Iodo-Gen® coated tubes were purchased from Thermo Scientific (Erembodegem, Belgium) and the radioactive sodium iodide solution (Na^{125}I) from Perkin Elmer (Zaventem, Belgium).

Animals

Female, Institute for Cancer Research, Caesarean Derived-1 (ICR-CD-1) mice (Harlan Laboratories, Venray, The Netherlands) of age 7-10 weeks and weighing 25-34 g, were used during the BBB-transport experiments. All animal experiments were performed in accordance with the Ethical Committee principles of laboratory animal welfare as approved by our institute (Ghent University, Faculty of Veterinary Medicine, number 2012-157).

Peptide selection

The currently known quorum sensing peptides are continuously collected into the Quorumpeps database (http://quorumpeps.ugent.be) [21]. To select chemically diverse quorum sensing peptides for BBB-permeability investigations, we optimized the three-dimensional structure of these 231 peptides (status in August 2014) and calculated over 3000 descriptors for each peptide. After removal of the constant descriptors, and correction for molecular weight, a final dataset of 1468 descriptors was retained. Multivariate data-analysis on this resulting 231x1468 data-matrix was performed using Principal Component Analysis (PCA) with SIMCA-P+ 12.0 (Umetrics, Sweden), and different clusters identified [22]. Finally, three chemically diverse quorum sensing peptides were selected to investigate their brain permeability characteristics.

Peptide handling

The quorum sensing peptides were purchased at GL Biochem (Shangai, China) and the positive control dermorphin at Bachem (Bubendorf, Switzerland). The peptide purity was determined to be ≥ 90%, based on UPLC-PDA analyses [23]. Prior to experimental use, the peptides were dissolved in phosphate buffer (25 mM) at a concentration of 1 µmol/ml.

Peptide ^{125}I radiolabeling and purification

Dermorphin and BIP-2 were labeled using the Iodogen method [24,25]. Briefly, 0.1 μmol of the lyophilized peptide was dissolved in 100 μl of phosphate buffer (pH 7.4, 25 mM). A Iodo-Gen® coated tube was previously rinsed with 1 ml of phosphate buffer. Subsequently, 50 μl of sodium iodide solution (1.1 μmol/ml) and 1 mCi of Na[125]I solution were transferred into this Iodo-Gen® coated tube. The oxidation reaction was allowed to proceed for six minutes at room temperature, after which the iodonium solution was transferred to 50 μl of peptide solution (1 μmol/ml). The iodination reaction of the peptide was allowed to proceed another six minutes at room temperature. Next, the reaction mixture was analysed by radio-HPLC and the eluting fractions determined for radioactive content (*i.e.* peptide concentration). The radio-HPLC apparatus consisted of a LaChrom Elite L-2130 pump with degasser (flow rate is 1 ml/min), a LaChrom Elite L-2300 column oven set at 30°C, a LaChrom Elite L-2400 UV-detector set at 215 nm (all Hitachi, Tokyo, Japan), a Rheodyne 7725i manual injector with 100 μl sample loop (Rheodyne, Rohnert Park, CA, USA), a Berthold LB500 HERM radioactivity detector (Berthold Technologies, Bad Wildbad, Germany) equipped with EZChrom Elite version 3.1.7 software for data acquisition (Scientific Software, Pleasanton, CA, USA) and a fraction collector FC 203 (Gilson International BV, Den Haag, The Netherlands). For separation, a Vydac Everest C_{18} (250 × 4.6 mm, 5 μm particle size) column (Grace, Baltimore, MD, USA) was coupled to the HPLC system. Mixtures of water (0.1% FA m/V) and acetonitrile (0.1% FA m/V) were used to create appropriate gradients for separation of peptides and their iodinated forms. The mono- (and di-) iodinated peptide fractions were then concentrated (if necessary) by nitrogen drying and the appropriate peptide concentrations prepared using Lactated Ringer's solution containing 1% of BSA. The negative control, *i.e.* BSA, was iodinated using the same procedure and the iodinated protein isolated from free iodine using an argent filter.

Peptides phrANTH2 and phrCACET1 were iodinated using the Chloramine-T method [26]: 50 μl of peptide solution (1 μmol/ml) was subsequently mixed with 20 μl of 4.5 mg/ml NaI in 100 mM phosphate buffer (phrANTH2) or 0.1% m/V formic acid in water (phrCACET1), 1 mCi of Na[125]I solution and 30 μl of a 4 mg/ml Chloramine-T in phosphate buffer solution (100 mM). For phrCACET1, 40 μl of 0.1% m/V formic acid in water was added before the Chloramine-T solution as well. The iodination reaction was continued for 120 (phrANTH2) or 40 (phrCACET1) seconds, after which 30 μl of sodium metabisulphite solution (8 mg/ml) was added to neutralize the oxidizing agent. Next, the reaction mixtures were analysed by radio-HPLC using the described procedures and the eluting fractions determined for radioactivity amount. Again, nitrogen drying was performed on the mono- (and di-) iodinated fractions and the solutions prepared using Lactated Ringer's solution containing 1% of BSA.

Multiple time regression analysis

In order to determine whether the peptides could enter the brain, *in vivo* multiple time regression analyses were performed. Therefore, ICR-CD-1 mice were anesthetized intraperitoneally using a 40% urethane solution (3 g/kg). Then, the jugular vein and carotid artery were isolated and 200 µl of the radiolabeled peptide solution, diluted to 30 000 cpm/µl using Lactated Ringer's solution containing 1% of BSA (LR/BSA), was injected into the jugular vein. At specified time points after injection (*i.e.* 1, 3, 5, 10, 12.5 and 15 min, with start and end in duplicates), blood was obtained from the carotid artery followed by decapitation of the mouse. The isolated brain was weighed and radioactivity measured in a gamma counter (Wallac Wizard automatic gamma counter, Perkin Elmer, Shelton, CT, USA), as well as from 50 µl serum, which was obtained by centrifuging the collected blood at 10 000 *g* for 15 min at 21°C. To evaluate the tissue distribution of the peptides during the BBB-experiments, seven other tissues, *i.e.* spleen, kidneys, lungs, heart, duodenum, muscles and liver, were collected immediately after decapitation of the mice at the last time point of 15 min. After weighing the tissues, the radioactivity was measured in a gamma counter.

The linear modeling of the multiple time regression analysis is based on the Gjedde-Patlak equation [27-29]:

$$\frac{A_m(t)}{C_p(t)} = K_{in}\Theta + V_i \quad \text{where} \quad \Theta = \int_0^T \frac{C_p(t) \cdot dt}{C_p(t)} \tag{1}$$

and where $A_m(t)$ is the amount of radioactivity in the brain at time t, $C_p(t)$ the amount of radioactivity in serum at time t, K_{in} the brain influx rate constant and V_i the initial brain distribution volume.

During the multiple time regression experiments, peptides are intravenously injected, which causes clearance by the organs. Therefore, the exposure time is used during the modeling of the brain influx of the peptides to account for the decreasing concentrations. The exposure time (Θ) represents the theoretical steady-state serum level of radiolabeled peptide at the serum concentration $C_p(t)$ and is defined as the integral of the serum radioactivity over time divided by the radioactivity at time t. The integral of radioactivity over time is represented by the area under the curve [27,30,31].

Finally, the brain/serum ratios (µl/g) were plotted versus the exposure time and the slope of this relationship measures the unidirectional influx rate (K_{in}) from blood to brain, whereas the intercept represents the initial brain volume of distribution (V_i).

For the evaluation of the tissue distribution of the radiolabeled peptides 15 min after IV-injection, the percentage of the injected dose for each isolated tissue is calculated as follows:

$$\% \ injected \ dose = \frac{A_{tissue}/w_{tissue}}{A_{injected}/w_{animal}} \ x \ 100 \tag{2}$$

where A_{tissue} and $A_{injected}$ are the measured activity of the isolated tissue and the activity of 200 µl of Multiple Time Regression solution, respectively, while w_{tissue} is the weight of the considered tissue and w_{animal} is the mass of the injected mouse. The results are the mean values of the duplicates.

Capillary depletion

This method was performed to determine whether the peptides, taken up by the brain, completely crossed the capillary wall instead of being trapped there. The method of Triguero et al., as modified by Gutierrez et al., was used [32,33]. Briefly, ICR-CD-1 mice were first anesthetized intraperitoneally using a 40% urethane solution (3 g/kg). After isolation of the jugular vein, 200 µl of the iodinated peptide solution, diluted to 10 000 cpm/µl using LR/BSA, was injected in the jugular vein. Ten minutes after injection, blood was collected from the abdominal aorta and the brain was perfused manually with 20 ml of Lactated Ringer's buffer after clamping the aorta and severing the jugular veins. Subsequently, the brain was collected, weighed and the radioactivity measured in the gamma counter. Then, the brain was homogenized with 0.7 ml of ice-cold capillary buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 and 10 mM D-glucose adjusted to pH 7.4) in a pyrex glass tube and mixed with 1.7 ml of 26% ice-cold dextran solution in capillary buffer. The resulting solution was weighed and centrifuged in a swinging bucket rotor at 5400 g for 30 min at 4°C, after measuring the radioactivity in the gamma counter. Pellet (capillaries) and supernatant (parenchyma and fat tissues) were collected, weighed and measured in a gamma counter. After centrifuging the obtained blood (10 000 g, 21°C, 15 min), the radioactivity of 50 µl serum was measured in a gamma counter as well.

Compartmental distribution was calculated as follows:

$$Fraction = \frac{CD_{tissue}}{\frac{A_{capillaries}}{A_{serum}} + \frac{A_{parenchym}}{A_{serum}}} \times 100 \tag{3}$$

where CD_{tissue} represents the ratio of the activity of the capillaries or parenchyma and the activity of serum for the fraction of radiolabeled peptide in the capillaries and parenchyma, respectively.

Brain-to-blood transport

This method was performed to quantify the amount of peptide pumped out of the brain by efflux transport as previously described [34]. ICR-CD-1 mice were anesthetized intraperitoneally using a 40% urethane solution (3 g/kg). Then, the skin of the skull was removed and a hole was made into the lateral ventricle using a 22 G needle marked with tape at 2 mm at the following coordinates: 1 mm lateral and 0.34 mm posterior to the bregma. The anesthetized mice received an intracerebroventricular injection of 1 µl of the diluted iodinated peptide solution using LR/BSA (25

000 cpm/µl) by pumping the peptide solution at a speed of 360 µl/h for 10 s using a syringe pump (KDS100, KR analytical, Cheshire, UK). At specified time points after intracerebroventricular injection (*i.e.* 1, 3, 5, 10, 12.5 and 15 min), blood was collected from the abdominal aorta and subsequently the mouse was decapitated. Then, the whole brain was collected, weighed and measured in a gamma counter, as well as from 50 µl of serum, which was obtained by centrifuging the collected blood at 10 000 *g* during 15 min at 21°C. The efflux half-life was calculated from the linear regression of the natural logarithm of the residual radioactivity in brain versus time as follows:

$$t_{1/2} = \frac{\ln(2)}{k_{out}}$$ (4)

where k_{out} is defined as the efflux rate constant calculated as the negative value of the slope of the linear regression, applying first order kinetics.

In vitro human plasma stability

In vitro metabolic stability of the quorum sensing peptides was determined in human plasma using previously described procedures [35]. In brief, 100 µg of non-radiolabeled peptide was incubated in 400 µl of Krebs-Henseleit buffer pH 7.4 and 500 µl of plasma at 37°C while shaking. At predetermined time intervals (*i.e.* 0, 30 and 120 minutes), aliquots were immediately transferred into microtubes containing 1:1 volume of 1% (V/V) trifluoroacetic acid solution in water. The enzyme reaction was further stopped by heating the solution at 95°C for 5 minutes. Next, the samples were centrifuged to precipitate the denatured proteins and the supernatant analysed using UPLC-PDA. The system consisted of a Waters Acquity H-Class Bio-samples Flow Through Needle (flow rate set at 0.5 ml/min), Waters Acquity H-Class BioQuaternary Solvent Manager, Waters Acquity H-Class column module (set at 30°C), Waters Acquity H-Class Photodiode Array Detector (PDA, quantification at 210 nm) or Waters Xevo TQ-S (Selected Ion Recording, SIR) and equipped with Waters Empower Pro software version 2 or MassLynx version 4.1 (Waters, Zellik, Belgium). Mixtures of water (0.1% FA m/V) and acetonitrile (0.1% FA m/V) were used to create appropriate gradients for separation of peptides and their metabolites. An appropriate placebo solution was similarly prepared. Assuming first-order kinetics, the rate constant k was obtained from

$$\ln\frac{P_t}{P_{t0}} = -kt$$ (5)

from which the half-life was determined as

$$t_{1/2} = \frac{\ln(2)}{k}$$ (6)

3. RESULTS

<u>Peptide selection</u>

The clustering results of the quorum sensing peptides are given in Figure 1: three main, chemically diverse clusters can be distinguished [22]. From each cluster, we selected one peptide resulting in three chemically diverse molecules, *i.e.* BIP-2 (Quorumpeps ID102), PhrANTH2 (Quorumpeps ID186) and PhrCACET1 (Quorumpeps ID206). BIP-2, or bacteriocin-inducing peptide 2, is synthesized by *Streptococcus pneumoniae*, a commensal of the human nasopharynx [36,37]. PhrANTH2 is produced by *Bacillus anthracis*, while PhrCACET1 is formed by *Clostridium acetobutylicum* [38].
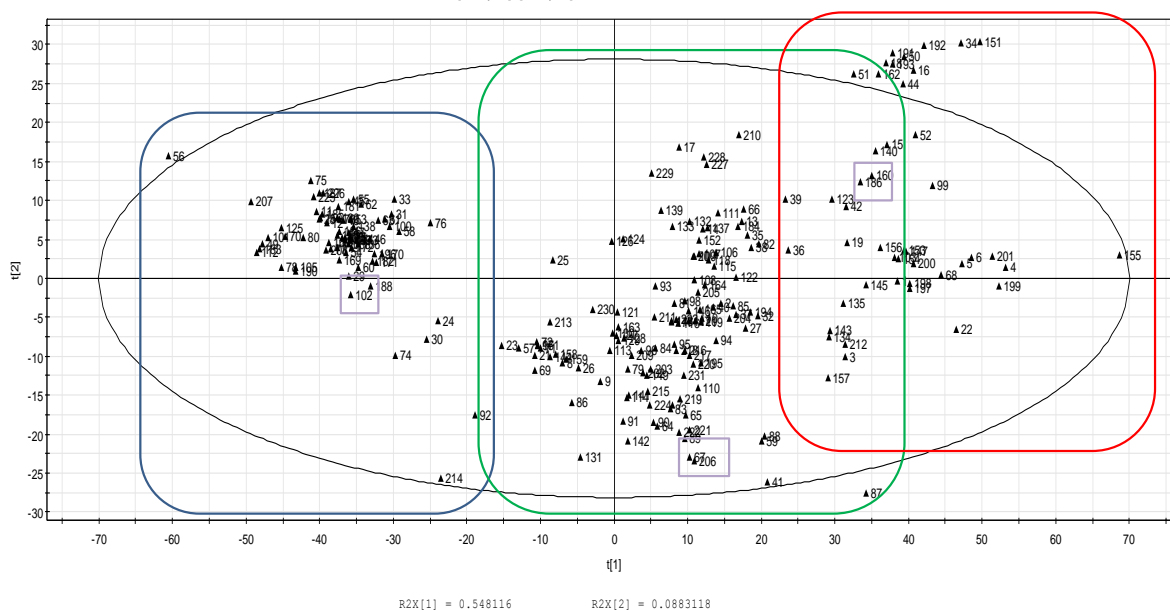
Figure 1. Score plot of the PCA analysis, distinguishing 3 main clusters. The selected peptides are indicated by the purple rectangles, *i.e.* Quorumpeps® ID102 (BIP-2), ID206 (PhrCACET1) and ID186 (PhrANTH2).

<u>Brain influx and tissue distribution</u>

Only 2 of the 3 investigated peptides showed a statistically significant influx into the mouse brain. In Figure 2, the ratio of the brain and serum radioactivity is plotted versus the exposure time; the quantitative influx parameters of the molecules are summarized in Table 1. The data were fitted using a simple linear regression model (equation 1).

Dermorphin clearly showed an influx: the calculated influx rate constant is 0.26 µl/(g×min). In literature, it is well established that BSA shows a very small, almost negligible brain influx in the Multiple Time Regression technique [39]. Here, we confirmed this small influx into the brain for BSA: a K_{in} of 0.12 µl/(g×min) was calculated. Both controls thus indicated a good performance of the brain influx experiments and their obtained values can be used to benchmark the influx results of the

quorum sensing peptides. Peptide PhrANTH2 showed a very small influx into the brain, comparable to BSA: a K_{in} of 0.18 µl/(g×min) is measured and found not to be statistically significantly higher than the K_{in} of BSA. Peptide PhrCACET1 shows a very high influx into the brain as the K_{in} was determined to be 1.33 µl/(g×min); the high V_i value indicates a rapid influx as well. BIP-2 showed a small influx into the brain: a K_{in} of 0.35 µl/(g×min) was measured and found to be statistically significantly higher than the K_{in} of BSA, comparable to dermorphin (not significantly different) but lower than the K_{in} of PhrCACET1.
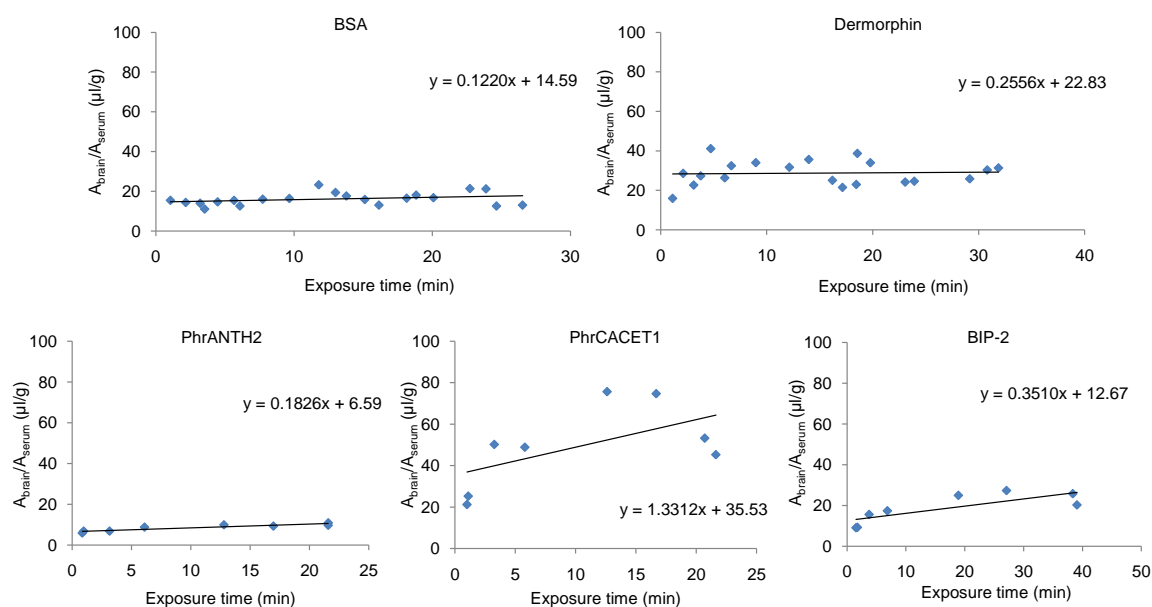
Figure 2. Brain influx of the selected quorum sensing peptides, using the simple linear regression method. BSA and dermorphin were used as the negative and positive controls, respectively. PhrANTH2 shows no significant brain influx, while the results of PhrCACET1 and BIP-2 indicate BBB permeability, which is the highest for PhrCACET1.

Table 1. Overview of the multiple time regression results using the linear regression model. Between brackets, the 65% confidence interval is mentioned (*i.e.* ± 1 SEM).

Peptide	K_{in} (µl/(g × min))	V_i (µl/g)
BSA	0.12 [0.040, 0.20]	14.59 [13.36, 15.81]
Dermorphin	0.26 [0.15, 0.36]	22.83 [21.25, 24.41]
PhrANTH2	0.18 [0.15, 0.22]	6.59 [6.12, 7.06]
PhrCACET1	1.33 [0.56, 2.11]	35.53 [25.41, 45.65]
BIP-2	0.35 [0.24, 0.47]	12.67 [10.07, 15.28]

It is also clear that dermorphin, PhrCACET1 and BIP-2 showed a biphasic brain influx model [40], characterized by a rapid initial uptake followed by a plateauing equilibrium. This initial brain uptake kinetics of BIP-2 (from 1 to 3 minutes) was similar to dermorphin (3.0 and 3.4 µl/(g×min), respectively), while PhrCACET1 showed a much higher initial unidirectional blood clearance (11.3 µl/(g×min)). These initial brain influx results thus correspond well with the overall classification conclusions: PhrCACET1 showed the highest brain influx, followed by BIP-2 and dermorphin.

The results of the capillary depletion study (Figure 3) at 10 min after injection validate the high brain influx of peptide PhrCACET1: the absolute amount of peptide in the brain (*i.e.* absolute y-axis values of Figure 3) is much higher for this peptide compared to the others. The relative amount of peptide that is effectively transferred into the brain showed a higher brain parenchyma (77% – 86%) versus a lower capillary retention (14% - 23%) for the three peptides.
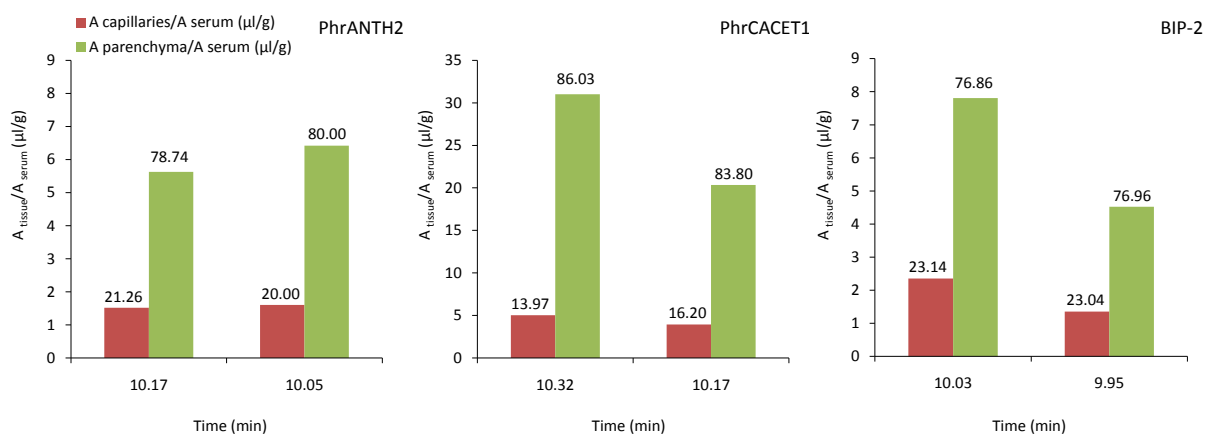
Figure 3. Capillary depletion results of the quorum sensing peptides.
The amount of peptide that is effectively transferred into the brain shows a higher brain parenchyma (green) versus a relatively low capillary (red) distribution for the three peptides. The capillary and parenchyma fraction (%) are indicated above the histogram.

In Figure 4, the relative concentrations in the different tissues, obtained from Multiple Time Regression experiments at the last time points (*i.e.* at 15 minutes post-injection) are graphically presented. BSA showed a clear liver distribution, while dermorphin was mainly distributed in the duodenum, followed by the liver and serum, 15 min after i.v. injection. The high duodenum accumulation is explained by the presence of peripheral mu opioid receptors for which dermorphin shows high affinity [41]. PhrANTH2 showed a low tissue distribution, while PhrCACET1 shows a higher distribution in the kidneys and duodenum. BIP-2 showed a very high liver accumulation.
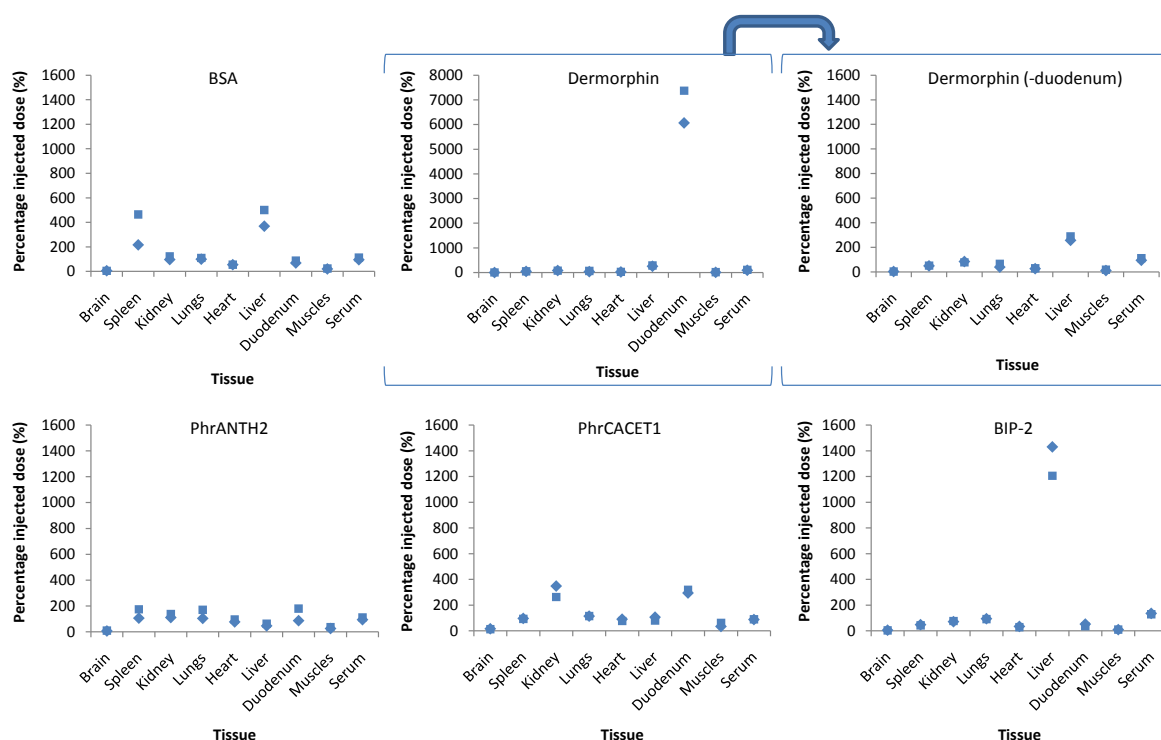
Figure 4. Tissue distribution of the quorum sensing peptides, 15 minutes after IV administration.
BSA shows a higher spleen and liver distribution, while dermorphin is massively accumulated in the mice
duodenum. PhrANTH2 shows no clear tissue distribution, while PhrCACET1 and BIP-2 are mainly distributed to
the kidneys/duodenum and liver, respectively.

Brain-to-blood transport

The natural logarithm of the measured residual radioactivity in the brain was plotted versus the time, to calculate the efflux constant k_{out}. No significant efflux was observed during the experimental time of 15 minutes for the three quorum sensing peptides (k_{out} BIP-2 = -0.06 ± 0.02; k_{out} PhrANTH2 = -0.05 ± 0.07; k_{out} PhrCACET1 = -0.02 ± 0.03).

In vitro human plasma stability

In order to correctly interpret the BBB permeability results, we investigated the stability of these non-radiolabeled quorum sensing peptides in human plasma. All three peptides were found to be stable during the experimental set-up time (*i.e.* maximum 15 minutes) although quite different metabolization kinetics were observed: a half-life value of 56.4, 23.6 and 320.7 minutes was obtained for PhrANTH2, PhrCACET1 and BIP-2, respectively.

4. DISCUSSION

This study demonstrates that the quorum sensing peptide PhrCACET1 (SYPGWSW) efficiently crosses the BBB, with a measured brain influx that is higher than that of dermorphin (positive control), which generally shows a low but significant influx into the brain. The brain influx permeability of BIP-2 (GLWEDLLYNINRYAHYIT) through the BBB is lower, *i.e.* higher than BSA but similar to the dermorphin brain influx values. In contrast, PhrANTH2 (SKDYN), shows no significant transport across the BBB in an *in vivo* mouse model. To evaluate these transport results, we compared them with the linear influx results of other investigated peptides, obtained using the same technique, for which the data are summarized in the Brainpeps® database [42]. The classification system of BBB influx data, using the BBB_{in} response proposed by Stalmans et al. [43], indeed indicated a 'very high influx' (*i.e.* class 5, BBB_{in} = 9) for the quorum sensing peptide PhrCACET1. A 'low influx' classification (*i.e.* class 2, BBB_{in} = 2) is obtained for both BIP-2 and PhrANTH2: despite the clear difference in brain influx values between these two peptides, they are both organized in the same class. However, these two peptides have K_{in} values that lie on the edges of the interval $]1.80 \times 10^{-4}; 3.68 \times 10^{-4}]$ ml/(g×min), with PhrANTH2 (1.83×10^{-4} ml/(g×min)) very close to 'very low influx' or class 1 and BIP-2 (3.51×10^{-4} ml/(g×min)) very close to 'median influx' or class 3. The brain influx values of dermorphin and BSA are correctly classified as 'low' and 'very low', respectively.

The brain influx of PhrCACET1 and BIP-2 showed a biphasic behavior: after a steep increase in the ratio of the brain and serum activity, the curves reached a plateau phase. For PhrCACET1, this can be explained by the saturation of the BBB transport mechanisms and/or accumulation into the kidneys and duodenum. For BIP-2, the high liver accumulation will most likely influence the brain influx behavior, together with a saturation of transport mechanisms. Metabolization of the peptides is limited during the experimental time frame and thus no primary cause of steady-state appearance.

BIP-2 is synthesized by the Gram-positive bacterium *Streptococcus pneumoniae*, a commensal of the nasopharynx in the majority of healthy children. Other commensal viridans streptococcal species, such as *Streptococcus mitis*, which are genetically highly related to *Streptococcus pneumoniae*, have commensal nasopharynx and oropharynx properties as well. Next to their commensal behavior, a variety of infectious complications can be assigned to these pathogens, including meningitis, endocarditis, bacteremia and septicemia [37,44]. Based on this bacterial presence, the quorum sensing peptides produced by these genetically related bacteria are expected to be available for transport to the brain, followed by BBB-penetration and subsequently biological activity. PhrCACET1 is produced by *Clostridium acetobutylicum*, a non-pathogenic spore-forming soil bacterium which is also used in the production of acetone, butanol and ethanol by large-scale industrial fermentation [45]. However, due to the genetic homology of *Clostridium acetobutylicum* with other pathogenic

Clostridium species, *i.e. Clostridium botulinum*, *Clostridium perfringens*, *Clostridium difficile* and *Clostridium tetani* [46,47], comparable peptides can probably also be synthesized in the human body by these bacteria. These quorum sensing peptides, when present in the blood, thus can reach the brain tissue by penetrating the BBB barrier and exert there their effects.

The three quorum sensing peptides mainly differ from each other in lipophilicity (principal component 2 in PCA plot) and size/compactness (principal component 1 in PCA plot): lipophilicity is determined by *e.g.* the clogP value, the number of hydrogen bonds (nHBonds) and the polarity of the molecule (TPSA), while size is evaluated by *i.a.* the WHIM size index and the Wiener W index. As BBB permeability generally increases with lipophilicity, *i.e.* PhrANTH2 < BIP-2 < PhrCACET1, this physicochemical descriptor can help in the prediction of BBB transport characteristics of quorum sensing peptides; these findings are thus in accordance with the current understandings in BBB-permeability of peptides [48], although further research is required to deepen these initial 'QSPR' relationships.

When we analyse the clustering results of all Quorumpeps peptides (n = 231, Figure 1) [21,22], and select the peptides with a comparable lipophilicity as PhrCACET1 (*i.e.* the peptide with the highest BBB permeability characteristics), we mainly find quorum sensing peptides that are synthesized by *Bacillus subtilis*, all of them containing an isoprenyl group on a tryptophan residue [49]. These peptides thus can, theoretically based on this *in silico* clustering, cross the blood-brain barrier and exert their biological effect in the brain. *Bacillus subtilis*, originally described as a soil organism, is also found in the human gut [50], so these PhrCACET1 lipophilicity-related quorum sensing peptides can be available for the brain once they have reached the blood circulation.

As our results indicate that certain quorum sensing peptides can pass the blood-brain barrier, future research should explore their effect on the brain tissue. Different studies already reported a possible association between the microbiota composition and different neurological disorders, without any clear explanation for these observations; the study about the role of the quorum sensing peptides in these pathologies is thus interesting. Previously, it was suggested that the predominant presence of *Clostridium spp.* in the human gut could be associated with autism in children, due to toxin production of this overgrown population [51]. As the quorum sensing peptide PhrCACET1 largely crosses the BBB, the neurological effect of this peptide should be investigated as well, since our results could potentially explain the mechanisms by which commensal flora trigger autism. Because the link between autism and gut microbes is still a topic of debate [52], the study towards the effect of quorum sensing peptides on autism disorders is of high interest. Next to autism, recent studies have also indicated that microbiota have dramatic effects on other central nervous dysfunctions as

well. For example, a shift in the gut microbial composition was demonstrated in hepatic encephalopathic patients, indicating the possible involvement of the microbiota in the pathophysiology of this disease [53]. Moreover, the human microbiome also seems to play a role in depression, anxiety and stress. With depression, a decrease in mood disorders was observed with probiotics containing Lactobacilli and Bifidobacteria [19]. The same beneficial effect of these probiotics is observed on anxiety as well; in contrary, infection with *Campylobacter jejuni* elevated anxiety-like behavior [19,54]. An altered gut microbiota composition was also associated with stress: a decreased abundance of *i.a. Bacteroides* and an increased abundance of *i.a. Clostridium* was observed after stress exposure [55]. Immune-mediated neuro-psychiatric disorders may be influenced by the microbiota as well, including multiple sclerosis, neuromyelitis optica and Guillain-Barré syndrome [19]. These recent findings, together with our results of BBB-penetration of quorum sensing peptides, thus excite the research on the influence of these signaling molecules on the development of central nervous system disorders.

5. CONCLUSIONS

We have demonstrated that some quorum sensing peptides (*e.g.* PhrCACET1 and BIP-2) can pass the blood-brain barrier when present in the blood circulation; BIP-2 demonstrated a high liver accumulation as well. Our results reported here, *i.e.* representing the first pharmacokinetic data of quorum sensing peptides, are awaiting for more information becoming available about the human microbiome (http://www.hmpdacc.org/) [56] as well as about the natural occurrence of quorum sensing peptides and their biological effects on mammalian cells, but already indicate a plausible, intriguing biological cross-talk mechanism.

6. REFERENCES

[1] Ryan RP, Dow JM. Diffusible signals and interspecies communication in bacteria. *Microbiology* 2008; **154**: 1845-1858.

[2] Ng WL, Bassler BL. Bacterial quorum-sensing network architectures. *Annu. Rev. Genet.* 2009; 43: 197-222.

[3] Costerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A common cause of persistent infections. *Science* 1999; **284**: 1318-1322.

[4] Kumari A, Pasini P, Daunert S. Detection of bacterial quorum sensing N-acyl homoserine lactones in clinical samples. *Anal. Bioanal. Chem.* 2008; **391**: 1619-1627.

[5] Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG. Pseudomonas aeruginosa quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. *Infect. Immun.* 2002; **70**: 1783-1790.

[6] Gitzinger M, Parsons J, Reski R, Fussenegger M. Functional cross-kingdom conservation of mammalian and moss (Physcomitrella patens) transcription, translation and secretion machineries. *Plant Biotechnol. J.* 2009; **7**: 73-86.

[7] Williams P. Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology* 2007; **153**: 3923-3938.

[8] Kumar S, Kolodkin-Gal I, Engelberg-Kulka H. Novel quorum-sensing peptides mediating interspecies bacterial cell death. *mBio* 2013; **4**: doi 10.1128/mBio.00314-13.

[9] Sifri CD. Quorum sensing: bacteria talk sense. *Clin. Infect. Dis.* 2008; **47**: 1070-1076.

[10] Fujiya M, Musch MW, Nakagawa Y, Hu S, Alverdy J, Kohgo Y, Schneewind O, Jabri B, Chang EB. The *Bacillus subtilis* quorum-sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. *Cell Host Microbe* 2007; **1**: 299-308.

[11] Wynendaele E, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Crosstalk between the microbiome and cancer cells by quorum sensing peptides. *Submitted for publication*.

[12] De Spiegeleer B, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, Wynendaele E. Quorum sensing peptides promote angiogenesis and invasion of breast cancer cells. *Submitted for publication*.

[13] Nassif X, Bourdoulous S, Eugène E, Couraud P-O. How do extracellular pathogens cross the blood-brain barrier? *TRENDS in Microbiol.* 2002; **10**: 227-232.

[14] Banks WA. Characteristics of compounds that cross the blood-brain barrier. *BMC Neurology* 2009; **9**: S3.

[15] Egleton RD, Davis TP. Development of neuropeptide drugs that cross the blood-brain barrier. *NeuroRx* 2005; **2**: 44-53.

[16] Stalmans S, Wynendaele E, Bracke N, Knappe D, Hoffmann R, Peremans K, Polis I, Burvenich C, De Spiegeleer B. Blood-brain barrier transport of short, proline-rich antimicrobial peptides. *Protein & Peptide Letters* 2014; **21**: 399-406.

[17] Kurihara A, Pardridge WM. Imaging brain tumors by targeting peptide radiopharmaceuticals through the blood-brain barrier. *Cancer Res.* 1999; **59**: 6159-6163.

[18] Deeken JF, Löscher W. The blood-brain barrier and cancer: transporters, treatment, and trojan horses. *Clin. Cancer Res.* 2007; **13**: 1663-1674.

[19]   Wang Y, Kasper LH. The role of microbiome in central nervous system disorders. *Brain Behav. Immun.* 2014; **38**: 1-12.

[20]   Kim KS. Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. *Nat. Rev. Neurosci.* 2003; **4**: 376-385.

[21]   Wynendaele E, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke F, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides. *Nucleic Acids Res.* 2013; **41**: D655-D659.

[22]   Wynendaele E, Gevaert B, Stalmans S, Verbeke F, De Spiegeleer B. Exploring the chemical space of quorum sensing peptides. *Submitted for publication*.

[23]   Wynendaele E, Verbeke F, Braet S, D'Hondt M, De Spiegeleer B. Quality evaluation of synthetic quorum sensing peptides used in R&D. *Accepted for publication*.

[24]   Baileys GS. The iodogen method for radiolabeling protein. In *The Protein Protocols Handbook* (Ed: Walker JM), Humana Press, New York City, **1996**, pp. 673-674.

[25]   Wynendaele E, Bracke N, Stalmans S, De Spiegeleer B. Development of peptide and protein based radiopharmaceuticals. *Curr. Pharm. Des.* 2014; **20**: 2250-2267.

[26]   Baileys GS. The chloramine T method for radiolabeling protein. In *The Protein Protocols Handbook* (Ed: Walker JM), Humana Press, New York City, **2002**, pp. 963-965.

[27]   Gjedde A. High- and low-affinity transport of D-glucose from blood to brain. *J. Neurochem.* 1981; **36**: 1463-1471.

[28]   Pan WH, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood-brain barrier. *Neuropharmacology* 1998; **37**: 1553-1561.

[29]   Patlak CS, Blasberg RG, Fenstermacher JD. Graphical evaluation of blood-to-brain transfer constants from multiple-time uptake data. *J. Cereb. Blood Flow Metab.* 1983; **3**: 1-7.

[30]   Gjedde A. Calculation of cerebral glucose phosphorylation from brain uptake of glucose analogs in vivo: a re-examination. *Brain Res.* 1982; **257**: 237-274.

[31]   Van Dorpe S, Adriaens A, Polis I, Peremans K, Van Bocxlaer J, De Spiegeleer B. Analytical characterization and comparison of the blood-brain barrier permeability of eight opioid peptides. *Peptides* 2010; **31**: 1390-1399.

[32]   Triguero D, Buciak J, Pardridge WM. Capillary depletion method for quantification of blood-brain-barrier transport circulating peptides and plasma-proteins. *J. Neurochem.* 1990; **54**: 1882-1888.

[33]   Gutierrez EG, Banks WA, Kastin AJ. Murine tumor-necrosis-factor-alpha is transported from blood to brain in the mouse. *J. Neuroimmunol.* 1993; **47**: 169-176.

[34]   Banks WA, Kastin AJ. Quantifying carrier-mediated transport of peptides from brain to the blood. *Method. Enzymol.* 1989; **168**: 652-660.

[35]   Vergote V, Van Dorpe S, Peremans K, Burvenich C, De Spiegeleer B. In vitro metabolic stability of obestatin: kinetics and identification of cleavage products. *Peptides* 2008; **29**: 1740-1748.

[36]   Knutsen E, Ween O, Havarstein LS. Two separate quorum-sensing systems upregulate transcription of the same ABC transporter in *Streptococcus pneumoniae*. *J. Bacteriol.* 2004; **186**: 3078-3085.

[37]   Henriques-Normark B, Normark S. Commensal pathogens, with a focus on *Streptococcus pneumoniae*, and interactions with the human host. *Exp. Cell Res.* 2010; **316**: 1408-1414.

[38]   Perego M, Brannigan JA. Pentapeptide regulation of aspartyl-phosphate phosphatases. *Peptides* 2001; **22**: 1541-1547.

[39]   Kastin AJ, Akerstrom V. Entry of exendin-4 into brain is rapid but may be limited at high doses. *Int. J. Obes.* 2003; **27**: 313-318.

[40]   Verbeken M, Wynendaele E, Mauchauffée E, Bracke N, Stalmans S, Bojnik E, Benyhe S, Peremans K, Polis I, Burvenich C, Gjedde A, Hernandez J-F, De Spiegeleer B. Blood-brain transfer and antinociception of linear and cyclic N-methyl-guanidine and thiourea-enkephalins. *Peptides* 2014; 63: 10-21.

[41]   Sternini C, Patierno S, Selmer IS, Kirchgessner A. The opioid system in the gastrointestinal tract. *Neurogastroenterol. Motil. Suppl.* 2004; **3**: 3-16.

[42]   Van Dorpe S, Bronselaer A, Nielandt J, Stalmans S, Wynendaele E, Audenaert K, Van De Wiele C, Burvenich C, Peremans K, Hsuchou G, De Tré G, De Spiegeleer B. Brainpeps: the blood-brain barrier peptide database. *Brain Struct. Funct.* 2012; **217**: 687-718.

[43]   Stalmans S, Gevaert B, Wynendaele E, Nielandt J, De Tré G, Peremans K, Burvenich C, De Spiegeleer B. Towards a unified blood-brain barrier influx response for peptides. *Submitted for publication*.

[44]   Mitchell J. *Streptococcus mitis*: walking the line between commensalism and pathogenesis. *Mol. Oral Microbiol.* 2011; **26**: 89-98.

[45]   Dürre P, Hollergschwandner C. Initiation of endospore formation in *Clostridium acetobutylicum*. *Anaerobe* 2004; **10**: 69-74.

[46]   Paredes CJ, Alsaker KV, Papoutsakis ET. A comparative genomic view of clostridial sporulation and physiology. *Nat. Rev. Microbiol.* 2005; **3**: 969-978.

[47]   Brüggemann H, Baumer S, Fricke WF, Wiezer A, Liesegang H, Decker I, Herzberg C, Martinez-Arias R, Merkl R, Henne A, Gottschalk G. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *PNAS* 2003; **100**: 1316-1321.

[48]   Begley DJ. The Blood-brain Barrier: Principles for targeting peptides and drugs to the central nervous system. *J. Pharm. Pharmacol.* 1996; **48**: 136-146.

[49]   Okada M, Yamaguchi H, Sato I, Cho SJ, Dubnau D, Sakagami Y. Structure-activity relationship studies on quorum sensing ComX(RO-E-2) pheromone. *Bioorg. Med. Chem. Lett.* 2009; **17**: 1705-1707.

[50]   Hong HA, Khaneja R, Tam NM, Cazzato A, Tan S, Urdaci M, Brisson A, Gasbarrini A, Barnes I, Cutting SM. *Bacillus subtilis* isolated from the human gastrointestinal tract. *Res. Microbiol.* 2009; **160**: 134-143.

[51]   Finegold SM, *et al.* Gastrointestinal microflora studies in late-onset autism. *Clin. Infect. Dis.* 2002; **35**: S6-S16.

[52]   Gondalia SV, Palombo EA, Knowles SR, Cox SB, Meyer D, Austin DW. Molecular characterization of gastrointestinal microbiota of children with autism (with and without gastrointestinal dysfunction) and their neurotypical siblings. *Autism Res.* 2012; **5**: 419-427.

[53]   Bajaj JS, Ridlon JM, Hylemon PB, Thacker LR, Heuman DM, Smith S, Sikaroodi M, Gillevet PM. Linkage of gut microbiome with cognition in hepatic encephalopathy. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2012; **302**: G168-175.

[54]   Foster JA, McVey Neufeld K-A. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci.* 2013; **36**: 305-312.

[55]   Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav. Immun.* 2011; **25**: 397-407.

[56]   Editorial. Microbiota meet big data. *Nat. Chem. Biol.* 2014; 10: 605.

CHAPTER VIII

IN VITRO CELL MEDIUM AND HUMAN PLASMA STABILITY, CACO-2 PERMEABILITY AND TOXICITY OF QUORUM SENSING PEPTIDES

"Live as if you were to die tomorrow. Learn as if you were to live forever."

Mahatma Gandhi

ABSTRACT

Quorum sensing peptides, *i.e.* signalling molecules synthesized by mainly Gram-positive bacteria, have varying effects, going from intra- and interspecies signalling to host crosstalk properties. Their biological significance for mammals is only beginning to be explored. Therefore, in this study, we investigated their stability in cell medium and their metabolisation kinetics in human plasma, next to their *in vitro* toxicity (*i.e.* cell viability and haemolysis) on healthy cells as well as on tumour cells. Using a Caco-2 model, it was found that some quorum sensing peptides can enter the blood circulation via intestinal absorption. No haemolytic activity after a 1 hour incubation period was demonstrated and remarkably high plasma stability values were observed for some peptides as well. The quorum sensing peptides demonstrated no toxic effects on both healthy and tumour cells using MTT; no *in silico* (tissue and organ) toxicity could be predicted as well. However, tumour-promoting effects cannot be excluded for some of the quorum sensing peptides, adding to the growing body of data that suggest that quorum sensing peptides can influence cancer fate.

CHAPTER VIII

IN VITRO CELL MEDIUM AND HUMAN PLASMA STABILITY, CACO-2 PERMEABILITY AND TOXICITY OF QUORUM SENSING PEPTIDES

Main focus in this chapter:

- To reflect the stability of quorum sensing peptides, in order to support the *in vitro* and *in vivo* significance of the obtained biological functionality results.

- To investigate if quorum sensing peptides, when present in the human gastrointestinal tract, can reach the general circulation.

- To denote the toxicity of quorum sensing peptides on eukaryotic cells.

1. INTRODUCTION

'Quorum sensing' is the process of cell-to-cell communication between bacteria by the use of signalling molecules regulating their gene expression. In contrast to Gram-negative bacteria, Gram-positive bacteria mainly use oligopeptides to activate this quorum sensing process, leading to *i.a.* biofilm formation with *Staphylococcus aureus*, competence induction in *Streptococcus* species (*e.g.* Competence Stimulating Peptide, CSP) and sporulation in *Bacillus subtilis* (*e.g.* Competence and Sporulation Factor, CSF) [1-3]. These Gram-positive bacteria are found throughout the human body, mainly occupying the gastrointestinal tract, skin, mouth, vagina and mammary gland, next to the occasional presence in the blood (bacteremia or septicemia). Although not yet investigated, the presence of quorum sensing peptides in these different tissues is very likely, given the widespread commensal or pathogenic presence of these Gram-positive bacteria in different areas of the human body and the physiological detection of other signalling molecules (*i.e.* acylhomoserine lacton

molecules from Gram-negative bacteria) in sputum, faeces and saliva [4,5]. The effects of the quorum sensing peptides can be diverse, going from intra-species communication to inter-species antimicrobial activity. Moreover, recent investigations from our group have revealed a selective crosstalk phenomenon between these quorum sensing peptides and mammalian cells, more specifically with human colon and breast cancer cells: some of the quorum sensing peptides were found to promote tumour cell invasion and angiogenesis, thereby influencing tumour metastasis [6,7]. These findings open new perspectives for understanding the role of the microbiome on our health as well as for clinical diagnostic and therapeutic applications.

In this study, we investigated the toxicity of the quorum sensing peptides on both healthy and cancerous cells, next to their effect on red blood cell haemolysis once they have reached the blood circulation (*e.g.* after intestinal absorption). Moreover, the stability of the peptides was investigated in cell medium as well as in human plasma, in order to support the *in vitro* and *in vivo* significance of the biological functionality results.

2. MATERIALS AND METHODS

Peptides

Based on the clustering results of the first 231 quorum sensing peptides of the Quorumpeps® database (http://quorumpeps.ugent.be) [8,9], 98 of these peptides were selected and purchased from different suppliers. The peptide purity was determined using UPLC-PDA analyses [10]. An overview of these peptides is given in Table 1; the numbering used in the Quorumpeps database is retained in this table.

Table 1. Peptide information.

Quorumpeps ID	Sequence	Solvent
2	FNTIPSY	Water
5	Ac-CGSLF, thiolacton linkage between C1 and F5	Water + 50% DMSO
7	FNTWPSY	Water
10	ADLPFEF	Water
11	AGTKPQGKPASNLVECVFSLFKKCN	Water
13	AIFILAS	Water + 58% DMSO
14	AITLIFI	Water + 60% DMSO
15	AKDEH	Water
16	AKTVQ	Water
17	ALILTLVS	Water + 60% DMSO
18	ARNQT	Water
19	NNWNN	Water
22	CVGIW, thiolacton linkage between C1 and W5	Water + 50% DMSO
24	CTFTLPGGGGVCTLTSECIC	Water + 1% DMSO
25	CVFSLFKKCN	Water
28	DIRHRINNSIWRDIFLKRK	Water
30	DLRGVPNPWGWIFGR	Water
31	DLRNIFLKIKFKKK	Water
32	DMCNGYF, thiolacton linkage between C3 and F7	Water + 50% DMSO
34	DRVGA	Water
40	DSVCASYF, thiolacton linkage between C4 and F8	Water + 50% DMSO
42	DWRFLNSIRDLIFPKRK	Water
44	EKMIG	Water
45	EMRISRIILDFLFLRKK	Water
46	EMRKSNNNFFHFLRRI	Water
47	EMRLPKILRDFIFPRKK	Water
49	EQLSFTSIGILQLLTIGTRSCWFFYCRY	Water + 17% DMSO

Table 1. Peptide information (continued).

Quorumpeps ID	Sequence	Solvent
50	ERGMT	Water
51	ERNNT	Water
52	ERPVG	Water
53	ESRLPKILLDFLFLRKK	Water
54	ESRLPKIRFDFIFPRKK	Water
55	ESRVSRIILDFLFQRKK	Water
56	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	Water
58	DSRIRMGFDFSKLFGK	Water
62	ESRISDILLDFLFQRKK	Water
71	QNCPNIFGQWM, lacton linkage between S3 and M11	Water + 50% DMSO
75	SINSQIGKATSNLVECVFSLFKKCN	Water
76	SNLVECVFSLFKKCN	Water
81	FNTIPKY	Water
82	NTIPKY	Water
84	FFNTCPSY	Water
85	FNTCPSY	Water
92	FHWWQTSPAHFS	Water
93	FLVMFLSG	Water + 33% DMSO
97	QNSPNIFGQWM, lacton linkage between S3 and M11	Water + 50% DMSO
99	GKAEF	Water
100	GKATSSISKCVFSFFKKC	Water + 33% DMSO
101	GLWEDILYSLNIIKHNNTKGLHHPIQL	Water
102	GLWEDLLYNINRYAHYIT	Water + 33% DMSO
103	GNWNN	Water
105	GSQKGVYASQRSFVPSWFRKIFRN	Water
107	GVNACSSLF, thiolacton linkage between C5 and F9	Water + 50% DMSO
111	GWWEDFLYRFNIIEQKNTKGFYQPIQL	Water + 50% DMSO
121	ILSGAPCIPW	Water

Table 1. Peptide information (continued).

Quorumpeps ID	Sequence	Solvent
123	IRFVT	Water
125	KSSAYSLQMGATAIKQVKKLFKKWGW	Water
132	LFSLVLAG	Water + 33% DMSO
133	LFVVTLVG	Water + 60% DMSO
134	LPFEF	Water + 50% DMSO
135	LPFEH	Water
137	LVTLVFV	Water + 50% DMSO
138	MAGNSSNFIHKIKQIFTHR	Water + 17% DMSO
140	MKAEH	Water
143	MPFEF	Water
146	NEVPFEF	Water
147	NGWNN	Water
148	YSTCDFIM, thiolacton linkage between C4 and M8	Water + 50% DMSO
151	NNGNN	Water
152	NNNWNNN	Water
153	NNWGN	Water
154	NNWNG	Water
155	NWN	Water
156	FNTIP	Water
157	FNTWP	Water
160	QKGMY	Water
162	QRGMI	Water
164	SDLPFEH	Water
165	SDMPFEF	Water
166	SGSLSTFFLLFNRSFTQALGK	Water
174	SGSLSTFFRLFNRSFTQALG	Water
176	SGSLSTFFRLFNRSFTQALGK	Water
177	SGSLSTFFRLFNRSFTQALGKIR	Water
180	SGSLSTFFRLFNRSQTQALGK	Water

Table 1. Peptide information (continued).

Quorumpeps ID	Sequence	Solvent
184	SIFTLVA	Water + 33% DMSO
186	SKDYN	Water
188	SLSTFFRLFNFSFTQALG	Water + 33% DMSO
191	SRKAT	Water
192	SRNAT	Water
193	SRNVT	Water
206	SYPGWSW	Water
207	TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK	Water
208	TNRNYGKPNKDIGTCIWSGFRHC	Water
210	VAVLVLGA	Water + 33% DMSO
212	VPFEF	Water
214	WPFAHWPWQYPR	Water
215	FNTWPKY	Water
218	YNPCSNYL, thiolacton linkage between C4 and L8	Water + 50% DMSO

Cell culture

MCF-7/AZ (human breast adenocarcinoma), HCT-8/E11 (human colorectal adenocarcinoma) and HEK-293 (human embryonic kidney) cells were grown in high-glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% (V/V) foetal bovine serum (FBS), 1% (w/V) L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen/GIBCO, Gent, Belgium) in a humidified atmosphere of 10% CO_2 at 37°C.

Caco-2 cells, originating from a human colorectal carcinoma, were cultured in high-glucose Dulbecco's Modified Eagle's Medium with L-glutamine, supplemented with 10% (V/V) foetal bovine serum (FBS), 1% of nonessential amino acids (100x), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen/GIBCO, Gent, Belgium) in a humidified atmosphere of 10% CO_2 at 37°C.

Caco-2 permeability assay

For transport studies, Caco-2 cells were seeded at a density of 300 000 cells for each Transwell® (Corning Costar, New York, USA) membrane insert filter (0.4 μm pore size, 12 mm filter diameter). Cell culture medium was changed every second day until monolayers were formed (day 21-29); monolayer integrity was evaluated using resistance measurements. Transport experiments were performed in both the apical-to-basolateral and basolateral-to-apical direction in Hanks' balanced salt solution (HBSS), according to Hubatsch *et al.* [11]. Peptide solutions (ID19, ID44 and ID155, each 10 μM in HBSS) were added to the donor compartment and aliquots taken from the receiving solution after 30, 60, 90 and 120 minutes of incubation. Aliquots of the donor solutions after 120 minutes were taken as well for calculation of the mass balance. Atenolol (50 μM) and propranolol (20 μM) were used as the low- and high-permeability control, respectively [12]. Peptide samples were analysed using UPLC-ESI/MS in selected ion monitoring (SIM, monoisotopic mass of the peptides) mode for the peptide analyte, with limits of detection of 0.56 – 0.58 pmol/ml. The system consisted of a Waters Acquity H-Class Bio-samples Flow Through Needle (flow rate set at 0.5 ml/min), Waters Acquity H-Class BioQuaternary Solvent Manager, Waters Acquity H-Class column module (set at 30°C) and Waters Xevo TQ-S, equipped with Waters Empower Pro software version 2 and MassLynx version 4.1 (Waters, Zellik, Belgium). Mixtures of water (0.1% formic acid m/V) and acetonitrile (0.1% formic acid m/V) were used to create appropriate gradients (95% to 80% of water in 3 minutes) and ACQUITY UPLC BEH300 C_{18} column (100 mm × 2.1 mm, 1.7 μm) for analysing the peptide solutions (5 μl injection volume). PepT1 transport (apical-to-basolateral direction) was investigated using the dipeptide LY (Leu-Tyr, 10 μM) substrate in combination with the quorum sensing peptide.

The apparent permeability coefficient (P_{app} in cm/s) was determined from the amount of compound transported per time and was calculated as follows (sink conditions): $P_{app} = (dQ/dt) / (A \times C_0)$, where dQ/dt is the steady-state flux (pmol/s), A is the surface area of the filter (cm^2) and C_0 is the initial concentration of analyte in the donor chamber (pmol/ml).

In vitro cell medium and human plasma stability

In vitro chemical and metabolic stability of the quorum sensing peptides was determined in cell medium and human plasma, respectively, using previously described procedures [13]. In brief, 100 µl of peptide solution (Table 1, 1 µg/µl) was incubated in 400 µl of Krebs-Henseleit buffer pH 7.4 and 500 µl of cell medium or plasma at 37°C while shaking. At predetermined time intervals (*i.e.* 0, 6 and 24 hours for cell medium; 0, 30 and 120 minutes for plasma), aliquots were immediately transferred into microtubes containing 1:10 volume of 1% (V/V) trifluoroacetic acid solution in water. Metabolism was quenched by heating the solution at 95°C for 5 minutes. The samples were centrifuged to precipitate the denatured proteins and the supernatant was quantitatively analysed for the peptide assay using UPLC-PDA or UPLC-ESI/MS. The same system as described for Caco-2 permeability measurements was used, adding the Waters Acquity H-Class Photodiode Array Detector (PDA, quantification at 210 nm) for these analyses as well. Mixtures of water (0.1% FA m/V) and acetonitrile (0.1% FA m/V) were used to create appropriate gradients for separation of peptides and their metabolites [10]. An appropriate placebo solution was similarly prepared. Assuming first-order kinetics, the rate constant k was obtained from $\ln(P_t/P_{t0}) = -kt$, from which the half-life was determined as $t_{1/2} = [\ln(2)]/k$. If a peptide was found to be stable at the end of the experiment, *i.e.* > 90% relative to t_{0min} at 24 hours for cell medium and 120 min for plasma, then the minimal half-life was calculated using the 90% value: $t_{1/2}$ of minimum 157.9 hours for cell medium and minimum 13.2 hours for plasma.

Haemolysis assay

A 10 µM stock solution is prepared for each quorum sensing peptide using water, with or without DMSO for solubility reasons, as described in Table 1. 40 µl of this stock solution is then diluted with 280 µl of 100 mM sodium phosphate buffer, pH 7.4 to obtain the peptide sample. To prepare the red blood cell solution, freshly obtained human blood was centrifuged at 1500 rpm for 15 minutes and the precipitate washed three times using freshly prepared 150 mM NaCl solution. After the final centrifugation step, the precipitate was resuspended into 100 mM sodium phosphate buffer pH 7.4 and mixed by inversion. The red blood cell solution was then diluted 1 to 10 with the same solvent to obtain the diluted red blood cell solution. To examine the haemolytic properties of the quorum

sensing peptides, 320 µl of the peptide sample was mixed with 80 µl of the diluted red blood cell solution to obtain a final volume of 400 µl and a final peptide concentration of 1 µM. Samples were incubated for 1 hour at 37°C while gently shaking. 100 mM sodium phosphate buffer pH 7.4 and 1% (V/V) Triton X-100 were chosen as the negative and positive control solutions, respectively. Blank solution was obtained by mixing 40 µl of water or aqueous DMSO (60%) with 280 µl of 100 mM sodium phosphate buffer pH 7.4 and 80 µl of diluted red blood cell solution. After 1 hour of incubation, all samples were centrifuged at 20 000 g for 5 minutes. The absorbance of the supernatant was measured at 405 nm and corrected for the blank absorbance. The percent haemolysis was then calculated using the following equation: $[(A_{sample} - A_{blank}) / (A_{positive\ control} - A_{blank})] \times 100$

In silico toxicity screening

The geometrical structure of the quorum sensing peptides was drawn using HyperChem 8.0 (Hypercube, Gainesville, FL, USA) as previously described [9]. Next, these peptide structures were loaded into the Derek Nexus 2.0 (Lhasa Limited, Leeds, United Kingdom) software program and the toxicity analysed using its toxicity knowledgebase.

MTT cell viability assay

HEK-293, HCT-8/E11 and MCF-7/AZ cells were suspended at 1×10^5 cells/ml, 2×10^4 cells/ml and 1×10^4 cells/ml, respectively. 90 µl of this cell suspension was transferred to a well of a 96-well plate. After an overnight incubation at 37°C and 10% CO_2, 10 µl of the selected peptide samples (10 µM, 1 µM and 100 nM) was added to the wells. Blank wells only contained cell medium, while positive and negative controls contained 90 µl of cell suspension and 10 µl of DMSO or water, respectively. The cells were again incubated at 37°C and 10% CO_2 for 24 hours. Next, 20 µl of a 12 mM MTT reagent solution was added to each well and the incubation continued for 2 to 4 hours. The cells were periodically viewed under the inverted microscope for presence of intracellular purple precipitates. When the purple precipitate was clearly visible, the cell medium was removed and 100 µl of DMSO added to each well and swirled gently. The 96-well plate with cover was left in the dark for 10 minutes at 37°C. The absorbance of the solutions was measured at 570 nm in a microtiter plate reader. The number of viable cells was then calculated using the following equation: Viable cells (%) = $[(A_{sample} - A_{blank})/(A_{negative\ control} - A_{blank})] \times 100$.

3. RESULTS

Quorum sensing peptides may pass the intestinal barrier

Extracellular Death Factor (EDF) ID19 (NNWNN), which is synthesized by the human gut commensal *Escherichia coli*, was found to be impermeable through the human epithelial enterocytes under our experimental Caco-2 conditions (*i.e.* $P_{app} < 2.9 \times 10^{-9}$ cm/s as LoD = 0.58 nM). However, its tripeptide analogue ID155 (NWN) shows low intestinal permeability kinetics (Figure 1): a permeability coefficient ($P_{app,ab}$) of 1.41×10^{-7} cm/s was calculated for the apical-to-basolateral transport. The efflux ratio ($P_{app,ba}/P_{app,ab}$) of 1.10 is doubled using a PepT1 substrate, *i.e.* dipeptide LY: the $P_{app,ab}$ decreases to 6.94×10^{-8} cm/s, indicative for the active proton-dependent transport mechanism for ID155 peptide absorption. *In vivo - in vitro* correlation studies demonstrated that these *in vitro* P_{app} values correspond with the absorption of about 5-10% in humans (*in vivo*) [11,14].

Quorum sensing peptide ID44 (PhrG, EKMIG), produced by *Bacillus subtilis*, only shows very small blood efflux (basolateral-to-apical) transport properties after 120 minutes (1.37×10^{-8} cm/s), without significant Caco-2 influx kinetics (LoD = 0.56 nM, so $P_{app} < 6.9 \times 10^{-9}$ cm/s).
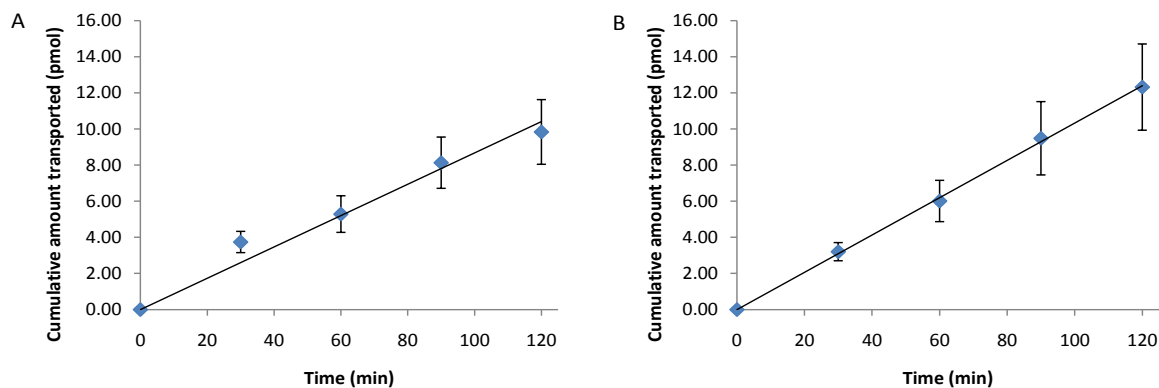
Figure 1. Intestinal permeability of quorum sensing peptide ID155.
(A) Mean (n = 5) apical-to-basolateral transport (*i.e.* intestinal absorption) of the EDF-analogue ID155 (NWN) through a Caco-2 monolayer; (B) mean (n = 6) basolateral-to-apical transport (*i.e.* blood efflux) of the ID155 peptide. Error bars represent SEM values.

Remarkably high cell medium and plasma stability half-life values are observed for some quorum sensing peptides

An overview of the estimated half-life values in cell medium and human plasma is given in Table 2. The half-life values of the quorum sensing peptides in cell medium range from relatively short (*i.e.* less than 36 minutes) to very high (*i.e.* more than 160 hours). The same is true for the human plasma stability with $t_{1/2}$ values ranging from less than 3 minutes to over 13 hours.

Almost 70% of the selected peptides have an *in vitro* plasma half-life value of 1 hour and more, with half of the peptides even having a half-life of more than 2.5 hours. More than 50% of the peptides have stability half-life values of over 24 hours in cell medium, which is the incubation period used during our cell viability assay. Most of the investigated quorum sensing peptides are thus relatively stable in cell medium and human plasma.

Table 2. Plasma and cell medium half-life values of the quorum sensing peptides.

Quorumpeps ID	Plasma half-life (h)	Medium half-life (h)
2	4.4	22.0
7	2.2	5.6
10	9.2	> 160[2]
13	2.2	51.0
16	0.8	22.5
17	2.5[1]	29.8
18	0.3[1]	10.3
19	> 13[1][2]	16.2[1]
28	> 13[1][2]	42.8
30	-[3]	20.8
34	> 13[1][2]	> 160[1][2]
42	> 13[1][2]	52.2
44	> 13[2]	> 160[1][2]
45	-[3]	33.1
46	8.7	45.8
47	0.4	> 160[1][2]
50	> 13[1][2]	> 160[1][2]
51	-[3]	18.3
52	> 13[1][2]	> 160[1][2]
54	8.8	> 160[1][2]
55	9.4	> 160[1][2]
58	1.3	10.9
62	12.7	> 160[1][2]
76	-[3]	25.0[1]
81	0.9	85.8[1]
84	0.5	8.4[1]
85	< 0.05[1][4]	< 0.6[1][4]
92	0.4	10.4
93	-[3]	45.3
99	0.4	62.1
101	-[3]	10.5
102	-[3]	26.9[1]
103	2.1	13.6
105	9.3	> 160[1][2]
111	-[3]	51.1
123	0.4	4.2
132	9.3	13.5[1]
133	9.0	12.7
135	0.2[1]	17.3[1]

Table 2. Plasma and cell medium half-life values of the quorum sensing peptides (continued).

Quorumpeps ID	Plasma half-life (h)	Medium half-life (h)
137	8.0	24.6[1]
138	1.5	36.0
143	0.6	54.6
146	> 13[2]	21.5[1]
147	> 13[1][2]	5.8
151	-[3]	16.2
152	> 13[2]	15.8
153	> 13[1][2]	5.8
154	> 13[2]	8.6
155	4.5	42.0
156	0.9	11.2
157	1.0	19.0
160	0.3[1]	9.9
162	1.5	2.7[1]
164	> 13[1][2]	75.6[1]
165	1.8	129.9
166	-[3]	34.0
174	-[3]	> 160[1][2]
176	-[3]	> 160[2]
180	12.4	> 160[1][2]
184	6.1	11.4[1]
186	0.3 [1]	18.4[1]
188	-[3]	31.0[1]
192	1.2	51.1
193	0.7	34.5
206	0.4	6.9
208	< 0.05[1][4]	4.9[1]
210	3.9	5.5[1]
212	0.2[1]	26.0
215	1.5	20.1

[1] -: the half-life is calculated using 2 time points.

[2] The calculated half-life is 13.2 hours and 157.9 hours for plasma and medium, respectively.

[3] The half-life could not directly be calculated, due to co-elution of plasma or cell medium components in the applied LC-method.

[4] At t = 6 h (cell medium) or t = 30 min (plasma), the peptide was no longer detectable. Under assumption that degradation follows first-order kinetics, at 10 times the $t_{1/2}$ the peptide is considered to be completely degraded. Thus, $t_{1/2}$ is considered to be < 0.6 h in cell medium and < 0.05 h in plasma.

The investigated quorum sensing peptides are not haemolytic

All investigated quorum sensing peptides were found to be non-haemolytic, *i.e.* haemolysis values are below the permissible level of 0.8% according to the Council of Europe guidelines or 1% as per the US FDA guidelines for medical devices (Figure 2) [15]. No significant difference was observed between the water and DMSO blank solutions, indicating that aqueous DMSO, used for dissolution of the peptide, could not be responsible for eventually observed haemolysis of the red blood cells. The negative control solution gave a mean haemolysis value of 0.07%. Peptide ID99 showed the highest percentage of haemolysis, but it is still limited to 0.39% after a 1 hour incubation period. The 3-sigma upper control limit was 0.48%, which is still below the permissible level. The results of this *in vitro* toxicity test on red blood cells, used as a model system [16], thus indicate that the investigated quorum sensing peptides are not toxic against normal eukaryotic cells.
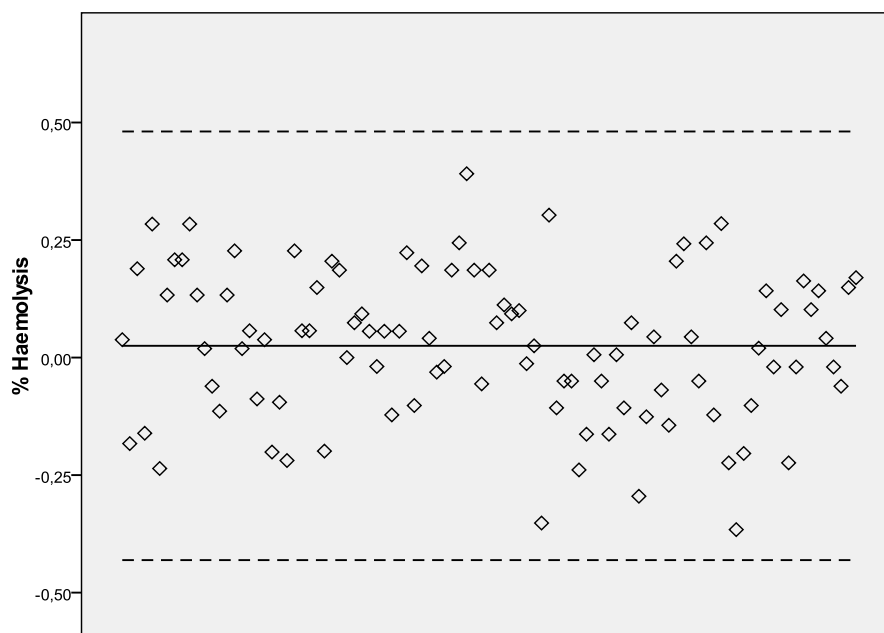
Figure 2. Haemolysis values of the investigated quorum sensing peptides.
All 99 quorum sensing peptides were found to be non-haemolytic at a peptide concentration of 1 µM.
Upper Control Limit (UCL) = 0.48; average = 0.03; Lower Control Limit (LCL) = -0.43.
Control limits: 0.003 probability limits (3-sigma).

In silico tissue and organ toxicity of quorum sensing peptides

The *in silico* toxicity evaluation of the quorum sensing peptides using Derek Nexus 2.0 is given in Table 3. There are no peptides found in the toxicity class '≥ probable', so none of the selected quorum sensing peptides have a level of likelihood of 'probable' for at least one endpoint. The most frequent *in silico* toxicities observed are hepatotoxicity, chromosome damage *in vitro* and skin

sensitisation. Some peptides (36 out of 99) belong to the class 'plausible', having a level of likelihood of 'plausible' for at least one endpoint. This means that most of the selected quorum sensing peptides (63 out of 99) have a level of likelihood of equivocal or lower. The quorum sensing peptides show no *in silico* toxicity in bacterial species (class 'impossible').

No direct cell-killing effect is observed with the quorum sensing peptides

The selected quorum sensing peptides do not show a cell-killing effect on MCF-7/AZ and HCT-8/E11 tumour cells, neither on healthy kidney cells (HEK-293) after 24 hours of treatment (Figure 3). Peptide ID76 (100 nM), in contrast to the other investigated quorum sensing peptides, induced an increased proliferation of HCT-8/E11 cells. The same is true for ID177 (1 µM), ID131 (100 nM), ID10 (100 nM) and ID186 (10 nM) on the HEK-193 cells. These positive outliers indicate that some quorum sensing peptides could promote the proliferation of cells and therefore require further investigations.
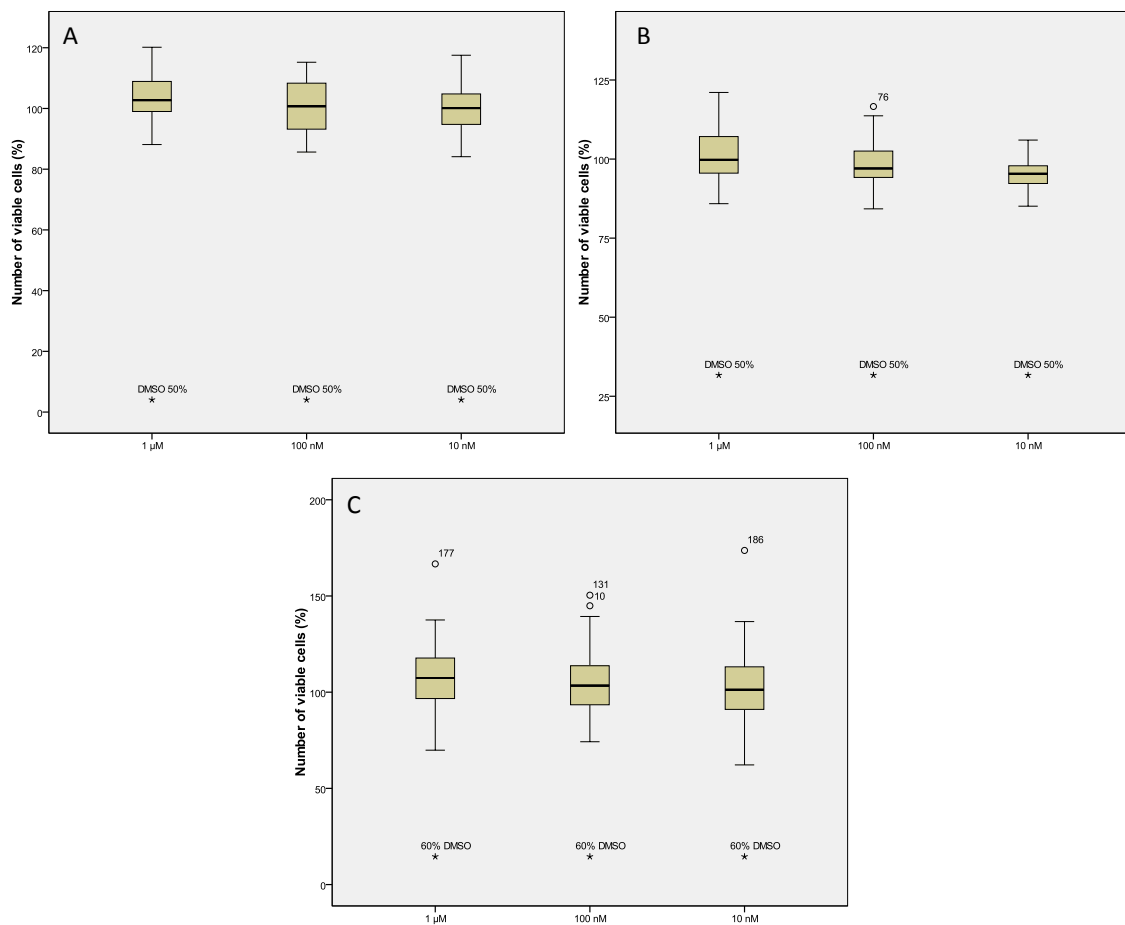

Figure 3. Boxplot of the mean cell viability results after quorum sensing peptide treatment during 24 hours. The investigated quorum sensing peptides do not directly kill the (A) MCF-7/AZ (n = 2), (B) HCT-8/E11 (n = 4) and (C) HEK-293 (n = 2) cells at the three investigated concentrations (*i.e.* 1 µM, 100 nM and 10 nM). DMSO (50% or 60% solution) is used as the positive control.

Table 3. *In silico* toxicity results of the quorum sensing peptides.

Endpoint	Phospholipidosis		hERG channel inhibition *in vitro*		Chromosome damage *in vitro*		Respiratory tract irritation		Skin sensitisation		Hepatotoxicity		RP hepatotoxicity		RP nephrotoxicity		RP chromosome damage		α-2-μ globulin nephropathy		Oestrogenicity		Photo allergenicity	
ID	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B
2					red	green			orange	green	red	green	orange	green	orange	green			blue	green			blue	green
5									orange	green									blue	green			blue	green
7					red	green			orange	green	red	green	orange	green	orange	green			blue	green			blue	green
10									blue	green			orange						blue	green			blue	green
11		green							red	green			orange	green					blue	green		ltgreen		
13									blue	green									blue	green			blue	green
14									blue	green	red	green							blue	green			blue	green
15		green							blue	green									blue	green			blue	green
16									blue	green									blue	green			blue	green
17									blue	green									blue	green			blue	green
18									blue	green									blue	green			blue	green
19									blue	green									blue	green			blue	green
22									red	green									blue	green				
24									blue	green									blue	green	ltgreen	green	blue	green
25		green							orange										blue	green	ltgreen	green	blue	green
28		green							blue	green									blue	green			blue	green
30									blue	green			orange	green					blue	green			blue	green
31		green							blue	green									blue	green			blue	green
32					red	green			orange	green	red	green			orange	green			blue	green			blue	green
34									blue	green									blue	green			blue	green
40					red	green			orange	green	red	green			orange	green			blue	green			blue	green
42									red	green									blue	green			blue	green
44									blue	green									blue	green			blue	green
45		green																	blue	green	ltgreen	green		
46		green									red	green	red	green					blue	green	ltgreen	green		
47		green											orange	green					blue	green	ltgreen			
49						green			red	green	red	green			orange	green			blue	green	ltgreen	green		
50									blue	green									blue	green			blue	green
51									blue	green									blue	green			blue	green
52									blue	green			orange						blue	green			blue	green
53		green											orange						blue	green	ltgreen	green		
54		green											orange						blue	green	ltgreen			
55		green																	blue	green	ltgreen			
56		green				green			red	green	red	green	orange	green	orange	green			blue	green	ltgreen			
58		green																	blue	green	ltgreen			
62		green																	blue	green	ltgreen			
69									blue	green							orange	green	blue	green			blue	green
75		green							red	green									blue					
76		green																	blue	green			blue	green
81					red	green			orange	green	red	green	orange	green	orange	green			blue		blue	green	blue	green

Table 3. *In silico* toxicity results of the quorum sensing peptides (continued).

Endpoint	Phospholipidosis		hERG channel inhibition *in vitro*		Chromosome damage *in vitro*		Respiratory tract irritation		Skin sensitisation		Hepatotoxicity		RP hepatotoxicity		RP nephrotoxicity		RP chromosome damage		α-2-μ globulin nephropathy		Oestrogenicity		Photo allergenicity	
ID	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B
82																								
84																								
85																								
92																								
93																								
97																								
99																								
100																								
101																								
102																								
103																								
105																								
107																								
111																								
121																								
123																								
125																								
131																								
132																								
133																								
134																								
135																								
137																								
138																								
140																								
143																								
146																								
147																								
148																								
151																								
152																								
153																								
154																								
155																								
156																								
157																								
160																								
162																								
164																								
165																								
166																								
174																								

Table 3. *In silico* toxicity results of the quorum sensing peptides (continued).

Endpoint	Phospholipidosis		hERG channel inhibition *in vitro*		Chromosome damage *in vitro*		Respiratory tract irritation		Skin sensitisation		Hepatotoxicity		RP hepatotoxicity		RP nephrotoxicity		RP chromosome damage		α-2-μ globulin nephropathy		Oestrogenicity		Photo allergenicity	
ID	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B
176		green																	blue	green	cream	green		
177					red	green			orange	green	red	green			orange	green			blue	green			blue	green
180		green																	blue	green	cream	green		
184									blue	green									blue	green			blue	green
186		green			red	green			orange	green	red	green			orange	green			blue	green			blue	green
188																					cream	green		
191									blue	green									blue	green			blue	green
192									blue	green									blue	green			blue	green
193									blue	green									blue	green			blue	green
206					red	green			orange	green	red	green	orange	green	orange	green			blue	green			blue	green
207		green									red	green	orange	green					blue	green	cream	green		
208		green				green			red	green	red	green	orange	green	orange	green			blue	green	cream	green		
210									blue	green									blue	green			blue	green
212													orange	green										
214					red	green			orange	green	red	green	orange	green					blue	green	cream	green	blue	green
215		green			red	green			orange	green	red	green	orange	green	orange	green			blue	green			blue	green
218					red	green			orange	green	red	green	orange	green	orange	green			blue	green			blue	green

The level of likelihood, a qualitative indication for the toxicity prediction of a chemical, is given as follows (in descending probability): Plausible [red] ; Equivocal [orange] ; Doubted [blue] ; Improbable [cream] ; Impossible [green] .
Empty cells in the matrix indicate that there is no evidence of toxicity, nor evidence of non-toxicity.
M: mammal; B: bacterium

4. DISCUSSION

The vast majority of bacteria inhabit the human intestine, with as many as 10^{12} bacterial cells per gram of the average human faeces [17]. Quorum sensing peptides produced by these commensal or pathogenic bacteria thus can enter the blood circulation after passing the intestinal mucosa. As indicated by our Caco-2 permeability results, some of these peptides indeed cross the intestinal barrier: quorum sensing peptides originating from intestinal bacteria can thus be found in the blood, after which they can exert their effect at distant sites of the human body. Despite the presence of a PepT1 transporter protein at the apical membrane of enterocytes, responsible for the absorption of di- or tripeptides [18], intestinal permeability is not limited to these small peptide structures: previous studies have indicated that larger peptides and proteins can also cross the normal intestine in an intact form (*e.g.* insulin, β-casein) [19-21]. Moreover, the use of permeability enhancers (*e.g.* bile salts, fatty acids or glycerides) can increase the intestinal absorption of the quorum sensing peptides as well [22]. Quorum sensing peptides can also enter the circulation through the skin or oral mucosa, seen the highly present bacterial population at these sites of the human body [23] and the skin and mouth mucosa permeability-data already described for peptides [24,25]. In general, the oral mucosa is considered intermediately permeable, situated between the epidermis and the intestinal mucosa [26].

Once the quorum sensing peptides have reached the blood circulation, either after intestinal absorption or using alternative absorption routes (*e.g.* skin or mouth mucosa), the quorum sensing peptides are prone to metabolic degradation. A wide distribution of *in vitro* half-life values has been obtained for the quorum sensing peptides in human plasma, with a number of peptides even being stable for 13 hours and more. The stability in cell medium varies largely as well, with some peptides being stable for minimum 160 hours. However, very unstable peptides are also identified in both cell medium and human plasma. The effects observed during *in vitro* cell-based assays thus can be due to degradants arising during the experiment. For the vast majority of the quorum sensing peptides, the *in vitro* stability half-life values are smaller in human plasma than in cell medium, due to the presence of peptidases in the former solution. However, for some of the Extracellular Death Factor (EDF) peptide-analogues (ID147, ID153 and ID154), the opposite is observed: the stability of this defined peptide group is higher in human plasma (*i.e.* $t_{1/2}$ > 13 hours) than in cell medium (*i.e.* $t_{1/2}$ values of around 7 hours).

The quorum sensing peptides (1 μM) were found to be non-haemolytic after a 1 hour incubation period with human red blood cells. Haemolysis is one of the main drawbacks during the development of peptide-based therapeutics, with the antimicrobial, anticancer and cell-penetrating peptides belonging to the main peptide groups with haemolytic characteristics [16,27-30].

The toxicity of the quorum sensing peptides at different sites of the human body was investigated using *in silico* screening methods: none of the selected quorum sensing peptides showed toxicity (*e.g.* hepatotoxicity or chromosome damage) with high probability. These observations are in accordance with other recent predictions on peptide toxicity, where the high abundance of cysteine (C), asparagine (N) and proline (P) amino acids is associated with increased toxicity [31]; as the investigated quorum sensing peptides do not predominantly contain these types of residues (average 19% of the peptide sequence), a low toxicity can thus be assumed. The ToxinPred (specific for peptides) toxicity predictions (http://crdd.osdd.net/raghava/toxinpred/index.html) of the peptides with the highest number of C, N or P amino acids, relative to the total amount of amino acids in the peptide sequence (*e.g.* ID152, ID22 and ID214) confirm the non-toxicity of the quorum sensing peptides [31].

The investigated quorum sensing peptides, which represent a large part of the quorum sensing peptide space [9], do not directly kill healthy kidney cells, cancerous colon cells or breast cancer cells. In contrast to the cell-killing properties, the cell viability assay indicated cell proliferative effects for some quorum sensing peptides on HCT-8/E11 and HEK-293 cells. These initial findings add to our previous evidence implicating the selective role of quorum sensing peptides on tumour cells [6,7].

5. CONCLUSIONS

In conclusion, some quorum sensing peptides, produced by different commensal or pathogenic bacteria, may pass the intestinal barrier, thereby reaching the blood circulation of the host and remain sufficiently stable. There, the peptides showed no haemolytic properties on red blood cells, so no primary toxicity on eukaryotic cells can be assigned to these molecules. Moreover, the peptides demonstrated no direct cell killing effect on other healthy cells or tumour cells as well, next to limited *in silico* probability of organ and tissue toxicity.

6.   REFERENCES

[1]   Heilmann C, Götz F, in *Bacterial Signaling* (Eds: Krämer R, Jung K) Wiley-VCH, Weinheim, **2010**, pp. 7-22.

[2]   Petersen FC, Fimland G, Scheie AA. Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in *Streptococcus mutans*. *Mol. Microbiol.* 2006; **61**: 1322-1334.

[3]   Pottathil M, Jung A, Lazazzera BA. CSF, a species-specific extracellular signaling peptide for communication among strains of *Bacillus subtilis* and *Bacillus mojavensis*. *J. Bacteriol.* 2008; **190**: 4095-4099.

[4]   Kumari A, Pasini P, Daunert S. Detection of bacterial quorum sensing N-acyl homoserine lactones in clinical samples. *Anal. Bioanal. Chem.* 2008; **391**: 1619-1627.

[5]   Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG. *Pseudomonas aeruginosa* Quorum-Sensing Systems May Control Virulence Factor Expression in the Lungs of Patients with Cystic Fibrosis. *Infect. Immun.* 2002; **70**: 1783-1790.

[6]   Wynendaele E, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Crosstalk between the microbiome and cancer cells by quorum sensing peptides. Manuscript submitted.

[7]   De Spiegeleer B, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, Wynendaele E. Quorum sensing peptides promote angiogenesis and invasion of breast cancer cells. Manuscript submitted.

[8]   Wynendaele E, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke F, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides. *Nucleic Acids Res.* 2013; **41**: D655-D659.

[9]   Wynendaele E, Gevaert B, Stalmans S, Verbeke F, De Spiegeleer B. Exploring the chemical space of quorum sensing peptides. Manuscript submitted.

[10]   Wynendaele E, Verbeke F, Braet S, D'Hondt M, De Spiegeleer B. Quality evaluation of synthetic quorum sensing peptides used in R&D. Accepted for publication.

[11]   Hubatsch I, Ragnarsson EGE, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.* 2007; **2**: 2111-2119.

[12]   Cattoor K, Bracke M, Deforce D, De Keukeleire D, Heyerick A. Transport of hop bitter acids across intestinal Caco-2 cell monolayers. *J. Agric. Food Chem.* 2010; **58**: 4132-4140.

[13]   Vergote V, Van Dorpe S, Peremans K, Burvenich C, De Spiegeleer B. In vitro metabolic stability of obestatin: kinetics and identification of cleavage products. *Peptides* 2008; **29**: 1740-1748.

[14]   Yee, S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man – Fact or myth. *Pharm. Res.* 1997; **14**: 763-766.

[15]   FDA summary basis of approval for red blood cells frozen and red blood cells deglycerolized (Reference number 86-0335). US License Number 635-10. Applicant-Department of the Navy, Navy Hospital, Bethesda, MD, 1986.

[16]   Gautam A, Chaudhary K, Singh S, Joshi A, Anand P, Tuknait A, Mathur D, Varshney GC, Raghava GPS. Hemolytik: a database of experimentally determined haemolytic and non-hemolytic peptides. *Nucleic Acids Res.* 2014; **42**: D444-D449.

[17]   Sommer F, Bäckhed F. The gut microbiota – masters of host development and physiology. *Nat. Rev. Microbiol.* **2013**; DOI: 10.1038/nrmicro2974.

[18]   Ziegler T, Fernández-Estívariz C, Gu LH, Bazargan N, Umeakunne K, Wallace TM, Diaz EE, Rosado KE, Pascal RR, Galloway JR, Wilcox JN, Leader LM. Distribution of the H$^+$/peptide transporter PepT1 in human intestine: up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. *Am. J. Clin. Nutr.* 2002; **75**: 922-930.

[19]   Ziv E, Bendayan M. Intestinal absorption of peptides through the enterocytes. *Microsc. Res. Tech.* 2000; **49**: 346-352.

[20]   Regazzo D, Mollé D, Gabai G, Tomé D, Dupont D, Leonil J, Boutrou R. The (193-209) 17-residues peptide of bovine β-casein is transported through Caco-2 monolayer. *Mol. Nutr. Food Res.* 2010; **54**: 1428-1435.

[21]   Antunes F, Andrade F, Ferreira D, Nielsen HM, Sarmento B. Models to predict intestinal absorption of therapeutic peptides and proteins. *Curr. Drug Metabolism.* 2013; **14**: 4-20.

[22]   Aungst BJ, Saitoh H, Burcham DL, Huang S-M, Mousa SA, Hussain MA. Enhancement of the intestinal absorption of peptides and non-peptides. *J. Control. Rel.* 1996; **41**: 19-31.

[23]   Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 2007; **449**: 811-818.

[24]   Taevernier L, Veryser L, Roche N, De Spiegeleer B. Human skin kinetics of cyclic depsipeptide mycotoxins. *J. Pept. Sci.* 2014; **20**: S299-S300.

[25]   Veuillez F, Kalia YN, Jacques Y, Deshusses J, Buri P. Factors and strategies for improving buccal absorption of peptides. *Eur. J. Pharm. Biopharm.* 2001; **51**: 93-109.

[26]   Squier CA, in *Drug targeting and delivery: concepts in dosage form design* (Ed: Junginger HE) Ellis Horwood Limited, England, **1992**, pp. 45-56.

[27]   Saar K, Langel U, in *Handbook of cell-penetrating peptides* (Ed: Langel U) CRC Press, Florida, **2007**, pp. 553-564.

[28]   Zhao J, Zhao C, Liang G, Zhang M, Zheng J. Engineering antimicrobial peptides with improved antimicrobial and hemolytic activities. *J. Chem. Inf. Model.* 2013; **53**: 3280-3296.

[29]   Yang Q.-Z, Wang C, Lang L, Zhou Y, Wang H, Shang D-J. Design of potent, non-toxic anticancer peptides based on the structure of the antimicrobial peptide, temporin-1CEa. *Arch. Pharm. Res.* 2013; **36**: 1302-1310.

[30]   Ruiz J, Calderon J, Rondón-Villarreal P, Torres R, in *Advances in Computational Biology* (Eds: Castillo LF, Cristancho M, Isaza G, Pinzón A, Rodríguez JMC) Springer, Londen, **2013**, pp. 253-257.

[31]   Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R, Open Source Drug Discovery Consortium, Raghava GPS. In silico approach for predicting toxicity of peptides and proteins. *Plos One* **2013**; DOI: 10.1371/journal.pone.0073957.

CHAPTER IX

DEVELOPMENT OF PEPTIDE AND PROTEIN BASED RADIO-PHARMACEUTICALS

"Friendship improves happiness, and abates misery,
by doubling our joys, and dividing our grief"

Marcus Tullius Cicero
(°106 B.C. - †43 B.C., Roman Statesman)

ABSTRACT

Radiolabelled peptides and proteins have recently gained great interest as theranostics, due to their numerous and considerable advantages over small (organic) molecules. Developmental procedures of these radiolabelled biomolecules start with the radiolabelling process, greatly defined by the amino acid composition of the molecule and the radionuclide used. Depending on the radionuclide selection, radiolabelling starting materials are whether or not essential for efficient radiolabelling, resulting in direct or indirect radioiodination, radiometal-chelate coupling, indirect radiofluorination or ^{3}H/^{14}C-labelling. Before preclinical investigations are performed, quality control analyses of the synthesized radiopharmaceutical are recommended to eliminate false positive or negative functionality results, *e.g.* changed receptor binding properties due to (radiolabelled) impurities. Therefore, radionuclidic, radiochemical and chemical purity are investigated, next to the general peptide attributes as described in the European and the United States Pharmacopeia. Moreover, *in vitro* and *in vivo* stability characteristics of the peptides and proteins also need to be explored, seen their strong sensitivity to proteinases and peptidases, together with radiolysis and trans-chelation phenomena of the radiopharmaceuticals. *In vitro* biomedical characterization of the radiolabelled peptides and proteins is performed by saturation, kinetic and competition binding assays, analysing K_D, B_{max}, k_{on}, k_{off} and internalization properties, taking into account the chemical and metabolic stability and adsorption events inherent to peptides and proteins. *In vivo* biodistribution can be adapted by linker, chelate or radionuclide modifications, minimizing normal tissue (*e.g.* kidney and liver) radiation, and resulting in favorable dosimetry analyses. Finally, clinical trials are initiated, eventually leading to the marketing of radiolabelled peptides and proteins for PET/SPECT-imaging and therapy of different clinical diseases.

Chapter IX

DEVELOPMENT OF PEPTIDE AND PROTEIN

BASED RADIOPHARMACEUTICALS

Main focus in this chapter:

- To give an overview of the techniques used for the radiolabelling of peptides and proteins.

- To summarize the different quality measures for peptide and protein radiopharmaceuticals and to indicate their importance to enhance the success rate of these theranostics.

- To review the available *in vitro* biomedical and *in vivo* pharmacokinetic assays.

1. INTRODUCTION

In the past decades, the use of radiolabelled peptides in research, diagnosis and/or therapy has increased enormously. In contrast to most small-molecule drugs, peptides demonstrate high affinity, strong selectivity and low toxicity. They can be easily produced and synthetically modified in order to optimize their affinity for a particular receptor and to display a more specific biodistribution pattern. Moreover, due to their small size, peptides show rapid tissue penetration, together with favourable pharmacokinetic properties, *i.e.* fast clearance from the blood and non-target tissues [1-3]. The main drawback of the use of peptide-based compounds is their low stability to peptidases and proteases found in most tissues [3]. However, metabolic stability can be increased by substitution of unnatural amino acids or D-isomers, amidation or acetylation of peptide termini or cyclisation, hereby increasing the probability of obtaining useful drugs, structurally related to the parent peptides [4,5]. Since the Food and Drug Administration (FDA) approved the use of insulin as a drug in 1982, (radiolabelled) protein therapeutics have found their way in therapy and diagnostics [6]. The main advantage of the protein scaffold over small molecules is that the contact interface of protein-protein interactions is large (1500-3000 $Å^2$), compared to the contact surface of protein-small molecule interactions (300-1000 $Å^2$). In addition, the contact surfaces of proteins that interact with other proteins are generally flat and often lack the grooves and pockets present at the surface that bind to small molecules [7]. Monoclonal antibodies for example show high affinity towards specific cell surface molecules, enabling high signal delivery to these targets. However, a major disadvantage of using intact

antibodies is that they generally have a long elimination half-life and usually circulate in the blood for several days, due to their large size. However, preliminary clinical studies with antibody fragments show promising pharmacokinetic results, encouraging the future use of radiolabelled antibody fragments [8]. Recent investigations of radiolabelled nanobodies, the smallest possible functional immunoglobulin-like antigen-binding fragments, have proven their potential use in *e.g.* mouse tumour models and for cardiovascular applications [9-12]. Pharmacokinetic and tumour targeting improvements are achieved through non-covalent interaction with albumin [13]. Recent studies indicate for example the potential use of ^{177}Lu-labelled anti-HER2 nanobodies in HER2-overexpressing tumour cells, showing high specific tumour uptake combined with low background uptake [14].

Currently, different radiolabelled peptides are marketed or investigated in (pre-)clinical trials for their use in *i.a.* oncology (*e.g.* ^{111}In-DTPA-Octreotide), inflammation and infection (*e.g.* ^{99m}Tc-RP128), thrombus imaging (*e.g.* ^{99m}Tc-Apcitide), atherosclerosis (*e.g.* ^{18}F-Endothelin-1), diabetes mellitus (^{18}F-labelled C-peptide from insulin) and Parkinson's (*e.g.* ^{18}F-DOPA) and Alzheimer's diseases (*e.g.* ^{125}I-amyloid β(1-40)). These peptides can be used as radiotherapeutic drugs or as diagnostics to evaluate the presence or progression of a disease. Currently, many tumour types show overexpression of all kinds of peptide-binding receptors, while some inflammatory diseases also demonstrate upregulation. Therefore, current research mainly focuses on the development of radiolabelled peptides and proteins in both these therapeutic areas [5,15-17].

To quantitatively detect the radiotracers, different molecular imaging modalities for preclinical research and clinical settings are available with different spatial resolutions and selectivity, of which positron emission tomography (PET) [18] and single photon emission computed tomography (SPECT) are commonly used. PET uses positron-emitting isotope (*e.g.* [18]F, ^{64}Cu, ^{68}Ga) labelled molecules, which produce two gamma-rays (about 180° apart) that are crucial for the PET coincidence detection and reconstruction of the PET images. Gamma-emitting isotopes (*e.g.* [99m]Tc, ^{123}I, ^{111}In) are used for SPECT imaging; here, the gamma-cameras are rotating around the patient and collimation of the gamma-rays is essential [19,20]. Dual-isotope imaging of different SPECT-radiotracers (*e.g.* [99m]Tc and ^{123}I) can be performed as well, yielding improved performance and reduced acquisition time, hereby reducing the phenomenon of cross-talk between the emitted gamma-ray photons [21,22].

Finally, radiolabelled peptides and proteins have to undergo several steps to confirm their promising use as theranostics. Therefore, after efficient and stable radiolabelling of the peptide or protein, *in vitro* characterization is necessary, determining receptor binding affinity, internalization and cell dissociation properties. Next, the pre-clinical *in vivo* characteristics are defined, evaluating animal biodistribution and tissue (*e.g.* tumour) targeting qualities. Potential molecules are then further evaluated during clinical studies in humans, determining safety, efficacy, dose and pharmacokinetics [23-25].

2. BASIC BUILDING BLOCKS

Amino acids for peptides and proteins

Twenty natural amino acids form the building blocks for peptides and proteins and their composition plays a key role in their radiolabelling opportunities and receptor binding characteristics. The diversity of the amino acids is distinguished by their different side chains R, dictating their unique physico-chemical properties. Table 1 shows the different classes of amino acids as well as their chemical structure, grouped by their dominant chemical property. Although many strategies have been proposed to divide the amino acids into classes, none has been fully satisfactory. By placing a set of amino acids in a defined order, called the primary sequence, the blueprint for its three-dimensional structure and function is created (*cfr.* Ramachandran plot [26]). In general, the Ramachandran plot shows which conformations are sterically allowed for each residue and a single point defines a certain secondary structure (*e.g.* α helix or β sheet). However, the more bulky the side chains are, the smaller the allowed region is. For example, glycine with its -H side chain allows more conformations than arginine, which can extend more than about 7 Å from the C_α atom. All specific structural features of peptides and proteins (on secondary, tertiary and/or quaternary level) are the result of physical forces acting on a polypeptide chain of a defined sequence. Three products of these forces are: (1) Hydrophobic groups tend to cluster together and become excluded from water by burial in the protein interior (*e.g.* π-π interactions between aromatic residues); (2) Polar atoms which become buried nearly always form hydrogen bonds or salt bridges with other buried polar atoms (*e.g.* salt bridge between polar charged residues); (3) The backbone and side chain conformations that are adopted, tend to be those which are favourable for isolated residues.

Some residues in peptides or proteins have essential structural or functional roles. When they are modified, the three-dimensional structure is changed or lost accompanied by a loss or gain of function, *e.g.* the peptide or protein is unable to bind to an interaction partner and perform its function. To demonstrate the importance of the amino acid composition and sequence in ligand-receptor interactions, Liapakis *et al.* reported the critical role of phenylalanine at position 2 of somatostatin hexapeptides for binding to the mutant somatostatine receptor subtype 1 (SSTR1$_{S305F}$) [27]. When this position was substituted by a tyrosine, binding to the SSTR1$_{S305F}$ receptor was hindered. The cannabinoid receptor on the other hand shows that an N-terminal extension of its peptide ligand changed the functionality from antagonist to agonist [28]. Also on protein level there have been different success stories reported based on these principles. Pegvisomant (Somavert) for example is a growth hormone receptor antagonist used in the treatment of acromegaly. By adding nine substitutions in the receptor binding site on human growth hormone, growth hormone receptor

dimerization was hindered and an antagonist was created [29]. In addition, these mutations had an additive effect on the binding affinity of this growth hormone analogue for site I on growth hormone receptor. However, not only substitutions can influence the structure and functionality of peptides and proteins, also residue modifications have been reported to (directly) interfere with the biological activity. For instance, ^{125}I-labelling of the angiotensin-(1-7) peptide revealed that the monoiodinated form had a biological activity identical to the native compound, whereas this was lost in the di-iodinated analogue [30]. Labelling of annexin V via amine-directed bifunctional agents, used for the detection of cell death, showed decreased biological activity [31]. Next, the reaction conditions used to introduce the label may promote some undesirable changes as well, such as oxidation, deamidation, side-chain isomerisation or aggregation [32].

Therefore, it is important to rationally choose a radiolabelling method for the subject peptide or protein (Table 1). Direct radio-iodination is possible through electrophilic aromatic substitution of an aromatic proton by radioiodine (I^+-form) at tyrosine and histidine moieties [33,34] while direct ^{99m}Tc-labelling is achieved by the reactive sulphide (thiol) groups of cysteine residues [5]. The amino groups of lysine and arginine are accessible for bifunctional chelating agent (BFCA) coupling and therefore also for radiolabelling. Fluorination of peptides is performed using ^{18}F-labelled prosthetic groups through fluoroalkylation, fluoroacylation and fluoroamidation on amino, thiol, hydroxyl or carboxyl groups [33].

Table 1. Natural amino acids, symbols, structural formula and properties.

Name, three-letter symbol and IUPAC one letter symbol	Structural formula side chain	$pK_1^{(a)}$ α-COOH	$pK_2^{(a)}$ α-NH$_3^+$	$pK_R^{(a)}$ side chain	pI$^{(b)}$	Residue Mass $^{(c)}$	Labelling properties of side chain
Amino acids with non-polar side chains							
Glycine Gly G		2.35	9.78	-	5.78	57.00	-
Alanine Ala A		2.35	9.87	-	5.98	71.1	-
Valine Val V		2.29	9.74	-	6.15	99.1	-
Leucine Leu L		2.33	9.74	-	6.14	113.2	-
Isoleucine Ile I		2.32	9.76	-	6.19	113.2	-
Methionine Met M		2.13	9.28	-	6.01	131.2	-
Proline Pro P		1.95	10.64	-	7.11	97.1	-
Phenylalanine Phe F (Aromatic)		2.20	9.31	-	5.96	147.2	-
Tryptophan Trp W (Aromatic)		2.46	9.41	-	597	186.2	-

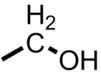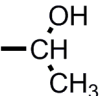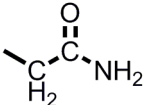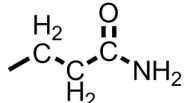**Table 1.** Natural amino acids, symbols, structural formula and properties (continued).
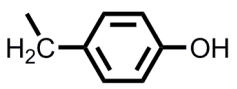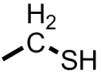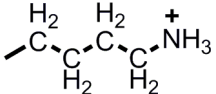
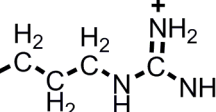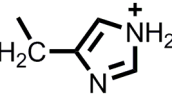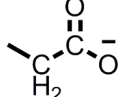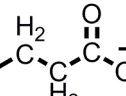| Name, three-letter symbol and IUPAC one letter symbol	Structural formula	$pK_1^{(a)}$ α-COOH	$pK_2^{(a)}$ α-NH$_3^+$	$pK_R^{(a)}$ side chain	pI[b]	Residue Mass [c]	Labelling properties of side chain
Amino acids with polar side chains (uncharged)							
Serine Ser S		2.19	9.21	-	5.69	87.1	Fluorination using prosthetic group on hydroxyl group
Threonine Thr T		2.09	9.10	-	5.60	101.1	Fluorination using prosthetic group on hydroxyl group
Asparagine Asn N		2.14	8.72	-	5.21	114.1	-
Glutamine Gln Q		2.17	9.13	-	5.73	128.1	-
Tyrosine Tyr Y (Aromatic)		2.20	9.21	10.46	5.51	163.2	Direct iodination at the ortho-position of the hydroxyl group; Fluorination using prosthetic group on hydroxyl group
Cysteine Cys C		1.92	10.70	8.37	5.67	103.1	Direct ^{99m}Tc-labelling via thiol group; Radiolabelling using prosthetic group on thiol group

Table 1. Natural amino acids, symbols, structural formula and properties (continued).

Name, three-letter symbol and IUPAC one letter symbol	Structural formula	$pK_1^{(a)}$ α-COOH	$pK_2^{(a)}$ α-NH$_3^+$	$pK_R^{(a)}$ side chain	$pI^{(b)}$	Residue Mass $^{(c)}$	Labelling properties of side chain
Amino acids with polar side chains (charged)							
Lysine Lys K		2.16	9.06	10.54	9.82	128.2	Radionuclide labelling via BFCA-coupling to amino group; Fluorination or iodination using prosthetic group on amino group
Arginine Arg R		1.82	8.99	12.48	10.77	156.2	Radionuclide labelling via BFCA-coupling to amino group; Fluorination using prosthetic group on amino group
Histidine His H		1.80	9.33	6.04	7.69	137.1	Direct iodination at the positions 2 and 5 of the imidazole-ring
Aspartic acid Asp D		1.99	9.90	3.90	3.42	115.1	Fluorination using prosthetic group on carboxyl group
Glutamic acid Glu E		2.10	9.47	4.07	2.80	129.1	Fluorination using prosthetic group on carboxyl group

$^{(a)}$ Data from Voet D, Voet JG, Pratt CW, Fundamentals of Biochemistry (2nd edition), pp76-93, John Wiley & sons, Inc. (2006) [35]

$^{(b)}$ Determined using ChemSketch 11.02 Software (ADCLabs, Frankfurt, Germany)

$^{(c)}$ Residue masses are given for the neutral residues. For the masses of the parental amino acids, the mass of water needs to be added (18).

BFCA: Bifunctional chelating agent

In addition to the twenty natural amino acids, unnatural amino acids are often incorporated in peptide sequences to improve the metabolic stability and/or the receptor binding properties, as well as to optimize the pharmacodynamics and bioavailability. A small selection of the wide variety of unnatural amino acids is given in Figure 1. Ligands in the D-amino acid configuration, which are naturally not incorporated by organisms into proteins, maintain their binding characteristics to their target protein of the natural handedness, while avoiding degradation by enzymes that act upon natural L-peptides [36]. For example, Octreotide (Sandostatin) is a synthetic somatostatin octapeptide analogue engineered with a tryptophan residue in the D-configuration that has a 50 times longer half-life than somatostatin [37,38]. The radioactive compound ^{111}In-DTPA-Octreotide (OctreoScan) has already been registered. The β-amino acids, which have the amino function bonded to the β-carbon atom, can introduce main-chain modifications when incorporated into peptides. Additionally, they are able to form stable and well-defined structures (*e.g.* helices [39]) and are stable against common peptidases [40]. [Dmt]DALDA is a synthetic peptide containing a dimethyltyrosine instead of the natural tyrosine that is responsible for a higher selectivity for the μ-opioid receptor [41]. Other examples from the biomedical research fields are amino acids containing immunogenic groups (*p*-nitrophenylalanine [pNO$_2$pa]) [42], chemical reactive groups (*p*-boronophenylalanine [pBO$_2$pa]) [43] and photocrosslinkers (*p*-azidophenylalanine [pAzpa]) [44].
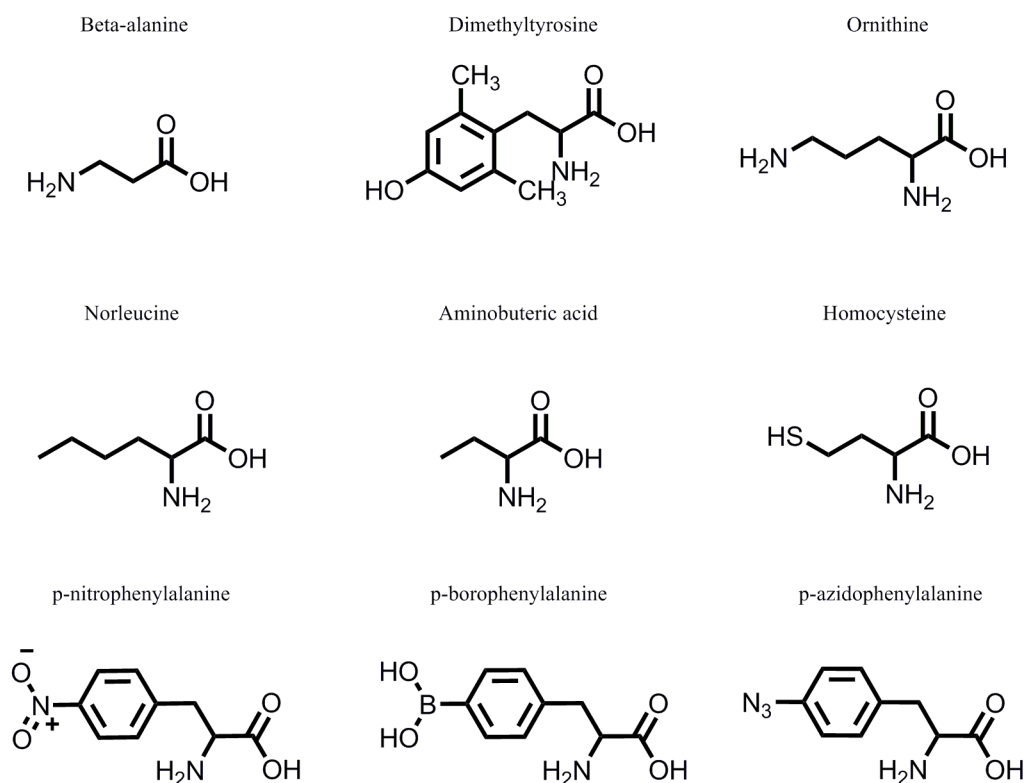

Figure 1. Selection of common unnatural amino acids.

Radionuclides

Radionuclides are typically classified by their physical characteristics: decay mode, half-life and energy transfer, which determine their application possibilities (Table 2). [111]In emits *i.a.* high linear energy transfer particles, *i.e.* Auger electrons, which are able to deliver high doses within a very short range (<10 μm) and thus can be used for radiotherapy. β[-]-emitters (*e.g.* [90]Y) emit energetic negatively charged β-particles, electrons, from the nucleus and are used for therapeutic purposes; β[+]-emitters (*e.g.* [18]F) use positively charged particles, named positrons and are used for PET imaging. γ-emitters (*e.g.* [99m]Tc) use electromagnetic radiation of high energy (photons), which are detected by a gamma camera in SPECT imaging. Finally, also α-particles ([4]He) can be emitted by α-emitting radionuclides (*e.g.* [213]Bi), offering tumour cell killing properties with minimal collateral damage to surrounding healthy tissue [24,45-50]. The physical half-life and radiation energy of the radionuclide is ideally related to the wanted *in vivo* pharmacokinetics of the radiopharmaceutical, *i.e.* tumour retention time and clearance [51].

Most radionuclides, used in nuclear medicine as diagnostics or therapeutics, are artificially produced by cyclotrons (charged particle bombardment), nuclear reactors (neutron bombardment) or radionuclide generators [52]. Radionuclide generators use a long-lived parent radionuclide (*e.g.* [99]Mo) which decays to a daughter radionuclide (*e.g.* [99m]Tc); the daughter isotope is then separated from the parent radionuclide by a chemical or physical process due to different characteristics (*e.g.* solubility) [38,47,53,54].

Table 2. Some radionuclides used in radiopharmaceutical tracers.

Radionuclide	Half-life	Decay mode	Energy (keV)	Application
^{99m}Tc	6.0 hours	Auger γ	15 (2%) 141 (89%)	Imaging
^{64}Cu	12.9 hours	β^+ β^-	653 (18%) 579 (39%)	Imaging and therapy
^{67}Cu	2.5 days	γ β^-	91 (7%), 93 (16%), 185 (49%) 392 (57%), 484 (22%), 577 (20%)	Therapy
^{123}I	13.3 hours	γ	529 (1.4%), 159 (84%)	Imaging
^{131}I	8.0 days	β^- γ	606 (90%), 334 (7%) 364 (82%), 284 (6%), 637 (7%), 80 (3%)	Therapy
^{111}In	2.8 days	Auger γ	19 (16%) 171 (90%), 245 (94%)	Imaging and therapy
^{67}Ga	3.3 days	γ	300 (17%), 185 (21%), 93 (39%)	Imaging
^{68}Ga	68 minutes	β^+ γ	2921 (9%), 1899 (88%) 1077 (3%)	Imaging
^{18}F	109.8 minutes	β^+	1655 (3%), 634 (97%)	Imaging
^{90}Y	2.7 days	β^-	2280 (100%)	Therapy
^{86}Y	14.7 hours	β^+ γ	2242 (13%), 1988 (4%), 1595 (5%), 1545 (6%), 1474 (9%), 1409 (14%), 1314 (7%), 1221 (12%) 1153 (31%), 1077 (83%), 627 (33%)	Imaging
^{177}Lu	6.7 days	γ β^-	113 (6%), 208 (11%) 498 (79%), 385 (9%), 177 (12%)	Imaging and therapy
^{125}I	60.1 days	γ	35 (7%)	Research
^{3}H	12.3 years	β^-	19 (100%)	Research
^{14}C	5730 years	β^-	156 (100%)	Research
^{213}Bi	45.6 minutes	α β^-	5870 (94%), 5549 (7%) 1427 (65%)	Therapy

Radiolabelling starting materials

Peptides and proteins can be directly **radioiodinated** on tyrosine and histidine amino acids. Indirect radioiodination can be achieved using the Bolton-Hunter reagent (*N*-succinimidyl 3-(4-hydroxyphenyl) propionate): this modifying group is incorporated via amide bond formation with a free amino group (*e.g.* lysine ε-amino functions and N-terminal α-amino groups). This technique is frequently used when direct I-labelling on tyrosine or histidine moieties is impossible, due to the absence of these necessary amino acids or when a modification diminishes receptor binding or bioactivity [55,56]. However, due to rapid *in vivo* dehalogenation of phenolic aromatic compounds, non-phenolic aromatic compounds have been developed to overcome this stability problem, *e.g. N*-succinimidyl-3-iodobenzoate (SIB) and *N*-succinimidyl-5-iodo-3-pyridinecarboxylate (SIPC). These radioiodinated prosthetic groups are again conjugated via a covalent bond to a free amino group of the peptide [57,58].

Radiometal complexation is the most frequently applied radiolabelling technique, using macrocyclic or acyclic chelators (Bifunctional Chelating Agent, BFCA, Figure 2), with different characteristics: charge, cavity size, denticity, chemical character of the donor binding groups and formation or dissociation rate of the complex [59]. **^{99m}Tc-labelling** of biomolecules is mostly achieved through a $[Tc=O]^{3+}$ core, forming square pyramidal Tc(V)-oxo complexes with tetradentate chelators, *e.g.* N_2S_2 diamidedithiols (DADS), N_3S triamidethiols, N_2S_2 monoamidemonoaminedithiols (MAMA) and N_2S_2 diaminedithiols (DADT). Besides, also $[Tc\equiv N]^{2+}$ or $[Tc(CO)_3]^{+}$ cores can be used to label pharmaceutical peptides or proteins. The use of 6-hydrazinonicotinamide (HYNIC) for ^{99m}Tc-labelling of biomolecules is also frequently reported. However, since HYNIC can only occupy one or two coordination sites, a coligand (*e.g.* tricine) is often needed to complete the coordination sphere of ^{99m}Tc [60]. Next, gallium (^{68}Ga), indium (^{111}In) or copper (^{64}Cu) labelling can be performed using the macrocyclic and acyclic chelating agents diethylenetriaminepentaacetic acid (DTPA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) [61]. Due to the high complex constants, dissociation of the radiometal from the complex is unfavourable, minimizing the accumulation of *e.g.* free ^{68}Ga and ^{111}In in liver and lungs caused by transferrin binding: formation constants (log K) of *e.g.* Ga^{3+} with transferrin equals 20.3, whereas with DOTA or NOTA, log K = 21.33 and 30.98, respectively [60,62-65]. However, it was shown that ^{64}Cu chelators such as ^{64}Cu-DOTA had disappointing kinetic stability due to dissociation and transchelation to other proteins. This led to the development of NOTA chelators which form five and six coordinate copper complexes with improved tumour specific uptake of *e.g.* bombesin analogues [66]. Moreover, ^{64}Cu-labelling of the anti-CD20 antibody rituximab with NOTA showed significant advantages over *e.g.*

DOTA and DTPA coupling, as radiolabelling could be performed rapidly at room temperature, under dilute conditions, resulting in high specific activity [67].

The coupling of large bulky BFCA's to the small peptides often significantly influences structure and therefore, also receptor binding properties, together with changed pharmacokinetic characteristics [3,5]. For that reason, linker molecules can be placed between the biomolecule and chelating agent, *e.g.* polyethylene glycol (PEG), hydrocarbon chain or hydrophilic or lipophilic amino acid sequence [57,60,68]. PEGylation of the radiopharmaceutical can improve pharmacokinetics and distribution patterns by increasing the molecular mass of proteins and peptides and shielding them from proteolytic enzymes [69,70].

Peptides or proteins cannot be **[18]F-labelled** by direct labelling procedures, but need the use of a prosthetic group. The most suitable group for fluorination is the *N*-succinimidyl-4-[18]fluorobenzoate ([^{18}F]SFB) molecule because of favourable *in vivo* stability characteristics and labelling yield [57].
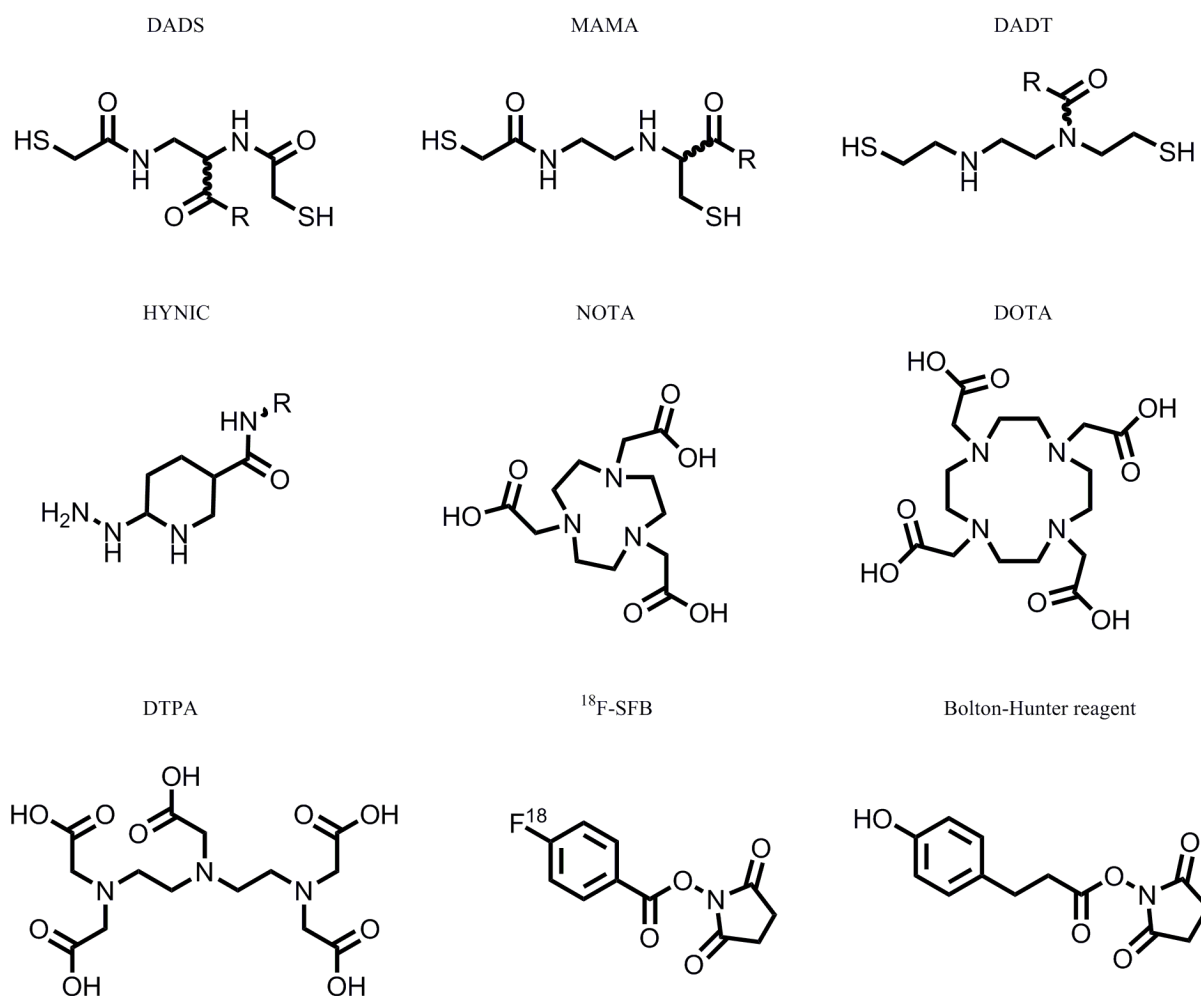

Figure 2. Bifunctional Chelating Agents (BFCA's) differing in chemical and physical properties (R: biomolecule).

3. PREPARATION OF RADIOTRACERS

Iodination

Direct iodination. Peptide radioiodination is a technique commonly used for *in vitro* radioligand binding studies as well as for medical imaging and therapy. Several iodination methods exist, mainly differing in the nature of oxidizing agent for converting I$^-$ into the reactive species, like I^2 or I$^+$ [71-73].

(1) Iodogen method: This iodination procedure was described by Salacinski *et al.* and uses iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril) (Figure 3) as a solid-phase oxidizing agent: the iodogen solution forms a film at the bottom of a polypropylene or glass vial, so reaction can easily be stopped by removing the reaction solution from the vial [74,75].

(2) Chloramine-T: The chloramine-T method, developed by Hunter and Greenwood in 1962 [76], is commonly used for protein and peptide radioiodination. However, Chloramine-T (Figure 3) is a powerful oxidizing agent with high chlorine potential, damaging certain peptides and proteins containing readily oxidizable groups, like thiols and thioethers. Oxidation reactions are then stopped by the addition of excess reductant (*e.g.* sodium metabisulphite) [72,77].

(3) Lactoperoxidase: The procedure, using the enzyme lactoperoxidase and the substrate hydrogen peroxide to iodinate proteins or peptides, is described by Marchalonis. Reaction is again stopped by adding an excess of reductant to the reaction mixture (*e.g.* 2-mercaptoethanol) [78,79].

(4) Iodo-Beads: This technique uses a chemical oxidizing reagent, *N*-chloro-benzenesulfonamide (Figure 3), which is covalently coupled to non-porous polystyrene spheres. Again, reaction is stopped by removing the reaction mixture from the beads in the reaction tube [80].

Comparing the different iodination techniques revealed that the Iodo-Beads method was inferior in terms of peptide recovery and formation of mono- and di-iodinated peptides. For iodination of peptides or proteins that are not sensitive to oxidation reactions, the chloramine-T technique was recommended based on its efficiency, simplicity and cost; peptides and proteins carrying oxidation-susceptible amino acids are preferably iodinated using the Iodogen method, due to the high iodination yield and peptide recovery [71,73,74].
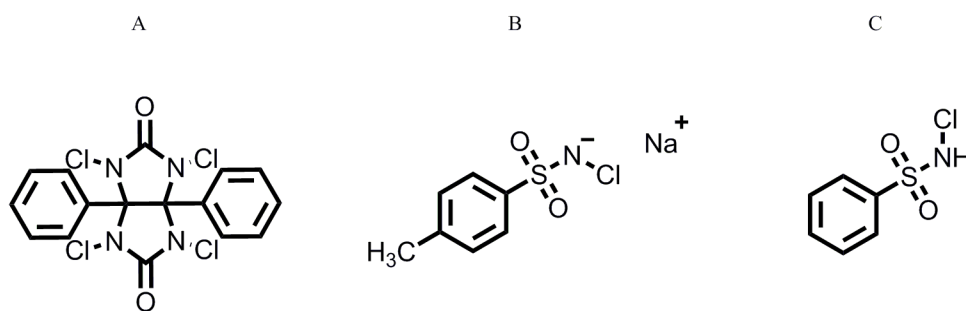
Figure 3. Oxidation reagens used in iodination reactions.

A: Iodogen; B: Chloramine-T; C: *N*-chloro-benzenesulfonamide

Indirect iodination. The Bolton-Hunter reagent, SIB or SIPC can be applied for indirect iodination of peptides or proteins, through acylation of a free amino group. The *N*-hydroxysuccinimide ester can be iodinated before or after covalent conjugation to the protein or peptide, in the absence of oxidizing agents to accelerate the conjugation reaction. With this method, biomolecules that are sensitive to oxidative damage can be radioiodinated [56,81].

Chelator coupling

Radiometal labelling is performed using the BFCA's, often involving several conjugation groups (*e.g.* hydrazides, esters, isothiocyanates), which can be radiolabelled before or after biomolecule coupling. An overview of typical conjugation reactions for reactive groups on the peptide or protein is given in Figure 4. The reactivity of an amine is mainly dependent on its basicity and class. Aliphatic amines such as the ε-amino group of lysine are moderately basic and reactive with most acylating reagents. However, the concentration of the free base form of aliphatic amines below pH 8 is very low, indicating that the kinetics of amine acylation by isothiocyanates, succinimidyl esters or other reagents are strongly pH-dependent; a pH of 8.5 to 9.5 is usually optimal for modifying lysine residues. In contrast, the α-amino group at the N-terminus usually has a pKa of ~7, so it can potentially be selectively modified by reaction at near neutral pH. Furthermore, although amine acylation should usually be carried out above pH 8.5, the acylation reagents tend to degrade in the presence of water, with the rate increasing as the pH increases. Protein modification by succinimidyl esters can typically be performed at pH 8.3, whereas isothiocyanates usually require a pH >9 for optimal conjugations; this high pH may be a factor when working with base-sensitive proteins. When performing amine coupling, avoiding amine containing buffers as TRIS and glycine is a prerequisite. Isothiocyanates form thioureas upon reaction with amines [82]. Direct NOTA- and DOTA-peptide conjugation is achieved by the formation of a stable peptide bond linkage between one of the carboxyl groups of NOTA or DOTA and a primary amine of the peptide. Therefore, the carboxyl group

is activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), affording an intermediate that can react with the different groups in the peptide [19,57] (Figure 4). Maleimide coupling is an alternative method that exploits thiol groups on the peptide or protein, resulting in a thioether bond [83] (Figure 4). However; the conjugation site is very important for maleimide coupling: highly solvent accessible sites rapidly loses their conjugator by exchange of the maleimide with thiol-reactive constituents; on the other hand, a partially accessible site with a positively charged environment has a positive effect by hydrolysis of the succinimide ring, thereby preventing the exchange reaction [84]. Recent studies have improved the chemical stability of maleimide linkers by modification of the succinimide ring [85]. HYNIC-peptide conjugates are produced via amide linkage formed through reaction of the active ester derivative of the ligand [62].

A. Isothiocyanates conjugation (amine group)
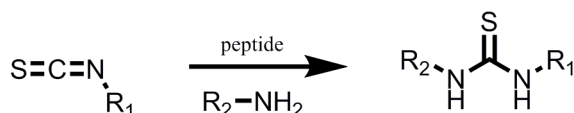
B. Hydroxysuccinimide active ester of BFCA (amine group)
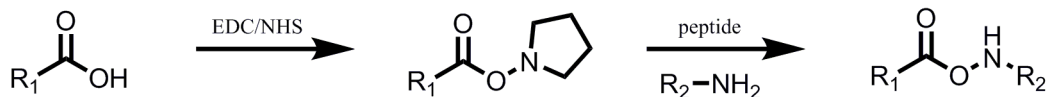
C. Maleimido group (thiol group)
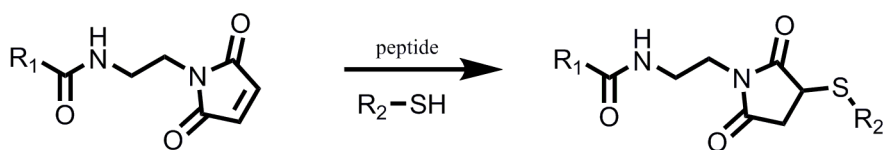
Figure 4. Conjugation reactions.
Conjugation groups are mentioned above each reaction as well as the functional group of the peptide.

Fluorination

[18]F-labelling can be achieved using a prosthetic group (*e.g.* [^{18}F]SFB), as described before. The labelling occurs through ^{18}F-fluoroalkylation, ^{18}F-fluoroacylation and ^{18}F-fluoroamidation of primary amino groups at the N-terminus or amino acid side chains or thiol functions on cysteine residues. The radiolabelling of peptides with [^{18}F]SFB usually occurs in four steps: (1) ^{18}F-fluorization, (2) acid or base hydrolization, (3) acid activation and (4) final acylation [19]. Recently, a one-step radiosynthesis

of ^{18}F was described by chelation of a NOTA-conjugated peptide (*e.g.* octreotide) with Al^{18}F; the ^{18}F-labelling of cyclic RGD-peptide derivatives using a specific arene group was also successfully applied [86-88]. Also the 'click chemistry' approach has been used for ^{18}F labelling of peptides, based on the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction (CuAAC) between terminal alkynes and azides resulting in 1,4-disubstituted 1,2,3-triazoles [89].

^{3}H/^{14}C labelling

The ^{3}H and ^{14}C isotopes are widely used in research studies, because of the frequent occurrence of these atoms in biomolecules. These radionuclides are incorporated into the peptide structure by acetylation using [^{14}C]- or [^{3}H]-acetic anhydride or reductive alkylation using *e.g.* [^{3}H]-sodium cyanoborohydride or [^{14}C]-formaldehyde. Both labelling reactions occur through binding on free amino groups of the peptide or protein structure. Next, ^{3}H- or ^{14}C-labelling at cysteine residues can take place as well, using [^{14}C]- or [^{3}H]-iodoacetic acid or [^{14}C]-iodoacetamide, resulting in a stable carboxymethylated cysteine amino acid. Radiolabelled peptides can also be produced using solid phase peptide synthesis with ^{3}H- or ^{14}C-labelled amino acids [90,91].

4. QUALITY CONTROL OF PEPTIDE AND PROTEIN RADIOPHARMACEUTICALS

General quality attributes

Because several peptides are already registered as active drugs in different clinical areas, the quality specifications of new drugs (*i.e.* quality attributes, procedures and acceptance criteria) are extremely important [92]. The same applies for radiopharmaceuticals (*e.g.* ^{99m}Tc-Annexin V): as site-specific radiolabelling of a protein or peptide is preferred for conservation of biological properties (*e.g.* receptor-binding), its quality control after the radiolabelling procedure is essential [5,30,31,93].

Vergote *et al.* suggest some typical peptide quality attributes to overcome toxicity or efficacy problems based on the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP). First, due to the production process of some proteins or peptides, contaminating substances from cells or culture medium can be present; therefore, extraction and purification procedures have to be performed. Next, the identity of the peptide or protein needs to be analysed as well, using *i.a.* peptide mapping for the determination of the amino acid composition or reference-comparison using chromatographic or spectrometric techniques [47,92]. The characterization of peptide impurities, originating from synthesis or instability problems [94], is of great importance too, as they

can be toxic or can have unexpected pharmacological effects compared to the native peptide: for example, crude peptide material of INSL6[151-161] was found to trigger a contractile response in guinea pig ileum longitudinal smooth muscle cells, while the 95% purity sample did not [29,95]. Besides, the radiopharmaceutical preparations for parenteral administration should meet the requirements for sterility testing as described in the Ph. Eur. and USP. However, due to small batch sizes and short half-life values, sterility validation of the production process, instead of the product, is often required. Finally, for certain radiopharmaceutical preparations, a test for bacterial endotoxins is prescribed [47].

Seen the increasing interest in peptide and protein radiopharmaceuticals, the European and US institutions are currently updating their guidelines regarding these theranostics. For peptide or protein radiopharmaceuticals, the guidelines from the International Atomic Energy Agency (IAEA), the World Health Organisation (WHO), the FDA and the European Association of Nuclear Medicine (EANM), concerning the requirements for registration of radiopharmaceuticals, are under discussion for harmonization [96]. Moreover, a draft guideline on radiopharmaceuticals based on monoclonal antibodies is planned for consultation in 2012 by the European Medicines Agency (EMA), taking into account both 'the guideline on Development, Production, Characterization and Specifications for Monoclonal Antibodies and Related Products', as well as 'the guideline on Radiopharmaceuticals' [97,98].

Radionuclidic purity

The radionuclidic purity represents the ratio, expressed as percentage, of the radioactivity of the desired radionuclide to the total radioactivity of the radiopharmaceutical preparation. The most generally useful technique to determine radionuclidic purity of γ-emitters is that of gamma spectrometry: the γ-energies on the spectra are identified. These radionuclidic impurities are limited in the individual monographs as these impurities can be responsible for unwanted increased radiation dose to the patient or obscure scintigraphic images [47,99,100]. For ^{111}In, photopeaks at energies of 171 and 245 keV are required, limiting ^{114m}In and ^{65}Zn impurities, *i.e.* the amount of ^{114m}In and ^{65}Zn is not greater than 3 kBq per MBq of ^{111}In in *e.g.* ^{111}In-pentetreotide parenteral injections [101]. Pure β-emitters can be evaluated for radionuclidic purity with a β-spectrometer or using a liquid scintillation counter [47].

In the past, radionuclidic impurities often occurred with generator-produced radionuclides due to breakthrough of the parent radionuclide during elution procedures of the daughter nuclide, *e.g.* ^{68}Ge or ^{99}Mo impurities in ^{68}Ga or ^{99m}Tc radionuclide samples, respectively. However, new developments have contributed to the production of generators with a satisfactory radionuclidic purity [54,102].

Radiochemical purity

The radiochemical purity represents the fraction (%) of the total radioactivity in the desired chemical form in the radiopharmaceutical. Therefore, determination of the radiochemical purity requires the separation of the different chemical substances containing the radionuclide. These radiochemical impurities can originate from different steps in the radiopharmaceutical development process: radionuclide production, chemical (radiolabelling) procedures, incomplete preparative separation or storage-related chemical degradation. The radiochemical purity is an important quality parameter for radiopharmaceuticals as it has a pronounced effect on the *in vivo* behaviour of the radiolabelled product, *e.g.* free ^{99m}Tc-pertechnetate or ^{111}In-DTPA molecules are observed to localize into stomach and bladder, respectively, resulting in altered biodistribution and potentially poor image quality [47,99,103,104].

To separate the different substances in the radiopharmaceutical preparation, any analytical separation method can be applied, *e.g.* paper chromatography (PC), thin-layer chromatography (TLC), electrophoresis, size-exclusion chromatography (SEC), gas chromatography and liquid chromatography (LC). For each EU-approved radiopharmaceutical product, the method of choice is given in the individual monograph in the European Pharmacopoeia or USP. In hospital environment, paper chromatography and instant thin-layer chromatography (ITLC) are frequently used, due to its simplicity and fast measurement procedures. The radioactive spots or areas are then detected by autoradiography or by measurement of radioactivity over the length of the chromatogram [47,104,105]. The radiochemical purity of ^{111}In-Ibritumomab Tiuxetan i.v. injection (Zevalin) for example, can be investigated by using a 1 x 8 cm instant silica gel strip as stationary phase, along with a 0.9% sodium chloride mobile phase solution. The distribution of radioactivity on the chromatogram is then investigated by scanning the strip with a suitable collimated radiochromatogram strip scanner; not less than 95% of the ^{111}In activity is present as a band between the R_f values of 0 and 0.1, corresponding to the ^{111}In-Ibritumomab Tiuxetan immunoconjugate [106].

Chemical purity

The chemical purity of a radiopharmaceutical is the fraction of the biomolecule in the desired chemical form. The determination of this quality factor thus requires quantification of the individual chemical impurities, as specified in the Ph. Eur. or USP monograph of the approved radiopharmaceutical. Chemical impurities may arise from instability problems of the material, before or after radiolabelling, as well as from the manufacturing process (*e.g.* residual solvents); these impurities can be responsible for *in vivo* adverse reactions and pharmacological or toxic effects by competing for active transport mechanisms and enzyme- or receptor-binding [47,95,99,100].

The identification and semi-quantification of the radiolabelled [18F]-6-fluorolevodopa, together with its impurities dopa, trimethyltin chloride (impurity A) and 6-hydroxydopa (impurity B), is performed using liquid chromatography on a spherical end-capped octadecylsilyl silica gel stationary phase column (4.0 x 250 mm). Based on the results with reference solutions for these impurities, identification and semi-quantification with a spectrophotometer (200 nm) and radioactivity detector is achieved: relative retention times (RRT), with reference to 6-fluorolevodopa (retention time = 6 min) (*i.e.* RRT_{dopa} = 0.8 and $RRT_{A/B}$ = 0.7), together with quantity limits (*i.e.* 6-fluorolevodopa = 15 mg/V, dopa = 1.0 mg/V, A = 0.5 mg/V, B = 0.025 mg/V and V being the maximum recommended dose in millilitres) are determined [107].

Assay: activity

Beta-emitting radionuclides are assayed with the use of a liquid-scintillation detector system, determining the disintegration rate of these radionuclides. This technique converts the radiation energy into fluorescence radiation using a liquid scintillator (*e.g.* 2,5-diphenyloxazole), after which the fluorescence wavelengths are detected by the multiplier phototubes [104]. Gamma photons interact with *e.g.* a NaI detector crystal by means of the Photoelectric Effect; the electrons released from this interaction then interact with the crystal to produce light (scintillation), which in turn is detected by the photo-multiplier tubes [48].

For some radiopharmaceutical preparations, a physiological distribution test is performed to evaluate the suitability and *in vivo* biodistribution of the product for the intended purpose. In general, each of three animals is injected intravenously with the preparation to be tested, after which they are placed in a separate cage, allowing individual excreta collections. At the specified time after injection, the animals are euthanized and dissected: the selected organs are assayed for their radioactivity and the distribution calculated (expressed as *e.g.* % of total injected dose or radioactivity per tissue mass). If the distribution of at least two of the three animals complies with the specifications, the radiopharmaceutical preparation meets the requirements of the physiological distribution test [47]. This biological distribution test is described in the USP and Ph. Eur. for *e.g.* 99mTc-labelled albumin aggregates, used as a scintigraphic imaging agent to evaluate pulmonary circulation [108-110]. In brief, a volume (not exceeding 0.2 ml) of 99mTc-macroaggregated albumin is intravenously injected into the caudal vein of each of three rats or mice (specified weight). After 5-15 minutes post injection, the animals are euthanized, after which the lungs, liver and (eventually) spleen are isolated and radioactivity measured, relative to the radioactivity of the remaining carcass. The percentage of radioactivity in the different organs is then calculated using the following formula: percentage radioactivity = (A/B) x 100, where A is the radioactivity in the organ of interest and B the

total radioactivity (carcass and isolated organs). To meet the specifications, at least 80% of the radioactivity should be found in the lungs and not more than a total of 5% is found in the liver and spleen, in not less than two of the three animals [108,109].

Specific activity

The specific radioactivity is the amount of radioactivity of a radionuclide per unit mass of the element or of the peptide or protein radiopharmaceutical concerned [47]. For example, for [^{18}F]-6-fluorolevodopa, the USP describes a specific activity specification of not less than 0.463 mCi per mg of L-fluorodopa [111]. For carrier-free radioisotopes, this quality parameter remains constant over time as a decay will alter the molecule as well, while it decreases for carrier-added elements or molecules; if non-radioactive isotopes of the same radionuclide are present, the mass of the element changes less than the radioactivity after radioactive decay (Figure 5) [104]. For peptide and protein radiopharmaceuticals, separation of the radiolabelled molecule from non-radiolabelled analogues is often difficult. Therefore, specific activity is usually calculated taking into account the radioactive concentration and the concentration of the chemical substance being studied, *i.e.* unmodified and/or modified peptide or protein assuming similar biological properties, after determining radionuclidic and radiochemical purity. This quality attribute is called 'effective specific activity' [112]. Contamination of the radiopharmaceutical with compounds of similar biological activity will therefore reduce the effective specific radioactivity *in vivo* [47,100].

To explain the progress of the effective specific activity, distinction must be made between the different labelling techniques: the radioisotope can be included into the peptide structure (*e.g.* ^{14}C, ^{11}C or ^{13}N-labelling) or can be attached to the peptide or protein without interfering with its basic structure (*e.g.* ^{68}Ga-NOTA-labelling). After radioactive decay of a ^{14}C-labelled molecule for example, the elemental composition and structure of the molecule significantly changes and therefore most probably influences its biological activity; in this case, both the amount of radioactivity and molecule concentration decrease in time. In case of chelated molecules, the peptide or protein structure is not directly modified after radioactive decay of the metal radionuclide, having the same biological activity as the starting radiopharmaceutical; the effective specific activity is then significantly decreased.

Moreover, the determination of synthetic peptide and protein concentrations is not straightforward, due to the presence of counter ions or salts, water or organic solvents. Due to these impurities, the actual peptide content can be reduced to 90 – 20% of the expected concentration based on mass, thus remarkably influencing specific activity calculations [92,113]. Peptide and protein content can be determined by quantitative amino acid analysis, which is not a routine quality assay [114,115].

Counter ions, salts and moisture content can be quantified using *e.g.* HPLC, gas chromatography, atomic spectroscopy or Karl Fisher methods [92,116,117].

In experiments involving saturable processes, the target-to-background ratio is often improved by increasing the specific activity of the radiopharmaceutical [118]. However, this phenomenon is not unambiguous: uptake of [111In-DTPA]octreotide in octreotide receptor-positive tissues is not maximal at the lowest possible peptide amount with maximum specific activity [119,120]. Moreover, also the labelling procedure influences specific activity: 111In-labelling of DTPA- and DOTA-conjugated compounds in MES or HEPES buffer instead of acetate buffer leads to the production of 111In-labelled compounds with higher specific activities [118].
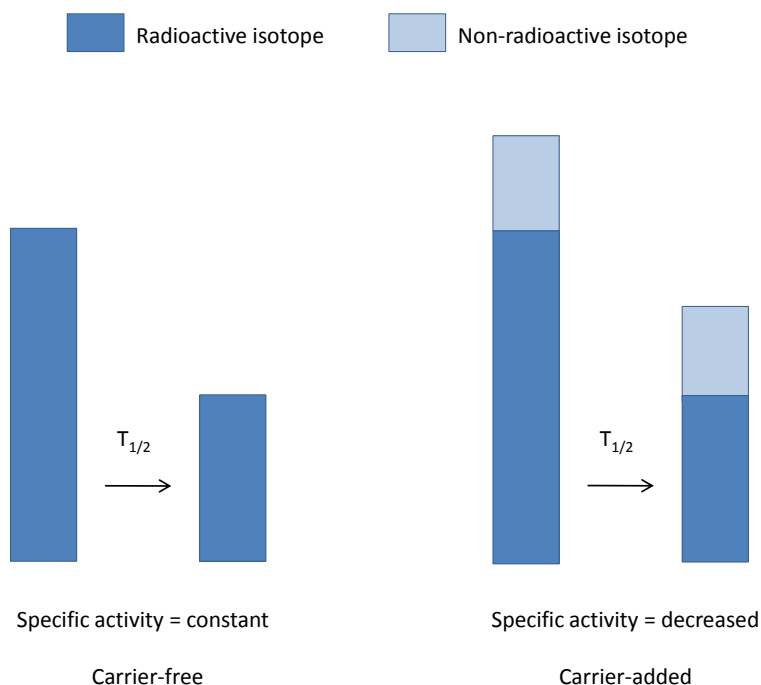
Figure 5. Specific activity of element

Stability and storage

Radiopharmaceutical stability is influenced by the specific activity of the radioactive material, the energy of the emitted radiation, the half-life of the radionuclide and the stability towards light, temperature variations and radiolysis of the peptide or protein itself [99]. For example, 90Y-labelled 2IT-BAD-Lym-1 monoclonal antibodies, formulated at a specific activity of 1-2 mCi/mg, showed good radiochemical purity (≥ 80%) and immunoreactivity (≥ 75%) over 3 days when stored at 4-8°C. However, increasing the specific activity to 4 and 9.4 mCi/mg revealed a decrease in radiochemical purity to 65% and 21%, respectively, while immunoreactivity even reduced to 3% with the highest

specific activity solution. This phenomenon was ascribed to the interaction of the moderate energy β-particles emitted by ^{90}Y with water molecules in the solution, generating highly reactive free radicals; these radicals can degrade the metal chelator as well as the biomolecules attached [121,122].

Radiopharmaceuticals should be stored in an airtight container, in a place that is sufficiently shielded to protect personnel from irradiation. The container itself should comply with the national and international regulations concerning the storage of radioactive substances. Interestingly, as containers may darken due to irradiation, this does not necessarily mean degeneration of the product [47].

During storage, the container should be appropriately labelled: name of product and manufacturer, identification number (marketing authorisation number), total radioactivity (MBq or mCi) at a stated date and time, total volume (if liquid or gaseous preparation), route of administration, expiry date, name and concentration of any added antimicrobial preservative and, if necessary, special storage conditions [47]. Next, the statement 'Caution – Radioactive material' is labelled onto the package of radiopharmaceuticals [101,106]. The labelling characteristics of a radiopharmaceutical are summarized in *e.g.* the European public assessment report (EPAR), describing the particulars to appear on the outer package, as well as the immediate packaging units of each vial of the reconstitution kit [123].

5. *IN VITRO* BIOMEDICAL CHARACTERIZATION

Chemical and metabolic stability

A main challenge for the use of peptides as radiopharmaceuticals is their low stability to peptidases and proteases found in most tissues, catalyzing the hydrolytic degradation and thus resulting in a short biological half-life. Consequently, the *in vivo* application of these metabolically unstable molecules is limited as insufficient amounts of peptide reach their target [23]. However, peptide stability can be improved by different methods: introduction of unnatural or D-amino acids, substitution of peptide bonds, terminal capping (N-acetylation or C-amidation) or cyclisation. [5].

Peptides can also be vulnerable to chemical and/or enzymatically catalyzed oxidation or reduction reactions. For example, oxidation by hydrogen peroxide (H_2O_2) of ^{99m}Tc-labelled radiopharmaceuticals can lead to increased levels of free ^{99m}Tc-pertechnetate [103]. The radiolytic generation of free radicals can add to the degradation of radiolabelled biomolecules as well, resulting in a decreased stability and purity profile of the radiopharmaceutical. These stability studies are of increasing importance, as denatured proteins can precipitate and localize in lungs, liver en spleen

tissues, leading in turn to adverse reactions *in vivo* [103,124,125]. Radiolysis however, can be prevented or diminished by the inclusion of radioprotectants (*e.g.* ascorbic acid or human serum albumin) in the formulation of ^{111}In-bombesin conjugates or other radiopharmaceuticals [122,125,126]. Moreover, freezing the radiolabelled biomolecules can minimize diffusion of free radicals in the aqueous solution, protecting the peptide or protein for radiolytic degradation: radiolytic damage of ^{131}I-labelled monoclonal antibodies was largely eliminated by freezing at -70°C, while 80-90% of immunoreactivity was lost after storage at 4°C for 1 to 12 days [122,127]. The phenomenon of trans-chelation, where the isotopic metal is released from the radiopharmaceutical also needs to be examined. For example, a metabolic study of a ^{64}Cu-labelled octreotide analogue demonstrated that Cu(II) dissociates from macrocyclic chelators and binds to superoxide dismutase in rat liver [128,129].

Both chemical and metabolic stability can be investigated by incubation in buffer, cell medium, enzyme solution, blood or plasma and tissue homogenates, after which the degradants, including the free radionuclide, can be detected, identified and quantified. This can be performed using *e.g.* chromatographic techniques coupled to spectrometric (Ultra-Violet (UV) spectrophotometer and mass spectrometer (MS)) and radioactivity detectors [130-132].

Peptides are traditionally separated using reversed-phase liquid chromatography (RP-LC) on an alkyl (C_{18}, C_8, C_4) bonded silica column and identified using mass spectrometry (MS), having good sensitivity and accuracy characteristics [133,134]. The most common RP-methods used for peptide separation involve isocratic or gradient elution, using a water and acetonitrile composing mobile phase in the presence of an acidic modifier (*e.g.* formic acid (FA) or trifluoroacetic acid (TFA)) [135,136]. Moreover, next to the alkyl functional groups, RP-LC can also be performed by linking other groups to the silica support: phenyl-hexyl linkers, ether-linked phenyl groups or polar functional groups (*e.g.* amide) within an alkyl chain, with different endcapping strategies [137]. Although RP-LC is a powerful separation technique, retention of polar biomolecules cannot be achieved using these RP-columns. Therefore, Hydrophilic-Interaction Chromatography (HILIC) was developed, which provides the separation of proteins and peptides using aqueous mobile phases and polar stationary phases [138,139]. Classical ion-exchange chromatographic procedures are based on the net charges of proteins and peptides, located either on a functional residue or on the N- or C-terminus. At acidic pH-values, peptide carboxyl groups are protonated and ion-exchange chromatography thus primarily depends on the number of basic amino acid residues (*i.e.* cation exchange chromatography) [136]. Size-exclusion chromatography can be used in the separation of polypeptides, based on differences in molecular weight. However, non-specific interactions can occur as well, *e.g.* hydrophobic or electrostatic effects. Therefore, spheric silica particles of various pore

sizes are coated with a hydrophilic film and are used for the separation of peptides and proteins, resulting in a good recovery and stability [136].

Receptor-binding characterization

Most radiolabelled peptides are currently marketed as diagnostics or therapeutics for their use in oncology. One of the main reasons for this prevalence of cancer-research, is the detection of peptide-receptor over-expression in many primary cancers, in comparison to their expression in normal tissues. These receptors (*e.g.* somatostatin, vasoactive intestinal peptide and bombesin receptors) can thus be used as molecular targets for the radiolabelled peptides and proteins. However, it should be noticed that species differences may exist for different receptors, emphasizing the importance of using native human receptors in *in vitro* characterization assays of radiopharmaceuticals for human use [140,141].

To determine the different receptor-binding properties of radiolabelled peptides and proteins, radioligand binding studies on receptor-membranes or receptor-overexpressing cells can be performed. Using these assays, the receptor affinity (K_D), maximum density of receptors (B_{max}) and the association (k_{on}) and dissociation (k_{off}) rate constants can be calculated to quantify the peptide/protein-receptor interaction. One method to determine these characteristics is by saturation analysis, where the amount of radioligand added is increased, while keeping the specific activity constant, or where the radioligand concentration is kept constant while decreasing the specific activity by the addition of unlabelled peptide. Non-specific binding is determined at each concentration by co-incubation with an excess (100- to 1000-fold) of unlabelled peptide. The amount of radioactivity bound to the cells is then verified using filtration methods after equilibrium conditions were reached, resulting in the determination of K_D and B_{max} values. The equilibrium constant K_D determines the strength of interaction of the radiolabelled biomolecule with the receptor; it is the ligand concentration that will occupy 50% of the receptors. B_{max} is expressed as amount of ligand bound per mass of protein. However, it is important to realize that the results obtained by filtration methods are highly dependent on the inherent adsorption and instability characteristics of the peptides, as well as the high response-sensitivity to operational conditions. Selection of appropriate ligand receptor binding conditions (*e.g.* temperature, time, buffer conditions) is therefore critical for peptides to avoid false negative results. Filter adsorption of *e.g.* ^{125}I-labelled vasoactive intestinal peptide (VIP) can be reduced by pretreatment procedures with polyethylenimine (PEI) and bovine serum albumin (BSA); the use of bacitracin protease inhibitor during incubation also decreased adsorption phenomena [142].

Next, kinetic binding experiments can be performed to determine the time course of ligand-receptor association (k_{on}) and dissociation (k_{off}), so the time period for reaching binding equilibrium is obtained. Having determined the K_D of a radiolabelled peptide for a target receptor in a saturation assay, the receptor binding potencies of (unlabelled) peptides can be compared by a competition binding assay: the displacement of radiolabelled peptide from the receptor is analysed after the addition of increasing concentrations of the unlabelled analogue [142-145].

Radioligand binding assays are widely performed by scientists in the biomedical and pharmaceutical research fields, for example to characterize and localize different subtypes of the endothelin receptor in tissue sections of the human atrioventricular conducting system [146]. Iodinated adrenomedullin derivates have been characterized for their effectiveness for lung nuclear medicine [147]. SOM230, a somatostatin peptidometic resulting from rational drug design, was characterized as potential drug as the compound had high affinity to the five human somatotropin release inhibiting factor receptor subtypes [148].

In addition to intact cells or membranes, isolated receptors can be analysed as well by immobilization on a sensor chip. Study of ligand-receptor interactions can then be performed using surface acoustic wave (SAW) or surface plasmon resonance (SPR) technologies, where label-free peptides/proteins can be used. SAW biosensors give information on affinity (K_D), kinetics (k_{on} and k_{off}), viscoelastic effects and conformational changes; using SPR optical sensors, the refractive index is linearly related to the deposited mass, but does not generally allow for the detection of conformational alterations [145,149].

Cell interactions

Radiolabelled biomolecules can interact with cells in different ways: (1) the receptors are used as binding sites for peptide analogues, after which internalization of the ligand-receptor complex, and therefore accumulation of radiotracer in the cell, takes place; (2) radioligands can bind with high affinity to peptide receptors without inducing cell-internalization, justifying their use as diagnostic tools for different clinical applications [140,141,150]. In general, agonistic peptides have the ability to internalize into tumour cells and desensitize the receptor. After internalization, the receptor can be recycled to the plasma membrane (resensitization) or degraded into the lysosomes (downregulation); this degradation phenomenon is aimed for radiotherapeutic peptides or proteins [151]. In contrast to agonists, antagonists show unsurpassed imaging characteristics: the somatostatine receptor antagonists ^{111}In-DOTA-sst2-ANT and ^{111}In-DOTA-sst3-ODN-8 showed extremely high tumour accumulation, due to a more than 15-fold increased number of accessible

binding sites per cell compared to their agonistic analogues [141]. However, although there is a consensus that antagonists generally do not trigger receptor-internalization, cholecystokinin, endothelin and neuropeptide Y antagonistic analogues are reported to stimulate internalization as well [152]. But what is the fate of the label when the protein or peptide is catabolised? Some labels are membrane permeant or are actively excreted from the catabolic cell, while others remain trapped inside the cell because of their charge and can persist for the lifetime of the cell [153]. The iodinated labels are catabolised to iodotyrosines, and leave the cell for recycling. The dehalogenase enzymes then scavenge the iodine for processing in the thyroid gland, especially iodine atoms present ortho- to a hydroxyl group on a phenyl ring, *i.e.* the product for most iodination labelling strategies. For the BFCA's, which make mainly use of lysines or cysteines, the label remains trapped inside the cell as charged lysine adducts upon catabolism [154].

Internalization assays are typically performed at 37°C, together with a low pH buffer (acid wash, *e.g.* glycine buffer pH 3) to dissociate surface-bound ligand after incubation. During the separation of bound and free radioligand, however, temperature is lowered because of a slower dissociation rate of the receptor-ligand complex [24,143].

Cell internalization of radiolabelled peptides or proteins can also be accomplished by the complexation or conjugation to cell-penetrating peptides, targeting intracellular, intranuclear or extracellular receptors for imaging or therapy. Kersemans *et al.* recently summarized the different strategies in molecular imaging using cell-penetrating peptides. To target the p21 cyclin-dependent kinase inhibitor in the nucleus of breast cancer cells, a TAT (trans-activating transcriptional activator) cell penetrating peptide was site-specifically conjugated to the Fc tail of anti-p21 antibodies and ^{123}I-labelled for imaging purposes. The higher uptake in the tumour compared to normal tissue is achieved because of the enhanced perfusion rate and hyperfenestration, together with an upregulation of the receptor in the tumour cells. Next, the radionuclide can directly be attached to the cell-penetrating peptide via a chelating group, so without conjugation to a specific biomolecule, *e.g.* ^{111}In-DOTA-TAT is internalized and transported to the nucleus of HeLa cells as well as mouse lymphocytes *in vitro* [155-157]. However, as different peptides and proteins (*e.g.* fibroblast growth factor A and B, epidermal growth factor, parathyroid hormone-related protein or angiotensin II) operate in part through an intracellular mode of action, transcellular transport is also possible without the use of cell-penetrating peptides. In some cases, *e.g.* for parathyroid hormone-related protein, association with the nucleus is reported, owing to the nuclear targeting sequence present in the native peptide or protein sequence [158-160].

6. *IN VIVO* ANIMAL-MODEL STUDIES

<u>Pharmacokinetics</u>

Next to the targeted tumour-uptake of radiopharmaceuticals and their metabolisation, their *in vivo* potential is also influenced by other pharmacokinetic properties of the molecules, *i.e.* non-tumour tissue distribution, non-specific binding to other organs and excretion patterns. These properties are influenced by the receptor expression in the different malignant and normal tissues, as well as by the metabolic and physicochemical characteristics (lipophilicity and charge) [141]. The *in vivo* tissue distribution is analysed using mice (or other animals), carrying specific tumours: at several time points after injection, biodistribution is measured using PET or SPECT imaging techniques or by organ radioactivity measurements after animal dissection. Moreover, excretion pathways into urine or via the hepatobilliary system can be analysed as well [24].

Radioiodination and radiofluorination usually increase lipophilicity of the molecule, leading to increased hepatobiliary clearance. The introduction of PEG molecules or carbohydrates and polar amino acids as a linker molecule, reduces tracer lipophilicity and thus induces renal excretion pathways [57,60,68,141]. For ^{111}In-labelled RGD peptides, Dijkgraaf *et al.* investigated the effect of linker variation on tumour and organ uptake: insertion of lysine caused enhanced kidney retention, while PEG showed the highest tumour-to-blood ratio and the lowest uptake in kidney and liver [68]. The addition of a charged amino acid spacer (*i.e.* –(arginine)$_3$- or –(ornithine)$_3$-) to a bombesin analogue was advantageous for biodistribution, pharmacokinetics and tumour targeting ability, because it reduced the upper abdominal radioactivity levels and increased tumour/normal tissue contrast ratios [161]. The pharmacokinetic properties of ^{99m}Tc-labelled peptides are predominantly influenced by the labelling-chemistry, generally leading to hydrophilic ^{99m}Tc-complexes that are excreted by the kidneys. Moreover, the pharmacokinetics of chelator-coupled peptides or proteins is greatly influenced by the type of chelator that is used: tumour and liver accumulation (whether or not by trans-chelation, see above [128,129]) and renal uptake can change between the different BFCA's. For example, changing the chelator for ^{111}In in the Tyr^3-octreotide analogue from DTPA to DOTA resulted in slightly increased tumour accumulation and significantly higher renal tracer uptake. Moreover, substituting ^{111}In by ^{68}Ga in the DOTA-derivative in turn led to an increased tumour uptake but reduced renal accumulation [141].

In vivo pharmacokinetic studies revealed the high renal activity concentration of most radiolabelled peptides and proteins, leading to severe nephrotoxicity. This clearance phenomenon occurs by glomerular filtration and reabsorption by the tubular cells of the kidneys, followed by lysosomal metabolisation. However, coinfusion of positively charged amino acids, *i.e.* lysine and arginine, can

block this tubular reabsorption process, without altering tumour uptake and biodistribution pattern. This was shown with ^{111}In-DTPA-octreotide, where coadministration with Lys and Arg led to a reduction in renal radio-accumulation of 43%. The coadministration of a gelatin-based plasma expander (*e.g.* Gelofusine) also reduced renal radiation of ^{111}In-labelled octreotide with 45%, without inducing any side effects [45,141,162-165].

Dosimetry

Before *in vivo* human studies can be performed, dosimetry of normal organs and tumour tissue is needed to avoid high radiation doses to non-target tissues (*e.g.* liver, kidney, bone marrow). One of the challenges in this drug development process is to deliver the highest activity to the tumour, while protecting normal tissue. Therefore, from the activity determined in the different organs using biodistribution studies and corresponding time-activity curves, the absorbed doses are estimated during *in vivo* studies [45,166,167].

Peptide radiopharmaceuticals can deliver high absorbed doses to the kidneys and may lead to permanent nephropathy. Reliable dosimetry of kidneys is thus crucial for safe and effective radiotherapy [168]. Besides the kidneys, the bone marrow is a potentially dose-limiting organ as well. The radiation dose to the bone marrow is usually calculated from the accumulated radioactivity of the radiopharmaceutical in the blood. However, as considerable variation in bone marrow absorbed dose between patients is reported, individual calculation of the bone marrow absorbed dose is necessary, leading to individual dose optimization [45,169].

To determine *e.g.* kidney dosimetry, the internal dosimetry scheme of the Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine can be applied. However, due to excessive interpatient variability of kidney absorbed doses, crucial improvements of this method are necessary; the inclusion of actual kidney masses and kidney regional distribution in the dosimetric estimates can prevent unexpected renal toxicity of radiopharmaceuticals [45,170,171]. Moreover, the effective dose-equivalent, describing the biological effect that can be observed for a specific organ, should be calculated using the current weighting factors established by the International Commission on Radiological Protection (ICRP) [172,173,98]. To obtain an indication of how exposure can effect overall health (*i.e.* effective dose), the equivalent dose can be multiplied by a tissue weighting factor related to the risk for a particular tissue or organ [174].

Moreover, the choice of radionuclide defines the radiation dose as well: [68]Ga, with a 68 minutes half-life, has a lower effective dose than those of other radioisotopes with longer half-lives; ^{11}C-labelled radiotracers have significantly lower doses than [68]Ga- and ^{18}F-labeled radiotracers [174].

7. *IN VIVO* HUMAN STUDIES AND APPLICATIONS

From animals to humans

Finally, the results of the *in vivo* animal studies have to be translated to human models to predict pharmacokinetics and pharmacodynamics of the radiopharmaceutical. This is not straightforward as species-specific physiological differences can lead to pharmacokinetic variations, indicating the increasing importance of data interpretation [175].

Phase 0 trials can be performed to evaluate these properties through administration of sub-pharmacological doses (microdoses, less than 1/100th of the dose calculated based upon animal data) for a short time period to a low number of humans, without any diagnostic or therapeutic purpose. Potential radiolabelled peptides or proteins then enter the Phase I – III clinical trials, where dose, safety and efficacy in humans are analysed. The Phase 0 clinical trials are designed to improve drug development: time periods from hit to first-in-human studies can markedly be reduced due to an earlier evaluation of human pharmacology and thus earlier selection of promising biomolecules for further development [176,177].

Oncology

The vast majority of radiopharmaceuticals is registered for cancer imaging or radiotherapy [178]. To date, the ^{111}In-DTPA-labelled somatostatin analogue, OctreoScan, is the most successful radiopeptide for tumour diagnosis, imaging several types of neuroendocrine tumours. Gastro-entero-pancreatic neuroendocrine tumours are relatively rare, with an estimated incidence of 3.6 to 13 cases per million population per year (in 2009) and a vast majority of the tumours not presenting clinically. Therefore, OctreoScan can be classified into the orphan medicinal products [23,179]. ^{90}Y-labelled Ibritumomab Tiuxetan (Zevalin), a recombinant murine IgG$_1$ kappa monoclonal antibody, is indicated for the treatment of CD20-positive follicular B-cell non-Hodgkin's lymphoma. However, as the CD20 antigen is located on the surface of malignant and normal B-lymphocytes, rituximab pretreatment is necessary to clear circulating B-cells, so ^{90}Y-Ibritumomab Tiuxetan delivers radiation more specifically to the lymphoma B-cells [180]. ^{131}I-Tositumomab (Bexxar), a murine IgG$_{2a}$ lambda monoclonal antibody, also binds to the CD20 antigen and can be used in the treatment of B-cell non-Hodgkin's lymphoma [181]. ^{99m}Tc-Arcitumomab (CEA-Scan) comprises a murine Fab' monoclonal antibody (IMMU-4) fragment that targets the carcinoembryonic antigen (CEA), secreted by tumour cells. This radiopharmaceutical is indicated for imaging of recurrent colorectal cancer, the fourth biggest cause of cancer deaths in 2008 (*i.e.* 608 000 deaths) [182,183]. Finally, ^{99m}Tc-Nofetumomab merpentan (Verluma) and ^{111}In-Capromab pendetide (ProstaScint) are also marketed for the imaging of (small

cell and non-small cell) lung carcinoma and prostate carcinoma, respectively [6,8,60]. An overview of these FDA- or EMA-approved radiolabelled peptides and proteins is given in Table 3 [123,184].

Other receptor-targeting radiolabelled peptides are currently under development as well, some of them already entering preclinical and clinical trials: cholecystokinin or gastrin analogues, glucagon-like peptide-1, bombesin, chemokine receptor CXCR4 targeting peptides and RGD peptides [60,185,186].

Receptor mediated tumour targeting is nowadays a hot topic in the field of molecular targeting. The molecular species involved are the peptides or antibodies (targeting agents) directed against cell membrane receptors present on tumours (targets). The targeting agents may itself evoke a therapeutic effect (simplex system) or it can act as a therapeutic carrier *e.g.* containing a chelator linked radiometal (duplex systems). Next to those systems already discussed in this review paper, three-component peptide heterodimer-effector conjugates have been described interacting with two targets while also a pretargeting/targeting-effector conjugate was published binding to one target (triplex system). Heterodimers has emerged as a promising strategy to improve peptide affinity. In a peptide heterodimer, two different peptides targeting different receptors are covalently attached by a linker (either flexible or rigid), as tumours co-express multiple peptide receptors [187]. Peptide homodimers on the other hand have been well documented to have higher avidity for targeting tumour cells than peptide monomers [188] or higher cellular uptake characteristics [189]. In addition, a target agent may be a peptide multimer (*e.g.* dendrimer) to which the therapeutic principle is attached. This multiplex system has already been described for the neurotensin peptide, which was synthesised as a tetrabranched form and linked to different units for therapy or diagnosis, while obtaining its biological activity and becoming resistant to proteolysis [190]. Also multiplex systems with dual-labelled imaging agents have been described, *e.g.* (^{111}In-DTPA)$_n$-trastuzumab-(IRDye 800CW)$_m$ which contains multiple γ-emitters and near infrared fluorescent dyes [191]. In some cases, receptor mediated tumour targeting is not an immediate option. Malignant gliomas are characterized by a high rate of local recurrence after surgery within the region of the original tumour, combined with very low incidence of distant metastases and poor prognoses of the patients. Therefore, a combination of gene therapy and receptor mediated tumour targeting are under investigation. Ter Horst *et al.* describe the locoregional delivery of adenoviral vectors encoding the somatostatin receptor subtype 2 (sst$_2$). Intravenous injection of ^{99m}Tc-labelled octreotate (^{99m}Tc-Dermotate 2) showed high sst$_2$ expression in the transfected xenograft tissue, underlining the potential of the increasing tumour sensitivity to targeted peptide radiotherapy [192].

Infection and inflammation

Next to tumour-targeted radiopharmaceuticals, research also focuses on peptide receptor radionuclide imaging (PRRI) of inflammatory markers (*e.g.* Vascular Adhesion Protein-1 (VAP-1)) or infection through leukocyte or microorganism binding [16,193]. As the tuftsin receptor is expressed by neutrophils, monocytes and macrophages, tuftsin analogues are currently investigated for their use in the diagnosis of infection or inflammation (*e.g.* ^{99m}Tc-RP128). Moreover, also chemotactic peptides targeting white blood cells, interleukin-8 derivatives and platelet factor 4 analogues are explored for their use in inflammation/infection imaging. Next, infection-selective agents, like *e.g.* defensin, ubiquicidin analogues, human lactoferrin peptides and alafosfalin are (pre)clinically investigated [16].

Osteomyelitis can already be located using either of the two authorized ^{99m}Tc-radiolabelled antibodies, *i.e.* LeukoScan or Scintimun. ^{99m}Tc-Sulesomab (LeukoScan), an IMMU-MN3 mouse IgG$_1$ Fab' monoclonal antibody fragment, is registered for diagnostic imaging, determining the location and extent of infection or inflammation in bone of patients with suspected osteomyelitis. This antibody recognizes the NCA-90 antigen of granulocytes [8,194]. Another radiolabelled monoclonal antibody, ^{99m}Tc-Besilesomab (Scintimun), targeting the NCA-95 antigen, is indicated for scintigraphic imaging of inflammation or infection in peripheral bone, in patients with suspected osteomyelitis [195] (Table 3).

Table 3. Examples of (FDA/EMA) approved radiopeptides and proteins.

Radioligand	Trade name	Target/Receptor	Application
^{111}In-DTPA-Pentetreotide	OctreoScan	SST2	Imaging of neuroendocrine tumours
[90]Y-Ibritumomab Tiuxetan	Zevalin	CD20	Therapy of non-Hodgkin's lymphoma
[131]I-Tositumomab	Bexxar	CD20	Therapy of non-Hodgkin's lymphoma
[99m]Tc-Arcitumomab	CEA-Scan	CEA	Imaging of colorectal carcinoma
[99m]Tc-Nofetumomab merpentan	Verluma	40-kDa glycoprotein	Imaging of small cell and non-small cell lung carcinoma
[99m]Tc-Sulesomab	LeukoScan	NCA-90	Imaging of osteomyelitis
[111]In-Capromab pendetide	ProstaScint	100-kDa glycoprotein	Imaging of prostate carcinoma
[99m]Tc-Besilesomab	Scintimun	NCA-95	Imaging of infection or inflammation (osteomyelitis)

SST: somatostatine receptor; CD20: B-lymphocyte antigen; GRP-R: gastrin-releasing peptide receptor; CEA: carcinoembryonic antigen; NCA-90: non-specific cross-reacting antigen 90; NCA-95: non-specific cross-reacting antigen 95

8. ALTERNATIVES FOR RADIOPEPTIDES AND THEIR APPLICATIONS

The concept of receptor mediated tumour targeting can also be applied in targeted chemotherapy, where small peptides are used to deliver cytotoxic drugs to tumours. In contrast to radiopeptides, cytotoxic peptides are hybrid molecules composed of (1) a peptide carrier, which binds to receptors on tumour cells, and (2) a cytotoxic moiety instead of a chelator-linked radiometal. Ideally, tumour cells that bind these peptides would be killed, while normal cells would be spared. Cytotoxic compounds (*e.g.* Arg-Gly-Asp molecules) linked to analogues of somatostatins have been developed for targeting somatostatin receptors and therefore are more selective for killing cancer cells [196]
Semi-conductor nanocrystal quantum dot labelled peptides, which serve as ligands for cellular receptors, provide an alternative to radionuclide imaging techniques [197]. These nanoparticles exhibit unique optical and spectroscopic properties, useful in imaging receptor distribution and targeting specific tissues and cell types. For example, quantum dot coated with a lung targeting peptide accumulated in the lungs of mice after intravenous injection [198].

9. CONCLUSIONS

Due to the over-expression of different peptide-receptors in several (malignant) tissues, radiolabelled peptides and proteins are currently developed as theranostics for a number of clinical applications (*e.g.* cancer, inflammation and infection). This is reflected by the numerous peptides and proteins that are currently under investigation in preclinical and even in clinical Phase I/II/III trials. However, quality control (*e.g.* radionuclidic, radiochemical and chemical purity) and subsequent *in vitro* biomedical and *in vivo* pharmacokinetic analyses are of increasing importance to enhance success rate of the preclinical-to-clinical transfer of radiopharmaceuticals.

10. REFERENCES

[1]   Bhutia SK, Maiti TK. Targeting tumors with peptides from natural sources. *Trends Biotechnol*. 2008; **26**: 210-217.

[2]   Jong M, Kwekkeboom D, Valkema R, Krenning EP. Radiolabelled peptides for tumour therapy: current status and future directions. Plenary lecture at the EANM 2002. *Eur. J. Nucl. Med. Mol. Imaging* 2003; **30**: 463-469.

[3]   Okarvi SM. Peptide-based radiopharmaceuticals and cytotoxic conjugates: Potential tools against cancer. *Cancer Treat. Rev.* 2008; **34**: 13-26.

[4]   Adessi C, Soto C. Converting a peptide into a drug: strategies to improve stability and bioavailability. *Curr. Med. Chem.* 2002; **9**: 963-978.

[5]   Okarvi SM. Peptide-Based Radiopharmaceuticals: Future Tools for Diagnostic Imaging of Cancers and Other Diseases. *Med. Res. Rev.* 2004; **24**: 357-397.

[6]   Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discov.* 2008; **7**: 21-39.

[7]   Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature* 2007; **450**: 1001-1009.

[8]   Olafsen T, Wu AM. Antibody Vectors for Imaging. *Semin. Nucl. Med.* 2010; **40**: 167-181.

[9]   Broisat A, Hernot S, Toczek J, De Vos J, Riou LM, Martin S, Ahmadi M, Thielens N, Wernery U, Caveliers V, Muyldermans S, Lahoutte T, Fagret D, Ghezzi C, Devoogdt N. Nanobodies Targeting Mouse/Human VCAM1 for the Nuclear Imaging of Atherosclerotic Lesions. *Circ. Res.* 2012; **110**: 927-937.

[10]   Vosjan MJWD, Vercammen J, Kolkman JA, Stigter-van Walsum M, Revets H, van Dongen GAMS. Nanobodies Targeting the Hepatocyte Growth Factor: Potential New Drugs for Molecular Cancer Therapy. *Mol. Cancer Ther.* 2012; **11**: 1017-1025.

[11]   Vaneycken I, D'Huyvetter M, Hernot S, De Vos J, Xavier C, Devoogdt N, Caveliers V, Lahoutte T. Immuno-imaging using nanobodies. *Curr. Opin. Biotechnol.* 2011; **22**: 877-881.

[12]   Gainkam LOT, Caveliers V, Devoogdt N, Vanhove C, Xavier C, Boerman O, Muyldermans S, Bossuyt A, Lahoutte T. Localization, mechanism and reduction of renal retention of technetium-99m labelled epidermal growth factor receptor-specific nanobody in mice. *Contrast Media Mol. Imaging.* 2011; **6**: 85-92.

[13]   Tijink BM, Laeremans T, Budde M, Stigter-van Walsum M, Dreier T, de Haard HJ, Leemans CR, van Dongen GAMS. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. *Mol. Cancer Ther.* 2008; **7**: 2288-2297.

[14]   D'Huyvetter M, Aerts A, Xavier C, Vaneycken I, Devoogdt N, Gijs M, Impens N, Baatout S, Ponsard B, Muyldermans S, Caveliers V, Lahoutte T. Development of 177Lu-nanobodies for radioimmunotherapy of HER2-positive breast cancer: evaluation of different bifunctional chelators. *Contrast Media Mol. Imaging* 2012; **7**: 254-264.

[15]   Koopmans KP, Glaudemans AW. Rationale for the use of radiolabelled peptides in diagnosis and therapy. *Eur. J. Nucl. Med. Mol. Imaging* 2012; **39**: S4-10.

[16]   Knight LC. Non-oncologic applications of radiolabeled peptides in nuclear medicine. *Q. J. Nucl. Med.* 2003; **47**: 279-291.

[17]   Vernon A, Ballard C, Modo M. Neuroimaging for Lewy body disease: Is the in vivo molecular imaging of α-synuclein neuropathology required and feasible? *Brain Res. Rev.* 2010; **65**: 28-55.

[18]   Cherry SR. The 2006 Henry N. Wagner Lecture: Of mice and men (and positrons) - advances in PET imaging technology. *J. Nucl. Med.* 2006; **47**: 1735-1745.

[19]   Chen K, Conti PS. Target-specific delivery of peptide-based probes for PET imaging. *Adv. Drug Deliv. Rev.* 2010; **62**: 1005-1022.

[20]   Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes. Dev.* 2003; **17**: 545-580.

[21]   Trott CM, Ouyang J, El Fakhri G. Comparison of simultaneous and sequential SPECT imaging for discrimination tasks in assessment of cardiac defects. *Phys. Med. Biol.* 2010; **55**: 6897-6910.

[22]   El Fakhri G, Moore SC, Maksud P, Aurengo A, Kijewski MF. Absolute Activity Quantitation in Simultaneous ^{123}I/^{99m}Tc Brain SPECT. *J. Nucl. Med.* 2001; **42**: 300-308.

[23]   Fani M, Maecke HR, Okarvi SM. Radiolabeled Peptides: Valuable Tools for the Detection and Treatment of Cancer. *Theranostics* 2012, **2**: 481-501.

[24]   Eberle AN, Mild G. Receptor-mediated tumor targeting with radiopeptides. Part 1. General principles and methods. *J. Recept. Signal Transduct. Res.* 2009; **29**: 1-37.

[25]   Verbruggen A, Coenen HH, Deverre J, Guilloteau D, Langstrom B, Salvadori PA, Halldin C. Guideline to regulations for radiopharmaceuticals in early phase clinical trials in the EU. *Eur. J. Nucl. Med. Mol. Imaging* 2008; **35**: 2144-2151.

[26]   Ramachandran GN, Sasisekharan V. Conformaton of polypeptides and proteins. *Adv. Protein Chem.* 1968, **23**: 283-438.

[27]   Liapakis G, Fitzpatrick D, Hoeger C, Rivier J, Vandlen R, Reisine T. Identification of Ligand Binding Determinants in the Somatostatin Receptor Subtypes 1 and 2. *J. Biol. Chem.* 1996; **271**: 20331-20339.

[28]   Gomes I, Grushko JS, Golebiewska U, Hoogendoorn S, Gupta A, Heimann AS, Ferro ES, Scarlata S, Fricker LD, Devi LA. Novel endogenous peptide agonists of cannabinoid receptors. *FASEB J.* 2009; **23**: 3020-3029.

[29]   Kopchick JJ, Parkinson C, Stevens EC, Trainer PJ. Growth hormone receptor antagonists: discovery, development and use in patients with acromegaly. *Endocr. Rev.* 2002; **23**: 623-646.

[30]   Loot AE, van Buiten A, Roks AJ, Henning RH. The suitability of iodinated Angiotensin-(1-7) peptides as pharmacological tools. *J. Pharmacol. Toxicol. Methods* 2005; **51**: 51-55.

[31]   Tait JF, Smith C, Levashova Z, Patel B, Blankenberg FG, Vanderheyden JL. Improved detection of cell death in vivo with annexin V radiolabeled by site-specific methods. *J. Nucl. Med.* 2006; **47**: 1546-1553.

[32]   Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JD, Shire SJ, Bjork N, Totpal K, Chen AB. Identification of multiple sources of charge heterogeneity in a recombinant antibody. *J. Chromatogr. B Biomed. Sci. Appl.* 2001; **752**: 233-245.

[33]   Fani M, Maecke HR. Radiopharmaceutical development of radiolabelled peptides. *Eur. J. Nucl. Med. Mol. Imaging.* 2012; **39**: S11-30.

[34]   Jakubowski N, Messerschmidt J, Anorbe MG, Waentig L, Hayen H, Roos PH. Labelling of proteins by use of iodination and detection by ICP-MS. *J. Anal. At. Spectrom.* 2008; **23**: 1487-1496.

[35]   Voet D, Voet JG, Pratt CW. *Fundamentals of Biochemistry,* Hoboken, Wiley, **2006**.

[36]   Schumacher TN, Mayr LM, Minor DL Jr, Milhollen MA, Burgess MW, Kim PS. Identification of D-peptide ligands through mirror-image phage display. *Science* 1996; **271**: 1854-1857.

[37]   Modlin IM, Pavel M, Kidd M, Gustafsson BI. Review article: somatostatin analogues in the treatment of gastroenteropancreatic neuroendocrine (carcinoid) tumours. *Aliment. Pharmacol. Ther.* 2010; **31**: 169-88.

[38]   Lawrence EO, inventor; Research Corporation N.Y., assignee. Method and apparatus for the acceleration of ions. **1934**, United States patent US 1948384.

[39]   Rathore N, Gellman SH, de Pablo JJ. Thermodynamic stability of beta-peptide helices and the role of cyclic residues. *Biophys. J.* 2006; **9**: 3425-3435.

[40]   Seebach D, Matthews JL. Beta-peptides: a surprise at every turn. *Chem. Comm.* 1997; **21**: 2015-2022.

[41]   Schiller PW, Nguyen TMD, Berezowska I, Dupuis S. Synthesis and in vitro opioid activity profiles of DALDA analogues. *Eur. J. Med. Chem.* 2000, **35**: 895–901.

[42]   Grünewald J, Hunt GS, Dong L, Niessen F, Wen BG, Tsao ML, Perera R, Kang M, Laffitte BA, Azarian S, Ruf W, Nasoff M, Lerner RA, Schultz PG, Smider VV. Mechanistic studies of the immunochemical termination of self-tolerance with unnatural amino acids. *Proc. Natl. Acad. Sci. U. S. A.* 2009; **106**: 4337-42.

[43]   Shirakawa M, Yamamto T, Nakai K, Aburai K, Kawatobi S, Tsurubuchi T, Yamamoto Y, Yokoyama Y, Okuno H, Matsumura A. Synthesis and evaluation of a novel liposome containing BPA-peptide conjugate for BNCT. *Appl. Radiat. Isot.* 2009; **67**: S88-90.

[44]   Bischoff R, Cordier Y, Rasmussen UB, Schlesinger Y, Gachet C, Jaquinod M, Tripet B, Chong PC, Pavirani A. Synthesis and characterization of photoactivatable peptide agonists of the human thrombin receptor. *FEBS Lett.* 1994; **349**: 301-306.

[45]   Cremonesi M, Ferrari M, Bodei L, Tosi G, Paganelli G. Dosimetry in Peptide Radionuclide Receptor Therapy: A Review. *J. Nucl. Med.* 2006; **47**: 1467-1475.

[46]   Kassis AI, Adelstein SJ. Radiobiologic Principles in Radionuclide Therapy. J Nucl Med 2005; 46: 4S-12S.

[47]   European Directorate for the Quality of Medicines and HealthCare, European Pharmacopoeia 7.5. Strassbourg: Council of Europe 2011.

[48]   Shwartz SC, inventor; Ohana I, inventor; U.C.G. Technologies, assignee. SPECT gamma camera. United States patent, 2005, US 6943355 B2.

[49]   Dadachova E. Cancer Therapy with Alpha-Emitters Labeled Peptides. *Semin. Nucl. Med.* 2010; **40**: 204-208.

[50]   LBNL Isotopes Project – LUNDS Universitet. WWW Table of Radioactive Isotopes [homepage on the Internet]. Firestone RB, Ekström LP [August 2012]. Available from: http://ie.lbl.gov/toi/index.asp

[51]   Mausner LF, Srivastava SC. Selection of radionuclides for radioimmunotherapy. *Med. Phys.* 1993; **20**: 503-509.

[52]   Bushberg JT, Seibert JA, Leidholdt EM, Boone JM. *The essential physics of medical imaging,* Lippincott Williams & Wilkins, Philadelphia, **2001**.

[53]   National Research Council (US) and Institute of Medicine (US) Committee on State of the Science of Nuclear Medicine. *Advancing Nuclear Medicine Through Innovation,* National Academies Press (US), Washington (DC), **2007**.

[54]   Lebowitz E, Richards P. Radionuclide Generator Systems. *Semin. Nucl. Med.* 1974; **4**: 257-268.

[55]   Miller BT. Acylation of Peptide Hydroxyl Group with the Bolton-Hunter Reagent. *Biochem. Biophys. Res. Commun.* 1996; **218**: 377-382.

[56]   Lane DJ, Richardson DR. William Hunter and radioiodination: revolutions in the labelling of proteins with radionuclides of iodine. *Biochem. J.* **2011**; doi: 10.1042/BJ20111293.

[57]   Khan IU, Beck-Sickinger AG. Targeted Tumor Diagnosis and Therapy with Peptide Hormones as Radiopharmaceuticals. *Anticancer Agents Med. Chem.* 2008; **8**: 186-199.

[58]   Wilbur DS, Hadley SW, Hylarides MD, Abrams PG, Beaumier PA, Morgan AC, Reno JM, Fritzberg AR. Development of a Stable Radioiodinating Reagent to Label Monoclonal Antibodies for Radiotherapy of Cancer. *J. Nucl. Med.* 1989; **30**: 216-226.

[59]   Brechbiel MW. Bifunctional Chelates for Metal Nuclides. *Q. J. Nucl. Med. Mol. Imaging* 2008; **52**: 166-173.

[60]   Liu S. Bifunctional coupling agents for radiolabeling of biomolecules and target-specific delivery of metallic radionuclides. *Adv. Drug. Deliv. Rev.* 2008; **60**: 1347-1370.

[61]   Bartholomä M.D. Recent developments in the design of bifunctional chelators for metal-based radiopharmaceuticals used in Positron Emission Tomography. *Inorganica Chim. Acta* 2012; **389**: 36–51.

[62]   Bartholomä MD, Louie AS, Valliant JF, Zubieta J. Technetium and Gallium Derived Radiopharmaceuticals: Comparing and Contrasting the Chemistry of Two Important Radiometals for the Molecular Imaging Era. *Chem. Rev.* 2010; **110**: 2903-2920.

[63]   Clarke ET, Martell AE. Stabilities of the Fe(III), Ga(III) and In(III) chelates of N,N',N''-triazacyclononanetriacetic acid. *Inorg. Chim. Acta* 1991; **181**: 273-280.

[64]   Byegard J, Skarnemark G, Skalberg M. The stability of some metal EDTA, DTPA and DOTA complexes: Application as tracers in groundwater studies. *J. Radioanal. Nucl. Chem.* 1999; **241**: 281-290.

[65]   Harris WR, Chen Y, Wein K. Equilibrium Constants for the Binding of Indium(III) to Human Serum Transferrin. *Inorg. Chem.* 1994; **33**: 4991-4998.

[66]   Craft JM, De Silva RA, Lears KA, Andrews R, Liang K, Achilefu S, Rogers BE. In vitro and in vivo evaluation of a 64Cu-labeled NOTA-Bn-SCN-Aoc-bombesin analogue in gastrin-releasing peptide receptor expressing prostate cancer. *Nucl. Med. Biol.* 2012; **39**: 609-616.

[67]   Cooper MS, Ma MT, Sunassee K, Shaw KP, Williams JD, Paul RL, Donnelly PS, Blower PJ. Comparison of 64Cu-Complexing Bifunctional Chelators for Radioimmunoconjugation: Labeling Efficiency, Specific Activity, and in Vitro/in Vivo Stability. *Bioconjugate Chem.* 2012; **23**: 1029-1039.

[68]   Dijkgraaf I, Liu S, Kruijtzer J, Soede AC, Oyen W, Liskamp R, Corstens F, Boerman O. Effects of linker variation on the in vitro and in vivo characteristics of an 111In-labeled RGD peptide. *Nucl. Med. Biol.* 2007; **34**: 29-35.

[69]   Autio A, Henttinen T, Sipilä HJ, Jalkanen S, Roivainen A. Mini-PEG spacering of VAP-1-targeting 68Ga-DOTAVAP-P1 peptide improves PET imaging of inflammation. *EJNMMI Res.* 2011; **1**: 1-7.

[70]   Harris JM, Chess RB. Effect of PEGylation on pharmaceuticals. *Nat. Rev. Drug Discov.* 2003; **2**: 214-221.

[71]   Vergote V, Bodé S, Peremans K, Vanbree H, Baert B, Slegers G, Burvenich C, De Spiegeleer B. Analysis of iodinated peptides by LC-DAD/ESI ion trap mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2007; **850**: 213-220.

[72]   Bailey GS. The Chloramine T Method for Radiolabeling Protein, In *The Protein Protocols Handbook* (Ed: Walker JM), Humana Press, Totowa, **2002**.

[73]   Vergote V, Baert B, Vandermeulen E, Peremans K, van Bree H, Slegers G, Burvenich C, De Spiegeleer B. LC-UV/MS characterization and DOE optimization of the iodinated peptide obestatin. *J. Pharm. Biomed. Anal.* 2008; **46**: 127-136.

[74]   Salacinski PR, McLean C, Sykes JE, Clement-Jones VV, Lowry P. Iodination of Proteins, Glycoproteins, and Peptides Using a Solid-Phase Oxidizing Agent, 1,3,4,6-Tetrachloro-3α,6α-diphenyl Glycoluril (Iodogen). *Anal. Biochem.* 1981; **117**: 136-146.

[75]   Bailey GS. The Iodogen Method for Radiolabeling Protein, In *The Protein Protocols Handbook* (Ed: Walker JM), Humana Press, Totowa, **2002**.

[76]   Hunter WM, Greenwood FC. Preparation of Iodine-131 Labelled Human Growth Hormone of High Specific Activity. *Nature* 1962; **194**: 495-496.

[77]   Yamada A, Traboulsi A, Dittert L, Hussain AA. Chloramine-T in Radiolabeling Techniques. III. Radioiodination of Biomolecules Containing Thioether Groups. *Anal. Biochem.* 2000; **277**: 232-235.

[78]   Marchalonis JJ. An Enzymic Method for the Trace Iodination of Immunoglobulins and other Proteins. *Biochem. J.* 1969; **113**: 299-305.

[79]   Huber RE, Edwards LA, Carne TJ. Studies on the Mechanism of the Iodination of Tyrosine by Lactoperoxidase. *J. Biol. Chem.* 1989; **264**: 1381-1386.

[80]   Markwell MA. A New Solid-State Reagent to Iodinate Proteins. *Anal. Biochem.* 1982; **125**: 427-432.

[81]   Bailey GS. The Bolton and Hunter Method for Radiolabeling Protein, In *The Protein Protocols Handbook* (Ed: Walker JM), Humana Press, Totowa, **2002**.

[82]   Meares CF, McCall MJ, Reardan DT, Goodwin DA, Diamanti CI, McTigue M. Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent addition of metal ions. *Anal. Biochem.* 1984; **142**: 68-78.

[83]   Tolmachev V, Xu H, Wållberg H, AhlgrenS, Hjertman M, Sjöberg A, Sandström M, Abrahmsén L, Brechbiel MW, Orlova A. Evaluation of a maleimido derivative of CHX-A" DTPA for site-specific labeling of Affibody molecules. *Bioconjug. Chem.* 2008; **19**: 1579–1587.

[84]   Shen BQ, Xu K, Liu L, Raab H, Bhakta S, Kenrick M, Parsons-Reponte KL, Tien J, Yu SF, Mai E, Li D, Tibbitts J, Baudys J, Saad OM, Scales SJ, McDonald PJ, Hass PE, Eigenbrot C, Nguyen T, Solis WA, Fuji RN, Flagella KM, Patel D, Spencer SD, Khawli LA, Ebens A, Wong WL, Vandlen R, Kaur S, Sliwkowski MX, Scheller RH, Polakis P, Junutula JR. Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol.* 2012; **30**: 184-189.

[85]   Palanki MS, Bhat A, Lappe RW, Liu B, Oates B, Rizzo J, Stankovic N, Bradshaw C. Development of novel linkers to conjugate pharmacophores to a carrier antibody. *Bioorg. Med. Chem. Lett.* 2012; **22**: 4249-4253.

[86]   Liu S, Liu H, Jiang H, Xu Y, Zhang H, Cheng Z. One-step radiosynthesis of 18F-AlF-NOTA-RGD2 for tumor angiogenesis PET imaging. *Eur. J. Nucl. Med. Mol. Imaging* 2011; **38**: 1732-1741.

[87]   McBride WJ, D'Souza CA, Sharkey RM, Goldenberg DM. The radiolabeling of proteins by the [18F]AlF method. *Appl. Radiat. Isot.* 2012; **70**: 200-204.

[88]   Jacobson O, Zhu L, Ma Y, Weiss ID, Sun X, Niu G, Kiesewetter DO, Chen X. A rapid and simple one-step F-18 labeling of peptides. *Bioconjug. Chem.* 2011; **22**: 422-428.

[89]   Campbell-Verduyn LS, Mirfeizi L, Schoonen AK, Dierckx RA, Elsinga PH, Feringa BL. Strain-promoted copper-free "click" chemistry for 18F radiolabeling of bombesin. *Angew. Chem. Int. Ed. Engl.* 2011; **50**: 11117-11120.

[90]   Chersi A, Trinca ML, Camera M. 14C-labeling of synthetic peptides. *J. Immunol. Methods* 1988; **110**: 271-273.

[91]   Schumacher TN, Tsomides TJ. In Vitro Radiolabeling of Peptides and Proteins. *Curr. Protoc. Protein. Sci.* **2001**; doi: 10.1002/0471140864.ps0303s00.

[92]   Vergote V, Burvenich C, Van de Wiele C, De Spiegeleer B. Quality specifications for peptide drugs: a regulatory-pharmaceutical approach. *J. Pept. Sci.* 2009; **15**: 697-710.

[93]   Eisenhut M, Haberkorn U. Molecular Position of Radiolabels and Its Impact on Functional Integrity of Proteins. *J. Nucl. Med.* 2006; **47**: 1400-1402.

[94]   Van Dorpe S, Verbeken M, Wynendaele E, De Spiegeleer B. Purity Profiling of Peptide Drugs. *J. Bioanal. Biomed.* 2011; **S6**: doi 10.4172/1948-593X.S6-003.

[95]   Verbeken M, Wynendaele E, Lefebvre RA, Goossens E, De Spiegeleer B. The influence of peptide impurity profiles on functional tissue-organ bath response: The 11-mer peptide INSL6[151-161] case. *Anal. Biochem.* 2012; **421**: 547-555.

[96]   ICH: harmonisation for better health [homepage on the Internet]. Topics under Harmonisation [August 2012]. Available from: http://www.ich.org/

[97]   European Medicines Agency: Science, Medicines, Health [homepage on the Internet]. Concept paper on the revision of the guideline on radiopharmaceuticals based on monoclonal antibodies [August 2012]. Available from: http://www.ema.europa.eu/

[98]   European Medicines Agency: Science, Medicines, Health [homepage on the Internet]. Guideline on radiopharmaceuticals [August 2012]. Available from: http://www.ema.europa.eu/

[99]   Farstad BS, Penuelas I. Radiopharmaceutical Manufacturing, In *Pharmaceutical Manufacturing Handbook: Production and Processes* (Ed: Gad SC), Wiley, Hoboken, **2007**.

[100]   Meyer G, Coenen HH, Waters SL, Langström RC, Strijckmans K, Vaalburg W, Halldin C, Crouzel C, Mazière B, Luxen A. Quality Assurance and Quality Control of Short-Lived Radiopharmaceuticals for PET, In *Radiopharmaceuticals for Positron Emission Tomography: Methodological Aspects* (Eds: Stöcklin G, Pike VW), Kluwer Academic Publishers, Dordrecht, **2010**.

[101]   The United States Pharmacopeia, USP 33/NF 28. The Standard of Quality. USP Monographs: Indium In 111 Pentetreotide Injection. Rockville: The United States Pharmacopeial Convention 2010.

[102]   Hnatowich DJ. A method for the preparation and quality control of 68Ga radiopharmaceuticals. *J. Nucl. Med.* 1975; **16**: 764-768.

[103]   Vallabhajosula S, Killeen RP, Osborne JR. Altered Biodistribution of Radiopharmaceuticals: Role of Radiochemical/Pharmaceutical Purity, Physiological, and Pharmacologic Factors. *Semin. Nucl. Med.* 2010; **40**: 220-241.

[104]   The United States Pharmacopeia, USP 33/NF 28. The Standard of Quality. Rockville: The United States Pharmacopeial Convention 2010.

[105]   Amin KC, Patel S, Doke A, Saha GB. An Alternative Method for Determining the Radiochemical Purity of 99mTc-Tetrofosmin. *J. Nucl. Med. Technol.* 2011; **39**: 51-54.

[106]   The United States Pharmacopeia, USP 33/NF 28. The Standard of Quality. USP Monographs: Indium In 111 Ibritumomab Tiuxetan Injection. Rockville: The United States Pharmacopeial Convention 2010.

[107]   European Directorate for the Quality of Medicines and HealthCare, European Pharmacopoeia 7.5. 01/2008:1918 Fluorodopa (18F) (prepared by electrophilic substitution) injection. Strassbourg: Council of Europe 2011.

[108]   European Directorate for the Quality of Medicines and HealthCare, European Pharmacopoeia 7.5. 01/2009:0296 Technetium (99mTc) macrosalb injection. Strassbourg: Council of Europe 2011.

[109]   The United States Pharmacopeia, USP 33/NF 28. The Standard of Quality. USP Monographs: Technetium Tc 99m Albumin Aggregated Injection. Rockville: The United States Pharmacopeial Convention 2010.

[110]   Petersson J, Sánchez-Crespo A, Larsson SA, Mure M. Physiological imaging of the lung: single-photon-emission computed tomography (SPECT). *J. Appl. Physiol.* 2007; **102**: 468-476.

[111]   The United States Pharmacopeia, USP 33/NF 28. The Standard of Quality. USP Monographs: Fluorodopa F 18 Injection. Rockville: The United States Pharmacopeial Convention 2010.

[112]   Lapi SE, Welch MJ. A historical perspective on the specific activity of radiopharmaceuticals: what have we learned in the 35 years of the ISRC? *Nucl. Med. Biol.* 2012; **39**: 601-608.

[113]   AltaBioscience [homepage on the Internet]. Introduction to Synthetic Peptides [October 2012]. Available from: http://www.altabioscience.com/

[114]   European Directorate for the Quality of Medicines and HealthCare, European Pharmacopoeia 7.5. 01/2010:20256 Amino acid analysis. Strassbourg: Council of Europe 2011.

[115]   The United States Pharmacopeia, USP 33/NF 28. The Standard of Quality. General Chapters: 1052 Biotechnology-derived articles: amino acid analysis. Rockville: The United States Pharmacopeial Convention 2010.

[116]   Food and Drug Administration. Guidance for Industry for the Submission of Chemistry, Manufacturing, and Controls Information for Synthetic Peptide Substances, 2004.

[117]   Swietlow A, Lax R. Quality control in peptide manufacturing: specifications for GMP peptides. *Pept. Chem.* 2004; **7**: 22-4.

[118]   Brom M, Joosten L, Oyen WJG, Gotthardt M, Boerman OC. Improved labelling of DTPA- and DOTA- conjugated peptides and antibodies with ^{111}In in HEPES and MES buffer. *EJNMMI Res.* **2012**; doi: 10.1186/2191-219X-2-4

[119]   Kwekkeboom D, Krenning EP, de Jong M. Peptide Receptor Imaging and Therapy. *J. Nucl. Med.* 2000; **41**: 1704-1713.

[120]   Breeman WAP, Kwekkeboom DJ, Kooij PPM, Bakker WH, Hofland LJ, Visser TJ, Ensing GJ, Lamberts SWJ, Krenning EP. Effect of Dose and Specific Activiy on Tissue Distribution of Indium-111-Pentetreotide in Rats. *J. Nucl. Med.* 1995; **36**: 623-627.

[121]   Salako QA, O'Donnell RT, DeNardo SJ. Effects of Radiolysis on Yttrium-90-Labeled Lym-1 Antibody Preparations. *J. Nucl. Med.* 1998; **39**: 667-670.

[122]   Reilly RM. *Monoclonal Antibody and Peptide-Targeted Radiotherapy of Cancer*, Wiley, Hoboken, 2010.

[123]   European Medicines Agency: Science, Medicines, Health [homepage on the Internet]. European public assessment reports [August 2012]. Available from: http://www.ema.europa.eu/

[124]   Liu S, Edwards DS. Stabilization of 90Y-Labeled DOTA-Biomolecule Conjugates Using Gentisic Acid and Ascorbic Acid. *Bioconjugate Chem.* 2001; **12**: 554-558.

[125]   Chakrabarti MC, Le N, Paik CH, De Graff WG, Carrasquillo JA. Prevention of Radiolysis of Monoclonal Antibody during Labeling. *J. Nucl. Med.* 1996; **37**: 1384-1388.

[126]   Pujatti PB, Massicano AV, Mengatti J, de Araújo EB. Preparation of [In-111]-labeled-DTPA-bombesin conjugates at high specific activity and stability: Evaluation of labelling parameters and potential stabilizers. *Appl. Radiat. Isot.* 2012; **70**: 856-863.

[127]   Wahl RL, Wissing J, del Rosario R, Zasadny KR. Inhibition of Autoradiolysis of Radiolabeled Monoclonal Antibodies by Cryopreservation. *J. Nucl. Med.* 1990; **31**: 84-89.

[128]   Ma D, Lu F, Overstreet T, Milenic DE, Brechbiel MW. Novel chelating agents for potential clinical applications of copper. *Nucl. Med. Biol.* 2002; **29**: 91-105.

[129]   Bass LA, Wang M, Welch MJ, Anderson CJ. In Vivo Transchelation of Copper-64 from TETA-Octreotide to Superoxide Dismutase in Rat Liver. *Bioconjugate Chem.* 2000; **11**: 527-532.

[130]   De Spiegeleer B, Van Dorpe S, Vergote V, Wynendaele E, Pauwels E, Van De Wiele C, Garcia-Solis P, Solis-Sainz JC. In vitro metabolic stability of iodinated obestatin peptides. *Peptides* 2012; **33**: 272-278.

[131]   Vergote V, Van Dorpe S, Peremans K, Burvenich C, De Spiegeleer B. In vitro metabolic stability of obestatin: Kinetics and identification of cleavage products. *Peptides* 2008; **29**: 1740-1748.

[132]   Karstad R, Isaksen G, Wynendaele E, Guttormsen Y, De Spiegeleer B, Brandsdal BO, Svendsen JS, Svenson J. Targeting the S1 and S3 subsite of trypsin with unnatural cationic amino acids generates antimicrobial peptides with potential for oral administration. *J. Med. Chem.* 2012; **55**: 6294-6305.

[133]   Mitulovic G, Mechtler K. HPLC techniques for proteomics analysis – a short overview of latest developments. *Brief Funct. Genomic. Proteomic.* 2006; **5**: 249-260.

[134]   Yoshida T. Peptide separation by Hydrophilic-Interaction Chromatography: a review. *J. Biochem. Biophys. Methods* 2004; **60**: 265-280.

[135]   Chakraborty AB, Berger SJ. Optimization of Reversed-Phase Peptide Liquid Chromatography Ultraviolet Mass Spectrometry Analyses Using an Automated Blending Methodology. *J. Biomol. Tech.* 2005; **16**: 325-333.

[136]   Stulik K, Pacakova V, Suchankova J, Claessens HA. Stationary phases for peptide analysis by high performance liquid chromatography: a review. *Anal. Chim. Acta* 1997; **352**: 1-19.

[137]   Mant CT, Cepeniene D, Hodges RS. Reversed-phase HPLC of peptides: Assessing column and solvent selectivity on standard, polar-embedded and polar endcapped columns. *J. Sep. Sci.* 2010; **33**: 3005-3021.

[138]   Van Dorpe S, Vergote V, Pezeshki A, Burvenich C, Peremans K, De Spiegeleer B. Hydrophilic interaction LC of peptides: Columns comparison and clustering. *J. Sep. Sci.* 2010; **33**: 728-739.

[139]   Alpert AJ. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* 1990; **499**: 177-196.

[140]   Reubi JC. Peptide Receptors as Molecular Targets for Cancer Diagnosis and Therapy. *Endocr. Rev.* 2003; **24**: 389-427.

[141]   Schottelius M, Wester H. Molecular imaging targeting peptide receptors. *Methods* 2009; **48**: 161-177.

[142]   Vergote V, Van Dorpe S, Verbeken M, Burvenich C, Van de Wiele C, Banks WA, De Spiegeleer B. Development of peptide receptor binding assays: Methods to avoid false negatives. *Regul. Pept.* 2009; **158**: 97-102.

[143]   Bylund DB, Toews ML. Radioligand binding methods: practical guide and tips. *Am. J. Physiol.* 1993; **265**: L421-L429.

[144]   Davenport AP, Russell FD. Radioligand binding assays: theory and practice, In *Current Directions in Radiopharmaceutical Research and Development,* (Ed: Mather SJ), Springer, Heidelberg, **1996**.

[145]   Hulme EC, Trevethick MA. Ligand binding assays at equilibrium: validation and interpretation. *Br. J. Pharmacol.* 2010; **161**: 1219-1237.

[146]   Molenaar P, O'Reilly G, Sharkey A, Kuc RE, Harding DP, Plumpton C, Gresham GA, Davenport AP. Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circ. Res.* 1993; **72**: 526-538.

[147]   Fu Y, Létourneau M, Chatenet D, Dupuis J, Fournier A. Characterization of iodinated adrenomedullin derivatives suitable for lung nuclear medicine. *Nucl. Med. Biol.* 2011; **38**: 867-874.

[148]   Bruns C, Lewis I, Briner U, Meno-Tetang G, Weckbecker G. SOM230: a novel somatostatin peptidomimetic with broad somatotropin release inhibiting factor (SRIF) receptor binding and a unique antisecretory profile. *Eur. J. Endocrinol.* 2002; **146**: 707-716.

[149]   Bender F, Roach P, Tsortos A, Papadakis G, Newton MI, McHale G, Gizeli E. Development of a combined surface plasmon resonance/surface acoustic wace device for the characterization of biomolecules. *Meas. Sci. Technol.* 2009; **20**: 1-6.

[150]   Ginj M, Zhang H, Waser B, Cescato R, Wild D, Wang X, Erchegyi J, Rivier J, Mäcke HR, Reubi JC. Radiolabeled somatostatin receptor antagonists are preferable to agonists for in vivo peptide receptor targeting of tumors. *Proc. Natl. Acad. Sci. U. S. A.* 2006; **103**: 16436-16441.

[151]   Bodei L, Paganelli G, Mariani G. Receptor Radionuclide Therapy of Tumors: A Road from Basic Research to Clinical Applications. *J. Nucl. Med.* 2006; **47**: 375-377.

[152]   Cescato R, Schulz S, Waser B, Eltschinger V, Rivier JE, Wester H, Culler M, Ginj M, Liu Q, Schonbrunn A, Reubi JC. Internalization of sst2, sst3, and sst5 Receptors: Effects of Somatostatin Agonists and Antagonists. *J. Nucl. Med.* 2006; **47**: 502-511.

[153]   Williams SP. Tissue distribution studies of protein therapeutics using molecular probes: molecular imaging. *AAPS J.* 2012; **14**: 389-399.

[154]   Perera RM, Zoncu R, Johns TG, Pypaert M, Lee FT, Mellman I, Old LJ, Toomre DK, Scott AM. Internalization, intracellular trafficking, and biodistribution of monoclonal antibody 806: a novel anti-epidermal growth factor receptor antibody. *Neoplasia* 2007; **9**: 1099-1110.

[155]   Costantini DL, Hu M, Reilly RM. Peptide Motifs for Insertion of Radiolabeled Biomolecules into Cells and Routing to the Nucleus for Cancer Imaging or Radiotherapeutic Applications. *Cancer Biother. Radiopharm.* 2008; **23**: 3-24.

[156]   Kersemans V, Cornelissen B. Targeting the Tumour: Cell Penetrating Peptides for Molecular Imaging and Radiotherapy. *Pharmaceuticals* 2010; **3**: 600-620.

[157]   Torchilin V. Intracellular delivery of protein and peptide therapeutics. *Drug Discov. Today Technol.* 2009; **5**: e95-103.

[158]   Re RN. On the Biological Actions of Intracellular Angiotensin. *Hypertension* 2000; **35**: 1189-1190.

[159]   Fiaschi-Taesch NM, Stewart AF. Minireview: Parathyroid Hormone-Related Protein as an Intracrine Factor – Trafficking Mechanisms and Functional Consequences. *Endocrinology* 2003; **144**: 407-411.

[160]   Aarts MM, Rix A, Guo J, Bringhurst R, Henderson JE. The Nucleolar Targeting Signal (NTS) of Parathyroid Hormone Related Protein Mediates Endocytosis and Nucleolar Translocation. *J. Bone Miner. Res.* 1999; **14**: 1493-1503.

[161]   Liolios CC, Fragogeorgi EA, Zikos C, Loudos G, Xanthopoulos S, Bouziotis P, Paravatou-Petsotas M, Livaniou E, Varvarigou AD, Sivolapenko GB. Structural modifications of 99mTc-labelled bombesin-like peptides for optimizing pharmacokinetics in prostate tumor targeting. *Int. J. Pharm.* 2012; **430**: 1-17.

[162]   Boerman OC, Oyen WJ, Corstens FH. Between the Scylla and Charybdis of peptide radionuclide therapy: hitting the tumor and saving the kidney. *Eur. J. Nucl. Med.* 2001; **28**: 1447-1449.

[163]   Rolleman EJ, Valkema R, de Jong M, Kooij PP, Krenning EP. Safe and effective inhibition of renal uptake of radiolabelled octreotide by a combination of lysine and arginine. *Eur. J. Nucl. Med.* 2003; **30**: 9-15.

[164]   Akizawa H, Uehara T, Arano Y. Renal uptake and metabolism of radiopharmaceuticals derived from peptides and proteins. *Adv. Drug Deliv. Rev.* 2008; **60**: 1319-1328.

[165]   Vegt E, Wetzels JF, Russel FG, Masereeuw R, Boerman OC, van Eerd JE, Corstens FH, Oyen WJ. Renal Uptake of Radiolabeled Octreotide in Human Subjects Is Efficiently Inhibited by Succinylated Gelatin. *J. Nucl. Med.* 2006; **47**: 432-436.

[166]   Siegel JA, Thomas SR, Stubbs JB, Stabin MG, Hays MT, Koral KF, Robertson JS, Howell RW, Wessels BW, Fisher DR, Weber DA, Brill AB. MIRD Pamphlet No. 16: Techniques for Quantitative Radiopharmaceutical Biodistribution Data Acquisition and Analysis for Use in Human Radiation Dose Estimates. *J. Nucl. Med.* 1999; **40**: 37S-61S.

[167]   Van de Wiele C, Dumont F, Dierckx RA, Peers SH, Thornback JR, Slegers G, Thierens H. Biodistribution and Dosimetry of ^{99m}Tc-RP527, a Gastrin-Releasing Peptide (GRP) Agonist for the Visualization of GRP Receptor-Expressing Malignancies. *J. Nucl. Med.* 2001; **42**: 1722-1727.

[168]   Baechler S, Hobbs RF, Boubaker A, Buchegger F, He B, Frey EC, Sgouros G. Three-dimensional radiobiological dosimetry of kidneys for treatment planning in peptide receptor radionuclide therapy. *Med. Phys.* 2012; **39**: 6118-6128.

[169]   Forrer F, Krenning EP, Kooij PP, Bernard BF, Konijnenberg M, Bakker WH, Teunissen JJM, de Jong M, van Lom K, de Herder WW, Kwekkeboom DJ. Bone marrow dosimetry in peptide receptor radionuclide therapy with [^{177}Lu-DOTA^{0},Tyr^{3}]octreotate. *Eur. J. Nucl. Med. Mol. Imaging* 2009; **36**: 1138-1146.

[170]   Wehrmann C, Senftleben S, Zachert C, Müller D, Baum RP. Results of Individual Patient Dosimetry in Peptide Receptor Radionuclide Therapy with 177Lu DOTA-TATE and 177Lu DOTA-NOC. *Cancer Biother. Radiopharm.* 2007; **22**: 406-416.

[171]   Watson EE, Stabin MG, Siegel JA. MIRD formulation. *Med. Phys.* 1993; **20**: 511-514.

[172]   McCollough CH, Schueler BA. Calculation of effective dose. *Med. Phys.* 2000; **27**: 828-837.

[173]   Stabin MG. Radiopharmaceuticals for Nuclear Cardiology: Radiation Dosimetry, Uncertainties, and Risk. *J. Nucl. Med.* 2008; **49**: 1555-1563.

[174]   Kim JH, Lee JS, Kang KW, Lee H, Han S, Kim T, Lee Y, Jeong JM, Lee DS. Whole-Body Distribution and Radiation Dosimetry of ^{68}Ga-NOTA-RGD, a Positron Emission Tomography Agent for Angiogenesis Imaging. *Cancer Biother. Radiopharm.* 2012; **27**: 65-71.

[175]   Martinez MN. Factors Influencing the Use and Interpretation of Animal Models in the Development of Parenteral Drug Delivery Systems. *AAPS J.* 2011; **13**: 632-648.

[176]   Marchetti S, Schellens JH. The impact of FDA and EMEA guidelines on drug development in relation to Phase 0 trials. *Br. J. Cancer* 2007; **97**: 577-581.

[177]   Kummar S, kinders R, Rubinstein L, Parchment RE, Murgo AJ, Collins J, Pickeral O, Low J, Steinberg SM, Gutierrez M, Yang S, Helman L, Wiltrout R, Tomaszewski JE, Doroshow JH. Compressing drug development timelines in oncology using phase '0' trials. *Nat. Rev. Cancer* 2007; **7**: 131-139.

[178]   Ambrosini V, Fani M, Fanti S, Forrer F, Maecke HR. Radiopeptide imaging and therapy in Europe. *J. Nucl. Med.* 2011; **52**: 42S-55S.

[179]   Medicare Service Advisory Committee's. OctreoScan scintigraphy for gastro-entero-pancreatic neuroendocrine tumours [August 2012]. Available from: http://www.health.gov.au/haf/msac

[180]   European Medicines Agency: Science, Medicines, Health [homepage on the Internet]. European public assessment reports: Zevalin [August 2012]. Available from: http://www.ema.europa.eu/

[181]   FDA, U.S. Food and Drug Administration. Protecting and Promoting Your Health [Homepage on the Internet]. Tositumomab and Iodine I 131 Tositumomab Product Approval Information – Licensing Action 6/27/03 [August 2012]. Available from: http://www.fda.gov/default.htm

[182]   Medicare Service Advisory Committee's. CEA-Scan for imaging recurrence and/or metastases in patients with histologically demonstrated carcinoma of the colon or rectum [August 2012]. Available from: http://www.otago.ac.nz/

[183]   World Health Organization (WHO) [homepage on the Internet]. Cancer, Fact sheet N°297 [updated 2012 February; August 2012]. Available from: http://www.who.int/

[184]   FDA, U.S. Food and Drug Administration. Protecting and Promoting Your Health [Homepage on the Internet]. FDA Approved Drug Products [August 2012]. Available from: http://www.fda.gov/default.htm

[185]   Laverman P, Sosabowski JK, Boerman OC, Oyen WJ. Radiolabelled peptides for oncological diagnosis. *Eur. J. Nucl. Med. Mol. Imaging* 2012; **39**: S78-92.

[186]   Zoller F, Eisenhut M, Haberkorn U, Mier W. Endoradiotherapy in cancer treatment – Basic concepts and future trends. *Eur. J. Pharmacol.* 2009; **625**: 55-62.

[187]   Reubi JC, Waser B. Concomitant expression of several peptide receptors in neuroendocrine tumours: molecular basis for in vivo multireceptor tumour targeting. *Eur. J. Nucl. Med. Mol. Imaging* 2003; **30**: 781-793.

[188]   Zhang X, Xiong Z, Wu Y, Cai W, Tseng JR, Gambhir SS, Chen X. Quantitative PET imaging of tumor integrin αvβ3 expression with 18F-FRGD2. *J. Nucl. Med.* 2006; **47**: 113-121.

[189]   Yu Z, Carlucci G, Ananias HJ, Dierckx RA, Liu S, Helfrich W, Wang F, de Jong IJ, Elsinga PH. Evaluation of a technetium-99m labeled bombesin homodimer for GRPR imaging in prostate cancer. *Amino Acids* 2012; **44**: 543-553.

[190]   Falciani C, Fabbrini M, Pini A, Lozzi L, Lelli B, Pileri S, Brunetti J, Bindi S, Scali S, Bracci L. Synthesis and biological activity of stable branched neurotensin peptides for tumor targeting. *Mol. Cancer Ther.* 2007; **6**: 2441-2448.

[191]   Sampath L, Kwon S, Ke S, Wang W, Schiff R, Mawad ME, Sevick-Muraca EM. Dual-labeled trastuzumab-based imaging agent for the detection of human epidermal growth factor receptor 2 overexpression in breast cancer. *J. Nucl. Med.* 2007; **48**: 1501-1510.

[192]   ter Horst M, Verwijnen SM, Brouwer E, Hoeben RC, de Jong M, de Leeuw BH, Sillevis Smitt PA. Locoregional delivery of adenoviral vectors. *J. Nucl. Med.* 2006; **47**: 1483-1489.

[193]   Roivainen A, Jalkanen S, Nanni C. Gallium-labelled peptides for imaging of inflammation. *Eur. J. Nucl. Med. Mol. Imaging* 2012; **39**: S68-77.

[194]   European Medicines Agency: Science, Medicines, Health [homepage on the Internet]. European public assessment reports: LeukoScan [August 2012]. Available from: http://www.ema.europa.eu/

[195]   European Medicines Agency: Science, Medicines, Health [homepage on the Internet]. European public assessment reports: Scintimun [August 2012]. Available from: http://www.ema.europa.eu/

[196]   Hofland LJ, Capello A, Krenning EP, de Jong M, van Hagen MP. Induction of apoptosis with hybrids of Arg-Gly-Asp molecules and peptides and antimitotic effects of hybrids of cytostatic drugs and peptides. *J. Nucl. Med.* 2005; **46**: 191S-198S.

[197]   Zhang Y, Wang TH. Quantum Dot Enabled Molecular Sensing and Diagnostics. *Theranostics* 2012; **2**: 631-654.

[198]   Akerman ME, Chan WCW, Laakkonen P, Bhatia SN, Ruoslahti E. Nanocrystal targeting in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 2002; **99**: 12617–12621.

SUMMARY & GENERAL CONCLUSIONS

"Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning."

Albert Einstein

SUMMARY & GENERAL CONCLUSIONS

As described in **Chapter I**, the human microbiome recently gathered great attention due to its possible role in the health of the host. The general aim of this research was to further explain this microbiome-host interaction. More specifically, we focussed on its possible association with cancer, a life-threatening and -debilitating pathology. Research in oncology mainly focused on the role of bacterial (geno)toxin production or inflammation-induced carcinogenesis, with the role of the quorum sensing peptides being highly neglected. However, previous results of other quorum sensing signal molecules (*i.e.* AHLs) indicate a possible interaction with mammalian cells, next to the strong correlation observed between the quorum sensing mechanisms and tumour metastasis. Hence, this work was intended to investigate, as an initial, exploratory proof-of-principle, if the human microbiome, through the use of quorum sensing peptides, can influence cancer.

Since many decades, the quorum sensing process is thoroughly described in literature, thereby focussing on *i.a.* molecule identification, pathway elucidation and structure-activity relationships (SAR). In **Chapter II**, this information was summarized through a comprehensive database (Quorumpeps®: http://quorumpeps.ugent.be), presenting the chemical information of the quorum sensing (derived) peptides, next to their species origin and quorum sensing functionality (*i.e.* methodology, target and activity). Currently, 231 quorum sensing peptides (or analogues thereof) are listed in this database, with the possibility of adding new peptide entities by the database users.

The structural information of these quorum sensing peptides, described in the Quorumpeps® database, served as a start of this study: the chemical space of the quorum sensing peptides was investigated using multivariate techniques, *i.a.* in order to select 'model peptides' to explore our crosstalk hypothesis. As described in **Chapter III**, the quorum sensing peptides were categorized in 3 chemically distinct clusters, which again could be subdivided in smaller clusters. Peptide size and compactness, lipophilicity, cyclisation and the presence of sulphur atoms and aromatic amino acids, were mainly found to determine this chemical classification. Moreover, it was noticed that most of the bacterial species produce chemically similar peptides (*i.e.* in same classification), except for *Bacillus subtilis*, for which peptides are described with structures covering the total chemical space of quorum sensing peptides.

Based on these clustering results, a selection of quorum sensing peptides was made to use for further functionality investigations. After chemical synthesis of these peptide structures, identity and

purity was screened in **Chapter IV** in order to correctly correlate peptide structure with the observed biological results. A large number of peptides did not meet our requested purity specifications (> 95% purity) and even contained peptide impurities "outperforming" the desired peptide concentration. Therefore, for these peptides, conclusions should be cautiously made.

These purity results were also used to select quorum sensing peptides for further functionality investigations. As this study was primarily intended to explore the microbiome-mammals crosstalk, and hence not meant to be exhaustive investigating all quorum sensing peptides in detail, we decided to use a purity cut-off value of 80% instead of the intended 95% purity level, *i.e.* peptides with a purity < 80% were excluded in our functionality evaluation.

In **Chapter V**, the interaction between these sufficiently pure quorum sensing peptides and human cancer cells, more particularly with colon cancer cells, was explored. It was clear that some quorum sensing peptides indeed interact with these cells, thereby promoting cancer cell invasion and angiogenesis. Moreover, an initial pathway map was suggested for this process: through the activation of *i.a.* the epidermal growth factor receptor (EGFR) or the IL-6 pathway, tumour progression can be initiated. These first results now open new perspectives on the role and applications of the microbiome on the guest's health, with the possibility of translating these findings into other biological and applied medical fields as well.

The same settings were used in **Chapter VI**, where the influence of quorum sensing peptides on breast cancer cells was further explored. Again, tumour invasion and angiogenesis were induced after quorum sensing peptide treatment, thereby confirming the existence of a crosstalk phenomenon previously observed with colon cancer cells.

Before investigating the effect of the quorum sensing peptides on brain cancer (and other central nervous system diseases), the permeability of these peptides through the blood-brain barrier, once they have reached the blood circulation, was studied. The results of this study are described in **Chapter VII**: some quorum sensing peptides indeed cross the blood-brain barrier, with the more lipophylic peptide showing the highest brain influx (K_{in} = 1.33 µl/(g×min)). These results can stimulate new research on exploring the effect of these quorum sensing peptides on brain (cancer) cells, based on the recent suggestions of a microbiome-brain disease correlation and our findings of tumour-promoting properties for some of these peptides.

In **Chapter VIII**, we analysed the permeability of some quorum sensing peptides through the intestinal barrier. Using a Caco-2 model, some peptides were found to cross this physiological barrier and reach the blood circulation, indicating a possible (tumour-promoting) effect throughout the human body for these quorum sensing peptides; the effects are thus not limited to the site of origin

and research should therefore expand to various cancer tissues. Next to the BBB-penetrating properties of these peptides, these initial intestinal permeability properties also stimulate in-depth pharmacokinetic (barrier) investigations, in order to link microbiota composition with different pathologies. Moreover, most of the quorum sensing peptides were found to remain sufficiently stable in cell medium and human plasma, thereby confirming the *in vitro* and *in vivo* functionality of the investigated peptides. No haemolytic and direct cell-killing effects were observed, next to limited *in silico* probability of organ and tissue toxicity.

Finally, within the DruQuaR tradition, the last chapter encompasses a regulatory-quality theme. **Chapter IX** gives an overview concerning the development of radiolabelled peptide and protein based radiopharmaceuticals. The increasing number of peptides and proteins in research as well as in clinical trial settings can benefit from their radiopharmaceutical equivalents. However, to increase their success rate as radiopharmaceuticals, a thorough and regulatory accepted quality control (including radionuclidic, radiochemical and chemical purity) and subsequent *in vitro* biomedical and *in vivo* pharmacokinetic analyses are indispensable.

Based on the results obtained during this study, our main research question, *i.e.* "Do quorum sensing peptides influence tumour cell behaviour?", could be answered: YES, quorum sensing peptides do interact with human cancer cells, thereby promoting cancer cell invasion and neo-angiogenesis. However, this is only the first step... Although we have laid the foundations for new research concerning the microbiome-disease association, even more questions remain unanswered, awaiting further investigations concerning chemical as well as *in vitro* and *in vivo* aspects.

First, we should investigate if the **other groups of quorum sensing peptides** (*i.e.* lantibiotics and isoprenyl modified peptides) demonstrate (similar) biological effects? As a proof-of-principle, in this initial exploratory study presented here, we only selected quorum sensing peptides from the linear and cyclic (thio)lacton peptide groups. However, the effect of other chemically diverse quorum sensing peptides should be investigated as well. This requires the availability of these chemically rather special peptides, which poses quite some synthetic challenges.

Moreover, the peptide purity is an underestimated concern in the biomedical research field, and we were (and are) still confronted with it in our research as well. Therefore, the investigated peptides with low purity levels should be resynthesized and/or purified at a sufficiently **high purity**, followed by further functional studies. Apart from the observed biological effects due to impurities (and not the main compound), it has also been demonstrated in other biological fields that peptide impurities can antagonize the effect of the main peptide itself, thereby rendering fals negative conclusions. On

SUMMARY & GENERAL CONCLUSIONS

the other hand, if this phenomenon is observed, these peptide impurities can be used as therapeutics to block the effect of the endogenous quorum sensing peptide; radiolabelled analogues of these peptide antagonists may be useful in the diagnosis of cancer.

As demonstrated during this study, quorum sensing peptides influence cancer cell behaviour *in vitro*, promoting cancer cell metastasis behaviour. The effect of the quorum sensing peptides on tumour cell morphology and invasion, revealed an **epithelial-to-mesenchymal (EMT)-like transition** of both the colon and breast cancer cells. These EMT-like characteristics are currently however only visually observed, and thus need to be confirmed biochemically by analysing *e.g.* E-cadherin, vimentin and N-cadherin concentrations after quorum sensing peptide treatment. Confirmation of this EMT behaviour thereby can strengthen our results of metastasis-promoting characteristics of some quorum sensing peptides.

In this research, we also found that some quorum sensing peptides can pass the blood-brain barrier. Therefore, it would be interesting to investigate if these peptides, in accordance with our results on colon and breast cancer cells, induce **brain tumour** cell invasion and angiogenesis. Moreover, the study about the causative relationships between the available quorum sensing peptides and different **neurological disorders** can contribute to the exciting microbiome-brain research as well. For example, the quorum sensing peptide PhrCACET1, which is synthesized by *Clostridium* species and largely passes the blood-brain barrier, and comparable quorum sensing peptide analogues, should be investigated for their effect on brain cancer and neurological disorders. The yet uninvestigated isoprenyl modified peptide structures, which are produced by *Bacillus* species and have similar lipophilic properties as PhrCACET1, should be investigated for their effect on brain (cancer) cells as well.

While we have currently limited our exploration to cell tests and initial kinetic data, we definitely need to know if and to what extent these quorum sensing peptides influence cancer *in vivo*? Therefore, a very burning question is: **Are quorum sensing peptides present in the human body**, under what conditions and to what extend? We already investigated the *in vitro* effect of quorum sensing peptides on human cancer cells and some initial ADME characteristics. However, the presence of these quorum sensing peptides in the human body is not yet demonstrated. Seen the *in vivo* presence of quorum sensing-mediated biofilms in the human body, and the observation of AHL signal molecules in diverse biological samples, the *in vivo* presence of quorum sensing peptides seems very likely. Therefore, to strengthen our obtained results, the *in vivo* existence of quorum sensing peptides should be investigated as well, requiring the analysis in *e.g.* faeces, skin, mucosa, sputum and plasma samples of different stratae of both healthy and diseased (cancer) patients.

Next to the *in vivo* quorum sensing peptide profiles, the **relation with the microbiome** should be clarified as well. It has become clear that quorum sensing peptides are produced under different conditions by different subspecies of bacteria, making this relation 'multi-variate' complex. Studying the microbiota composition, in relation with the quorum sensing peptide profiles, can broaden our knowledge, thereby extending the possible microbiome-cancer relationship already found in our *in vitro* research.

If quorum sensing peptides are found in the human body, it will thus be important to investigate their *in vivo* **behaviour** using appropriate animal models. First, the pro-metastatic effects of the quorum sensing peptides should be investigated after treatment of the mouse tumour model with the purified peptides themselves. Second, gnotobiotic mice should be screened for the presence of quorum sensing peptides, and eventually the effect of these bacteria and their peptides on cancer progression analysed. In a next step, clinical samples of healthy individuals and cancer patients, carrying either metastasized or non-metastasized tumour lesions, should be investigated over time for their quorum sensing peptide profiles, as well as microbiota composition to finally offer convincing and clinically useful insights on the microbiome-cancer outcome relationship.

Although we mainly obtained *in vitro* results during our exploratory research, which need to be confirmed *in vivo* as earlier described, our findings can potentially be of high importance for the patient. If our assumptions are valid (*i.e.* quorum sensing peptides are available *in vivo* in nano- or micromolar concentration and these peptides show pro-metastatic effects *in vivo*), the patient can rationally adapt his/her life style to combat the complex, heterogeneous and multi-variate disease that cancer is. The use of probiotics for example could help to suppress undesired bacteria in the human gut, and thereby diminish the synthesis of pro-metastatic quorum sensing peptides. However, the choice of probiotics should be well considered in function of the disease, as some of these bacteria can possibly synthesize undesirable quorum sensing peptides. The gut microbiota composition can be adapted by variations in diet as well, which in turn may influence the quorum sensing peptide profile of the patient. Hygienic measures can prevent changes in microbiota structure of the gut, next to other tissues like the skin, breast and mouth. Attention should thus be paid to avoid bacterial contaminations of food or materials, which can negatively influence the microbiome of the patient. Future studies should however prove and deepen these as yet hypotheses and reflections, in order to correctly inform, diagnose and/or treat the patients, the ultimate objective of our science.

SAMENVATTING & ALGEMENE CONCLUSIES

"Achter de veranderlijke vormen van de natuur ligt een onveranderlijke zuivere werkelijkheid."

Piet Mondriaan
(°1872 - †1944, Nederlandse schilder)

SAMENVATTING & ALGEMENE CONCLUSIES

Zoals beschreven in **Hoofdstuk I**, geniet het humaan microbioom heel wat aandacht voor zijn mogelijke rol in de beïnvloeding van de gezondheid van de gastheer. Het algemene doel van dit onderzoek bestond er dan ook in de interactie tussen dit microbioom en de gastheer na te gaan; meer specifiek hebben wij ons gericht op de mogelijke associatie met kanker, een levensbedreigende en slopende aandoening. Het algemeen onderzoek naar de rol van de bacteriën bij kanker focust zich momenteel op de invloed van bacteriële toxines of het effect van inflammatie op de tumorontwikkeling. De rol van quorum sensing peptiden, geproduceerd door deze bacteriën, werd echter nog niet onderzocht. Voorgaand onderzoek heeft reeds aangetoond dat bepaalde andere quorum sensing signaalmoleculen (AHLs) kunnen interageren met zoogdiercellen. Daarnaast worden ook heel wat gelijkenissen gezien tussen het quorum sensing mechanisme en het metastase proces bij kanker. Op basis van deze recente bevindingen stelden wij de hypothese dat het microbioom, met behulp van quorum sensing peptiden, het gedrag van kankercellen kan beïnvloeden. In deze thesis hebben we deze hypothese onderzocht, waarbij we voor het eerst de interactie tussen de quorum sensing peptiden en humane kankercellen wensen aan te tonen.

De voorbije decennia werd het quorum sensing proces reeds uitvoerig beschreven in de literatuur, waarbij voornamelijk gefocust werd op molecule identificatie, pathway opheldering en structuur-activiteitsrelaties (SAR) van deze signaalmoleculen bij bacteriën. In **Hoofdstuk II** werd deze uitgebreide informatie samen gebracht in een databank (Quorumpeps: http://quorumpeps.ugent.be), waarbij de chemische informatie van de quorum sensing peptiden (en analogen) wordt weergegeven, naast hun bacteriële oorsprong en quorum sensing functionaliteit (methodologie, receptor en activiteit). Momenteel zijn 231 quorum sensing peptiden in deze databank opgelijst, waarbij gebruikers de mogelijkheid hebben om nieuwe gegevens aan deze databank toe te voegen.

De structurele gegevens van de quorum sensing peptiden, beschreven in de Quorumpeps databank, werden vervolgens geanalyseerd en gebruikt als de start van dit onderzoek: de chemische ruimte van de quorum sensing peptiden werd onderzocht met behulp van multivariate data-analyse, waarna diverse 'modelpeptiden' werden geselecteerd voor verder functioneel onderzoek. Zoals beschreven in **Hoofdstuk III** kunnen de quorum sensing peptiden onderverdeeld worden in 3 chemisch verschillende groepen, waarbij deze zelf kunnen opgesplitst worden in diverse kleinere clusters. De peptide grootte en compactheid, lipofiliciteit, cyclisatie en de aanwezigheid van zwavelatomen en aromatische aminozuren zijn de bepalende factoren voor deze chemische classificatie. Daarenboven

werd opgemerkt dat de meeste bacteriële soorten chemisch gelijkende peptiden synthetiseren (zelfde groepering), met uitzondering van *Bacillus subtilis* waarbij quorum sensing peptiden werden beschreven met uiteenlopende chemische eigenschappen.

De geselecteerde quorum sensing peptiden werden vervolgens synthetisch aangemaakt, waarna de identiteit en zuiverheid werden onderzocht (**Hoofdstuk IV**). Deze zuiverheidsbepaling is noodzakelijk om de verkregen biologische functionaliteitsresultaten correct te correleren met het quorum sensing peptide; peptide onzuiverheden kunnen namelijk sterk de functionaliteit beïnvloeden. Uit de resultaten bleek dat een groot aantal van de aangemaakte quorum sensing peptiden niet de gewenste zuiverheid bevatten (> 95% zuiverheid), waarbij de concentratie aan onzuiverheden de gewenste peptide concentratie zelfs 'overtreft'. De biologische resultaten van deze peptiden dienen dus met voldoende voorzichtigheid te worden geïnterpreteerd.

Deze resultaten werden ook gebruikt bij de selectie van quorum sensing peptiden voor verdere functionaliteitsstudies. Gezien dit onderzoek de intentie had om een eerste inzicht te krijgen in de microbioom-zoogdier interactie, en dus geen allesomvattend onderzoek was waarbij alle quorum sensing peptiden in detail werden bestudeerd, werd een zuiverheidslimiet aangewend van 80%; de quorum sensing peptiden met een zuiverheid < 80% werden dus niet geïncludeerd tijdens de verdere functionaliteitsstudies.

In **Hoofdstuk V** werden de interacties tussen de weerhouden quorum sensing peptiden en humane kankercellen, meer bepaald colonkankercellen, onderzocht. Het was duidelijk dat een aantal quorum sensing peptiden inderdaad interageren met deze cellen, waarbij het invasieve karakter van de tumorcellen en de aanmaak van nieuwe bloedvaten (angiogenese) werd bevorderd. Daarenboven werden een aantal pathways voor de activatie van dit proces voorgesteld: na activatie van o.a. de epidermale groeifactor receptor (EGFR) of de interleukine-6 pathway wordt kanker progressie geïnitieerd. Deze resultaten openen dus nieuwe perspectieven over de rol en toepassingen van het microbioom op de gezondheid van de gastheer, met de mogelijkheid om deze bevindingen in andere biologische en medische gebieden aan te wenden.

Dezelfde benadering werd toegepast in **Hoofdstuk VI**, waarbij de invloed van de quorum sensing peptiden op borstkankercellen verder werd onderzocht. Er werd opnieuw een verhoogde tumorcelinvasie en angiogenese waargenomen na quorum sensing peptide behandeling, waarbij de microbioom-interactie, die initieel werd geobserveerd bij colonkankercellen, dus werd bevestigd.

Alvorens we de effecten van de quorum sensing peptiden onderzoeken op hersentumorcellen (en andere aandoeningen van het centraal zenuwstelsel), is het noodzakelijk om de permeabiliteit van deze peptiden, eens aanwezig in de bloedbaan, doorheen de bloed-hersenbarrière na te gaan. De

resultaten van deze studie worden weergegeven in **Hoofdstuk VII**: een aantal quorum sensing peptiden passeren effectief de bloed-hersenbarrière, waarbij het meest lipofiele peptide de hoogste herseninflux (K_{in} = 1.33 µl/(g×min)) vertoont. Deze resultaten kunnen dus het onderzoek naar de effecten van deze quorum sensing peptiden op hersen(tumor)cellen stimuleren; de herseninflux van bepaalde quorum sensing peptiden kan daarbij nieuwe inzichten bieden in het onderzoek naar de associatie tussen het microbioom en diverse hersenaandoeningen.

In **Hoofdstuk VIII** werd de permeabiliteit van enkele quorum sensing peptiden doorheen de darmwand onderzocht. Met behulp van een Caco-2 model werd aangetoond dat deze quorum sensing peptiden deze fysiologische barrière kunnen doorbreken en op die manier de bloedbaan bereiken. Deze quorum sensing peptiden kunnen dus hun mogelijks (tumor-bevorderend) effect op diverse plaatsen in het lichaam uitoefenen, waarbij hun functionaliteit dus niet wordt beperkt tot de plaats van aanmaak. Het kankeronderzoek naar deze peptiden dient dus te worden uitgebreid naar diverse weefsels, met aandacht voor farmacokinetische (barrière) studies; het onderzoek naar de associatie tussen het microbioom en diverse aandoeningen kan op die manier worden gestimuleerd. Daarenboven werd aangetoond dat de meeste quorum sensing peptiden voldoende stabiel blijven in celmedium en humaan plasma, wat de bekomen *in vitro* en *in vivo* resultaten van de onderzochte peptiden bevestigd. Er werden geen hemolytische en celdodende effecten geobserveerd voor de quorum sensing peptiden, evenals geen *in silico* orgaan- en weefseltoxiciteit.

Tenslotte werd, naar goede gewoonte bij DruQuaR, in het laatste hoofdstuk een regulatoir-kwaliteitsaspect bestudeerd. **Hoofdstuk IX** geeft een overzicht van de technieken die gebruikt worden bij de radiolabelling van peptiden en proteïnen (radiofarmaca). Het toenemend aantal peptiden en proteïnen in zowel onderzoek als klinische trials kan uitgebreid worden met hun radiogemerkte equivalenten. Om het slaagpercentage bij de ontwikkeling van radiofarmaca te verhogen zijn echter grondige en gereguleerde kwaliteitscontroles (inclusief radionuclidische, radiochemische en chemische zuiverheid) noodzakelijk, evenals geschikte *in vitro* biomedische en *in vivo* farmacokinetische testen.

Op basis van de bekomen resultaten kan onze belangrijkste onderzoeksvraag "Kunnen quorum sensing peptide het gedrag van kankercellen beïnvloeden?" worden beantwoord: JA, quorum sensing peptiden kunnen inderdaad interageren met humane kankercellen, waarbij deze de tumorcelinvasie en angiogenese bevorderen. Deze studie is echter slechts een eerste stap… Hoewel we de basis hebben gelegd voor nieuw onderzoek betreffende de associatie tussen het microbioom en diverse

ziekten, blijven er heel wat vragen onbeantwoord die via verdere chemische, *in vitro* en *in vivo* onderzoeken dienen te worden opgelost.

Vooreerst dienen we te onderzoeken of ook de **andere groepen van quorum sensing peptiden** (met name de lantibiotica en isoprenyl-gemodificeerde peptiden) vergelijkbare biologische effecten veroorzaken. Om onze hypothese initieel te verifiëren, hebben wij enkel de lineaire en cyclische quorum sensing peptiden onderzocht naar hun functionaliteit. De effecten van de andere groepen van quorum sensing peptiden dienen dus ook te worden geanalyseerd. De synthese van deze chemisch afwijkende peptiden brengt echter heel wat uitdagingen met zich mee.

De zuiverheid van peptiden is en blijft een onderschatte factor in het biomedisch onderzoek. Daarom zullen de onderzochte onzuivere peptiden opnieuw moeten worden aangemaakt en/of opgezuiverd met een voldoende hoge zuiverheid, waarna hun effect op de tumorcellen opnieuw dient te worden onderzocht. Naast de reeds gerapporteerde biologische effecten van peptide onzuiverheden, kan het effect van het quorum sensing peptide ook worden geblokkeerd door deze peptiden, waarbij dus vals-negatieve resultaten worden bekomen. Wanneer dit fenomeen wordt waargenomen kunnen deze peptide onzuiverheden echter verder worden onderzocht voor hun gebruik als therapeuticum om het effect van de endogeen aanwezig quorum sensing peptiden te blokkeren; radiogemerkte analogen van deze peptiden kunnen daarnaast gebruikt worden als diagnosticum bij kanker.

Zoals werd aangetoond in deze studie beïnvloeden bepaalde quorum sensing peptiden het gedrag van kankercellen *in vitro*, met mogelijks een bevordering van de tumormetastase. Het effect van de quorum sensing peptiden op de tumorcel morfologie en invasie toonde een **epitheliaal-mesenchymale verandering (EMT)** aan van zowel de colon- als borstkankercel. Deze EMT-karakteristieken werden echter enkel visueel waargenomen, waarbij het biochemische aspect (een verandering in o.a. E-cadherine, vimentine en N-cadherine expressie) nog verder dient te worden onderzocht. Een bevestiging van dit EMT-gedrag kan daarbij de metastase-bevorderende eigenschappen van bepaalde quorum sensing peptiden bekrachtigen.

In dit onderzoek werd ook aangetoond dat sommige quorum sensing peptiden de bloed-hersenbarrière kunnen passeren. Het is dus interessant om de **effecten van deze peptiden ook op hersentumorcellen** te onderzoeken. De studie naar het oorzakelijke verband tussen de beschikbare quorum sensing peptiden en diverse **neurologische aandoeningen** kan daarnaast ook bijdragen tot het microbioom-hersen onderzoek. Zo kan het effect van bijvoorbeeld PhrCACET1, een quorum sensing peptiden dat geproduceerd wordt door *Clostridium* species en sterk de bloed-hersenbarrière doorkruist, op hersentumorcellen en neurologische aandoeningen interessante informatie opleveren. De effecten van de isoprenyl-gemodificeerde quorum sensing peptiden, die geproduceerd

worden door *Bacillus* species en vergelijkbare lipofiele eigenschappen vertonen als PhrCACET1, dienen ook te worden onderzocht.

Hoewel wij ons in deze studie hebben beperkt tot celtesten en initiële kinetische studies, is het belangrijk om te onderzoeken of de quorum sensing peptiden ook het gedrag van de tumorcellen *in vivo* beïnvloeden. De vraag "**Zijn quorum sensing peptiden aanwezig in het lichaam**, onder welke omstandigheden en in welke mate?" dient dus absoluut te worden onderzocht. Gezien de *in vivo* aanwezigheid van quorum sensing gemedieerde biofilms en de reeds aangetoonde andere quorum sensing signaalmoleculen (AHL), lijkt ook de aanwezigheid van de quorum sensing peptiden in het lichaam zeer waarschijnlijk. Om onze *in vitro* resultaten kracht bij te zetten, zullen dus diverse biologische matrices (bv. feces, huid, sputum en plasma) van gezonde en zieke (kanker) patiënten moeten worden onderzocht naar de aanwezigheid van quorum sensing peptiden.

Naast de bepaling van het *in vivo* quorum sensing peptide profiel, dient ook de **relatie met het microbioom** te worden uitgeklaard. Het is duidelijk dat quorum sensing peptiden onder diverse omstandigheden en door diverse soorten bacteriën worden aangemaakt, waardoor deze relatie multi-variaat en complex is. Het onderzoek naar de samenstelling van het microbioom, en de verhouding met het quorum sensing peptide profiel, kan onze kennis verbreden en dus de mogelijke relatie tussen het microbioom en kanker uitbreiden.

Wanneer deze quorum sensing peptiden in het lichaam worden aangetoond, zal hun effect vervolgens ook *in vivo* worden onderzocht met behulp van geschikte diermodellen. Vooreerst dient het pro-metastaserend effect van het zuivere quorum sensing peptide in het muis kankermodel te worden nagegaan. Vervolgens moeten muizen met een gekend microbioom (gnotobiont) worden gescreend naar de aanwezigheid van quorum sensing peptiden, en het effect van deze bacteriën en peptiden op tumor progressie geëvalueerd. In een volgende stap zullen klinische stalen van gezonde individuen en kankerpatiënten (met en zonder metastase letsels) in functie van de tijd moeten worden geanalyseerd naar zowel hun quorum sensing peptide profiel als microbioom samenstelling, om uiteindelijk overtuigende en klinisch bruikbare inzichten te krijgen in de relatie tussen het microbioom en kanker.

Hoewel wij in deze studie enkel *in vitro* resultaten hebben verkregen, welke dus *in vivo* moeten worden bevestigd, kunnen onze resultaten mogelijks van betekenis zijn voor de patient zelf. Indien onze aannames geldig blijken (*i.e.* quorum sensing peptiden zijn aanwezig in het lichaam in nano- of micromolaire concentraties en deze peptiden vertonen ook pro-metastaserende effecten *in vivo*), zal de patiënt zijn levensstijl rationaal moeten wijzigen om de complexe, heterogene en multi-variate

ziekte die kanker is te verslaan. Zo zou bijvoorbeeld het gebruik van probiotica kunnen helpen bij de onderdrukking van ongewenste bacteriën in de darm, en op die manier de synthese van metastase-bevorderende quorum sensing peptiden helpen verminderen. De keuze van de probiotica dient echter goed te worden overwogen, gezien bepaalde van deze bacteriën zelf ongewenste quorum sensing peptiden kunnen aanmaken en op die manier de progressie van de tumor kunnen beïnvloeden. De samenstelling van het microbioom van de darm kan ook wijzigen na variaties in de voeding, welke dus opnieuw het quorum sensing peptide profiel van de patiënt kan aanpassen. Daarnaast kunnen hygiënische maatregelen een verandering in het microbioom van de darm verminderen, evenals de huid, de borst en de mond. Er dient dus voldoende aandacht te worden besteed aan hygiëne om veranderingen in het microbioom ten gevolgd van bacteriële contaminaties (voeding en materialen) te vermijden. Verdere studies dienen deze diepgaande gedachten echter uit te werken, zodat de patiënt correct kan worden geïnformeerd.

CURRICULUM VITAE

"Succes is not the key to happiness, happiness is the key to success."

Albert Schweitzer
(°1875 - †1965, German philosopher)

EVELIEN WYNENDAELE

PERSONALIA

Surname:	Wynendaele
First name:	Evelien
Nationality:	Belgian
Place & date of birth:	Zottegem, 17 April 1986
Private address:	Ledebergstraat 40
	9550 Hillegem
Professional address:	Drug Quality & Registration (DruQuaR) laboratory
	Department of Pharmaceutical Analysis
	Faculty of Pharmaceutical Sciences
	Ghent University
	Harelbekestraat 72
	9000 Ghent
	Belgium
Phone:	(+32) 9 264 80 94
E-mail:	Ejwynend.Wynendaele@UGent.be
URL:	http://www.ugent.be/DruQuaR

EDUCATION

1998 – 2004:	Science-Maths high school at Sint-Paulusinstituut, Herzele
2004 – 2009:	Master of Science in Drug Development at Ghent University
2010:	'Molecular simulations of biosystems and *in-silico* screening' at Ghent University
2011:	'Proefdierkunde voor Biotechniekers FELASA niveau B' at Hogeschool Gent

2011:   'Pharmaceutical Bioinformatics' at Uppsala University

2012:   'Your Research and Legal Rights: IP, contracts, copyright,…' at Ghent University

2013:   'Project Management' at Ghent University

2013:   'Personal Effectiveness' at Ghent University

2014:   'Module 1: Introduction to SAS' at Institute for Continuing Education in Science (ICES) of Ghent University

2014:   'Module 3: Introduction to R' at Institute for Continuing Education in Science (ICES) of Ghent University

PROFESSIONAL EXPERIENCE

August 2009 – present: Staff scientist at DruQuaR, Ghent University

Supporting master theses and practical courses, as well as industrial servicing activities.

Responsible person for ICT at DruQuaR, Ghent University

Januari 2012 – present: Responsible person for radioactivity at DruQuaR, Ghent University

PUBLICATIONS IN JOURNALS WITH PEER REVIEW

PUBLISHED AND/OR ACCEPTED

D'Hondt M, Demaré W, Van Dorpe S, Wynendaele E, Burvenich C, Peremans K, De Spiegeleer B. Dry heat stress stability evaluation of a casein peptide mixture. *Food Chemistry* 2011; **128**: 114-122.

Novoa A, Van Dorpe S, Wynendaele E, Chung N, Lemieux C, Schiller P, Tourwé D, De Spiegeleer B, Ballet S. Structural modification and biological evaluation of Dmt-DALDA analogues. *Pharmacological Reports* 2011; **63**: 231.

Van Dorpe S, Verbeken M, Wynendaele E, De Spiegeleer B. Purity profiling of peptide drugs. *Journal of Bioanalysis and Biomedicine* 2011; S6.

Wynendaele E, Pauwels E, Van De Wiele C, Burvenich C, De Spiegeleer B. The potential role of quorum-sensing peptides in oncology. *Medical Hypotheses* 2012; **78**: 814-817.

Verbeken M, <u>Wynendaele E</u>, Lefebvre R, Goossens E, De Spiegeleer B. The influence of peptide impurity profiles on functional tissue-organ bath response: the 11-mer peptide INSL6[151-161] case. *Analytical Biochemistry* 2012; **421**: 547-555.

Van Dorpe S, Bronselaer A, Nielandt J, Stalmans S, <u>Wynendaele E</u>, Audenaert K, Van De Wiele C, Burvenich C, Peremans K, Hsuchou H, De Tré G, De Spiegeleer B. Brainpeps: the blood-brain barrier peptide database. *Brain Structure and Function* 2012; **217**: 687-718.

De Spiegeleer B, Van Dorpe S, Vergote V, <u>Wynendaele E</u>, Pauwels E, Van De Wiele C, Garcia-Solis P, Solis-Sainz JC. In vitro metabolic stability of iodinated obestatin peptides. *Peptides* 2012; **33**: 272-278.

Karstad R, Isaksen G, <u>Wynendaele E</u>, Guttormsen Y, De Spiegeleer B, Brandsdal B-O, Svendsen J, Svenson J. Targeting the S1 and S3 sub-site of trypsin with unnatural cationic amino acids generates antimicrobial peptides with potential for oral administration. *Journal of Medicinal Chemistry* **2012**; DOI: 10.1021/jm3002058.

Novoa A, Van Dorpe S, <u>Wynendaele E</u>, Spetea M, Bracke N, Stalmans S, Betti C, Chung N, Lemieux C, Zuegg J, Cooper M, Tourwé D, De Spiegeleer B, Schiller P, Ballet S. Variation of the Net Charge, Lipophilicity, and Side Chain Flexibility in Dmt(1)-DALDA: Effect on Opioid Activity and Biodistribution. *Journal of Medical Chemistry* 2012; **55**: 9549-9561.

De Spiegeleer B, Stalmans S, <u>Wynendaele E</u>, Bracke N, Verbeken M, Peremans K, Polis I, Burvenich C. Receptor and Blood-brain Barrier Characterisation of Opioid Peptides in Drug Research & Early Development. *Journal of Peptide Science* 2012; **18**: S49.

<u>Wynendaele E</u>, Bronselaer A, Nielandt J, D'Hondt M, Stalmans S, Bracke N, Verbeke F, Van De Wiele C, De Tré G, De Spiegeleer B. Quorumpeps database: chemical space and functionality of quorum-sensing peptides. *Nucleic Acids Research* 2013; **41**: D655-D659.

D'Hondt M, Gevaert B, Stalmans S, Van Dorpe S, <u>Wynendaele E</u>, Peremans K, Burvenich C, De Spiegeleer B. Reversed-phase fused-core HPLC modeling of peptides. *Journal of Pharmaceutical Analysis* 2013; **3**: 93-101.

Suleman S, Vandercruyssen K, <u>Wynendaele E</u>, D'Hondt M, Bracke N, Duchateau L, Burvenich C, Peremans K, De Spiegeleer B. A rapid stability-indicating, fused-core HPLC method for simultaneous determination of β-artemether and lumefantrine in anti-malarial fixed dose combination products. *Malaria Journal* 2013; **12**: 1-11.

D'Hondt M, Verbeke F, Stalmans S, Gevaert B, <u>Wynendaele E</u>, De Spiegeleer B. Derringer desirability and kinetic plot LC column comparison approach for MS-compatible lipopeptide analysis. *Journal of Pharmaceutical Analysis* 2013; **4**: 173-182.

Stalmans S, <u>Wynendaele E</u>, Bracke N, Gevaert B, D'Hondt M, Peremans K, Burvenich C, De Spiegeleer B. Chemical-Functional Diversity in Cell-Penetrating Peptides. *Plos One* 2013; **8**: e71752.

Wynendaele E, Bracke N, Stalmans S, De Spiegeleer B. Development of peptide and protein based radiopharmaceuticals. *Current Pharmaceutical Design* 2014; **20**: 2250-2267.

Stalmans S, Wynendaele E, Bracke N, Knappe D, Hoffmann R, Peremans K, Polis I, Burvenich C, De Spiegeleer B. Blood-brain barrier transport of short, proline-rich antimicrobial peptides. *Protein & Peptide Letters* 2014; **21**: 399-406.

Bracke N, Van Poucke M, Baert B, Wynendaele E, De Bels L, Van den Broeck W, Peelman L, Burvenich C, De Spiegeleer B. Identification of a microscopically selected microorganism in milk samples. *Journal of Dairy Science* 2014; **97**: 609-615.

Bracke N, Wynendaele E, D'Hondt M, Haselberg R, Somsen GW, Pauwels E, Van de Wiele C, De Spiegeleer B. Analytical characterisation of NOTA-modified somatropins. *Journal of Pharmaceutical and Biomedical Analysis* 2014; **96**: 1-9.

Wynendaele E, De Spiegeleer A, Vandekerckhove M, Stalmans S, Boucart M, Van den Noortgate N, Venken K, Van Calenbergh S, Aspeslagh S, Elewaut D. An in silico approach for modelling T-helper polarizing iNKT cell agonists. *Plos One* 2014; **9**: e87000.

D'Hondt M, Wynendaele E, Vandercruyssen K, Bauters T, Vandenbroucke J, Mullens S, Vervaet C, Remon JP, De Spiegeleer B. Investigation of active pharmaceutical ingredient loss in pharmaceutical compounding of capsules. *Journal of Pharmaceutical and Biomedical Analysis* 2014; **96**: 68-76.

Veryser L, Wynendaele E, Taevernier L, Verbeke F, Joshi T, Tatke P, De Spiegeleer B. N-alkylamides: from plant to brain. *Functional Foods in Health and Disease* 2014; **4**: 264-275.

D'Hondt M, Bracke N, Taevernier L, Gevaert B, Verbeke F, Wynendaele E, De Spiegeleer B. Related impurities in peptide medicines. *Journal of Pharmaceutical and Biomedical Analysis* **2014**; DOI: 10.1016/j.jpba.2014.06.012.

Taevernier L, Wynendaele E, D'Hondt M, De Spiegeleer B. Analytical quality-by-design approach for sample treatment of albumin containing solutions. *Journal of Pharmaceutical Analysis* **2014**; DOI: 10.1016/j.jpha.2014.06.001.

Stalmans S, Wynendaele E, Bracke N, De Spiegeleer B. Do cell-penetrating peptides cross the blood-brain barrier? *Journal of Peptide Science* 2014; **20**: S211-212.

Verbeken M, Wynendaele E, Mauchauffée E, Bracke N, Stalmans S, Bojnik E, Benyhe S, Peremans K, Polis I, Burvenich C, Gjedde A, Hernandez J-F, De Spiegeleer B. Blood-brain transfer and antinociception of linear and cyclic *N*-methyl-guanidine and thiourea-enkephalins. *Peptides* 2014; **63**: 10-21.

Suleman S, Gemechu Z, Habtewold D, Zeleke M, Duchateau L, Levecke B, Vercruysse J, D'Hondt M, Wynendaele E, De Spiegeleer B. Quality of medicines commonly used in the treatment of soil transmitted helminthes (STH) and giardia in Ethiopia: a nationwide survey. *Plos Neglected Tropical Diseases* **2014**; DOI: 10.1371/journal.pntd.0003345.

Wynendaele E, Verbeke F, Braet S, D'Hondt M, De Spiegeleer B. Quality evaluation of synthetic quorum sensing peptides used in R&D. *Journal of Pharmaceutical Analysis* **2014**.

SUBMITTED

Wynendaele E, Gevaert B, Stalmans S, Verbeke F, De Spiegeleer B. Exploring the chemical space of quorum sensing peptides.

Wynendaele E, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Crosstalk between the microbiome and cancer cells by quorum sensing peptides.

De Spiegeleer B, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, Wynendaele E. Quorum sensing peptides promote angiogenesis and invasion of breast cancer cells.

Wynendaele E, Stalmans S, Gevaert B, Verbeke F, Lauwers M, Van De Wiele C, Peremans K, Burvenich C, De Spiegeleer B. Quorum sensing peptides selectively penetrate the blood-brain barrier.

Wynendaele E, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, De Wever O, Bracke M, De Spiegeleer B. *In vitro* cell medium and human plasma stability, Caco-2 permeability and toxicity of quorum sensing peptides.

Bracke N, Barhdadi S, Wynendaele E, Gevaert B, D'Hondt M, De Spiegeleer B. Surface acoustic wave biosensor as a functional quality method in pharmaceutics.

Suleman S, Verheust Y, Dumoulin A, Wynendaele E, D'Hondt M, Vandercruyssen K, Veryser L, Duchateau L, De Spiegeleer B. Gas chromatographic method for the determination of lumefantrine in anti-malarial finished pharmaceutical products.

Stalmans S, Gevaert B, Wynendaele E, Nielandt J, De Tré G, Peremans K, Burvenich C, De Spiegeleer B. Towards a unified blood-brain barrier influx response for peptides.

IN PREPARATION

Gevaert B, Stalmans S, Wynendaele E, Taevernier L, Bracke N, De Spiegeleer B. Functional chemical characterisation of the peptide-drug space.

Stalmans S, Wynendaele E, Gevaert B, Bracke N, Peremans K, Burvenich C, De Spiegeleer B. Cell-penetrating peptides selectively cross the blood-brain barrier in vivo.

Yao H, Vancoillie J, D'Hondt M, <u>Wynendaele E</u>, Bracke N, De Spiegeleer B. An analytical quality-by-design (aQbD) approach for L-asparaginase activity.

Gevaert B, <u>Wynendaele E</u>, Stalmans S, Bracke N, D'Hondt M, Maes K, Smolders I, Van Eeckhaut A, De Spiegeleer B. Blood-brain barrier characteristics of four neuromedin peptides.

PRESENTATIONS AT (INTER)NATIONAL CONFERENCES

<u>Wynendaele E</u>, Pauwels E, Van Dorpe S, Vergote V, Van De Wiele C, De Spiegeleer B. HPLC-MS characterisation of chelate modified somatropin, *10th Flemish Youth Conference of Chemistry*, 1-2 March 2010, Blankenberge, Belgium.
Poster presentation

<u>Wynendaele E</u>, Pauwels E, Van De Wiele C, Van Dorpe S, De Spiegeleer B. LC-MS quality control of modified pharmaceutical proteins: the somatropin case, *Annual BSMS meeting on Mass spectrometry*, 16 April 2010, Woluwe, Belgium.
Oral presentation

<u>Wynendaele E</u>, Wiskerke D, De Spiegeleer B. Characterisation of modified pharmaceutical proteins: the somatropin case, *Scientific Afternoon of Faculty of Pharmaceutical Sciences*, 12 May 2010, Ghent, Belgium.
Poster presentation

<u>Wynendaele E</u>, Mertens K, Pauwels E, Van De Wiele C, De Spiegeleer B. LC-MS characterization and cell-binding properties of chelate modified somatropin. *Drug Analysis*, 21-24 September 2010, Antwerp, Belgium.
Poster presentation

Novoa A, Van Dorpe S, <u>Wynendaele E</u>, Chung N, Lemieux C, Schiller PW, Tourwé D, De Spiegeleer B, Ballet S. Structural modification and biological evaluation of Dmt1-DALDA analogues, *8th meeting of European Opioid Conference*, 13-15 April 2011, Krakow, Poland
Poster presentation

D'Hondt M, <u>Wynendaele E</u>, Bauters T, Vandenbroucke J, Robays H, De Spiegeleer B. Influence of variability in starting materials quality on stability of finished drug products : a quality-by-design factor and response, *13th International symposium on Oncology Pharmacy Practice*, 9-11 May 2012, Melbourne, Australia.
Poster presentation

Van Dorpe S, <u>Wynendaele E</u>, Burvenich C, Novoa A, Tourwé D, Ballet S, De Spiegeleer B. Blood-brain barrier and ADME properties of selected DALDA-peptide derivatives, *15th forum of Pharmaceutical Sciences*, 12-13 May 2011, Spa, Belgium.
Oral presentation

Wynendaele E, Pauwels E, Van De Wiele C, Bronselaer A, De Tré G, De Spiegeleer B. Classification of quorum-sensing peptides, *15th forum of Pharmaceutical Sciences*, 12-13 May 2011, Spa, Belgium.
Oral presentation

Wynendaele E, Bronselaer A, De Tré G, Pauwels E, Boucart M, Van De Wiele C, De Spiegeleer B. Development of a quorum-sensing peptide database: quorumpeps. *Scientific Afternoon of Faculty of Pharmaceutical Sciences*, 26 May 2011, Ghent, Belgium.
Poster presentation

Van Dorpe S, Vandenbroeck I, Wynendaele E, Verbeken M, Novoa A, Tourwé D, Ballet S, De Spiegeleer B. ADME behaviour of some Dmt-DALDA-peptide derivatives, *Scientific Afternoon of Faculty of Pharmaceutical Sciences*, 26 May 2011, Ghent, Belgium.
Poster presentation

Stalmans S, Wynendaele E, De Spiegeleer B. Exploring the chemical-functional space of cell-penetrating peptides, *Scientific Afternoon of Faculty of Pharmaceutical Sciences*, 16 May 2012, Ghent, Belgium.
Poster presentation

De Spiegeleer B, D'Hondt M, Stalmans S, Wynendaele E, Van Dorpe S. Blood-brain barrier modelling of bioactive peptides, *13th symposium on Bioactive peptides*, 7-10 June 2012, Naples, Italy.
Oral presentation

De Spiegeleer B, Stalmans S, Wynendaele E, Bracke N, Verbeken M, Peremans K, Polis I, Burvenich C. Receptor and blood-brain barrier characterisation of opioid peptides in drug research and early development, *32nd European Peptide Symposium*, 2-7 September 2012, Athens, Greece.
Oral presentation

Stalmans S, D'Hondt M, Wynendaele E, Bracke N, De Spiegeleer B. Exploring the Brainpeps database, *15th International symposium on Signal Transduction in the Blood-Brain Barriers*, 13-16 September 2012, Potsdam, Germany.
Poster presentation

Bracke N, Wynendaele E, Van De Wiele C, De Spiegeleer B. Surface acoustic wave technology as a tool for functional quality characterization of new compounds and biosimilars, *24th International symposium on Pharmaceutical and Biomedical Analysis*, 30 June – 3 July 2013, Bologna, Italy.
Oral presentation

De Spiegeleer A, Wynendaele E, Stalmans S, Van den Noortgate N, Vandekerckhove M, Van Calenbergh S, Aspeslagh S, Venken K, Elewaut D. A computational high-throughput screening approach of iNKT-agonists: a novel tool to find optimized iNKT cell ligands, *7th International symposium on CD1 and NKT Cells*, 13-19 September 2013, Tours, France.
Poster presentation

Bracke N, <u>Wynendaele E</u>, Haselberg R, Somsen GW, Barhdadi S, De Spiegeleer B. Somatropin (rhGH) functional quality characterization by LC/CE-MS and SAW biosensor techniques, *Biopharmacy day*, 18 December 2013, Ghent, Belgium.
Poster presentation

Veryser L, <u>Wynendaele E</u>, Taevernier L, Verbeke F, Joshi T, Tatke P, De Spiegeleer B. Are plant N-alkylamide cosmenutriceuticals also active in the central nervous system?, *15th International Conference of Functional Food Center*, 10-11 May 2014, Regensburg, Germany.
Oral presentation

Bracke N, <u>Wynendaele E</u>, Barhdadi S, Van De Wiele C, De Spiegeleer B. The possible role of somatropin derivatives as a theranostic in oncology, *Oncopoint*, 6 February 2014, Ghent, Belgium.
Oral presentation

Bracke N, <u>Wynendaele E</u>, Gevaert B, De Spiegeleer B. Surface acoustic wave biosensor as a functional-quality method in pharmaceutics, *24th World Anniversary Congress on Biosensors*, 27-30 May 2014, Melbourne, Australia.
Oral presentation

Stalmans S, <u>Wynendaele E</u>, De Spiegeleer B. How the exploration of the chemical space of cell-penetrating peptides helps to understand their functionality, *14th Naples workshop on Bioactive Peptides*, 12-14 June 2014, Naples, Italy.
Oral presentation

<u>Wynendaele E</u>, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Crosstalk between mammalian cells and the microbiome through quorum sensing peptides, influencing cancer metastasis, *14th Naples workshop on Bioactive Peptides*, 12-14 June 2014, Naples, Italy.
Oral presentation

Bracke N, <u>Wynendaele E</u>, Gevaert B, De Spiegeleer B. Surface acoustic wave biosensor as an analytical tool in pharmaceutics: a quality by design approach, *10th International Symposium on Drug Analysis*, 22-25 June 2014, Liege, Belgium.
Oral presentation

Gevaert B, <u>Wynendaele E</u>, Verbeken M, D'Hondt M, Stalmans S, Peremans K, Burvenich C, Delesalle C, De Spiegeleer B. Exploration of the peptide drug space, *33rd European Peptide Symposium*, 31 August – 5 September 2014, Sofia, Bulgaria.
Poster presentation

Stalmans S, <u>Wynendaele E</u>, Bracke N, De Spiegeleer B. Do cell-penetrating peptides cross the blood-brain barrier?, *33rd European Peptide Symposium*, 31 August – 5 September 2014, Sofia, Bulgaria.
Poster presentation

<u>Wynendaele E</u>, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. How does the human microbiome affect cancer outcome?, *1ˢᵗ Peptides in Paris Symposium*, 5-8 October 2014, Paris, France.
Oral presentation

<u>Wynendaele E</u>, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Quorum sensing peptides: missing link between microbiome and health, *2ⁿᵈ World Congress on Targeting Microbiota*, 16-17 October 2014, Paris, France.
Poster presentation

<u>Wynendaele E</u>, Verbeke F, D'Hondt M, Hendrix A, Van De Wiele C, Burvenich C, Peremans K, De Wever O, Bracke M, De Spiegeleer B. Cross-talk between human microbiome and mammalian cells: quorum-sensing peptides do influence metastasis of cancer cells, *Conference "Bacterial Signaling from bench to clinical application"*, 17 November 2014, Oslo, Norway.
Invited oral presentation

RESEARCH VISITS

12 August – 23 August 2013:	Training on isolation and immune-modulation of bone marrow derived macrophages (BMDM) and dendritic cells (BMDC), University of Leipzig, Germany.
8 October – 10 October 2014:	Peptide & Protein Chemistry & Biology Training Workshop, University of Cergy-Pontoise, France