Frontpage: Antibodies
Successful use of high-resolution native mass spectrometry for analysing co-occurring proteoforms in therapeutic antibodies (see article p. 8).

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Welcome

This issue of NPC Highlights presents a number of current developments from the Dutch proteomics community: new research initiatives, high-level technological and bio-inspired projects, valorisation approaches and news from the Dutch proteomics community. Moreover, special attention is paid to the plenary speakers of our latest Progress Meeting.

This year’s edition, with the theme ‘Next Generation Proteomics - Proteomics Next Generation’, proved to be a very successful one. Next to the presentations of some internationally leading researchers (see also page 20-23), several young scientists had the possibility to feature their research. Furthermore, three PhD students were awarded a Poster Prize, made possible by Advion. Meanwhile, during poster sessions and social breaks there was ample opportunity to meet and interact with old and new colleagues in the field. I look back with great satisfaction and pride and I hope we can count on your support and contributions for the next editions.

The following NPC Progress Meeting will take place on 25-26 January 2015, in collaboration with the European large-scale facility PRIME-XS. Speakers that already confirmed are Juergen Cox, Bernd Bodenmiller, Jesper Olsen and Paola Piccoti. So mark your calendar!

With the recent closing of NGI in mind, it is good to announce new initiatives related to our field, e.g. the new Institute for Chemical Immunology, made possible by a NWO gravity grant. Headed by Sjaak Neefjes from the Netherlands Cancer Institute, the institute combines forces of some leading Dutch research groups in the areas of chemistry, biology and immunology. This also nicely demonstrates the role of proteomics in current disease-oriented research approaches. Please see pages 6-7 for more information.

But I hope you also enjoy the contributions from some cutting-edge research projects, as presented by Sara Rosati and Yang Yang about fine-tuning native mass spectrometry technology, by Christine Mummery about using proteomics for personalized therapies and by Michiel Vermeulen about studying the epigenome, as well as the experiences of Tokameh Mahmoudi and Arzu Umar in taking their results towards valorisation. Last but not least, Bert Poolman shares his view on the Groningen Membrane NPC Research Hotel over the last decade.

Albert Heck, scientific director NPC
The Royal Netherlands Academy of Arts and Sciences (KNAW) has appointed Albert Heck as new member. Academy members are prominent researchers active in all the disciplines. New members are nominated by peers from within and outside the Academy and are appointed for life.

Furthermore, Heck is one of the newly elected EMBO members, the European Molecular Biology Organisation for life scientists. In total 106 outstanding researchers in the life sciences were newly elected on the occasion of the 50th anniversary of EMBO. EMBO Members make invaluable contributions to the organization by providing suggestions and feedback on the activities of EMBO. They serve on selection committees for EMBO programmes and mentor young scientists. Their input has helped to promote excellence in life sciences since 1964.

The Netherlands Organisation for Scientific Research (NWO) has awarded 88 innovative scientists a Vidi grant. With this grant NWO gives talented researchers the opportunity to develop their own line of research and to build up their own research group. Each scientist receives a maximum amount of € 800,000.

Vidi is aimed at excellent researchers who have gained several years of research experience after obtaining their PhDs. The scientists belong to the best ten to twenty percent in their discipline. A Vidi grant funds their research for a period of five years. Vidi is part of the NWO Talent Scheme, which consists of the Veni, Vidi and Vici grants.

NPC affiliated researchers who received a Vidi grant are:

Simone Lemeer, Utrecht University, Chemistry
“Een navigatiesysteem in iedere cel”

Jurgen Marteijin, Erasmus MC, Genetics
“DNA-reparatie onder de loep”

NPC theme leader Shabaz Mohammed (Oxford University and Biomolecular Mass Spectrometry and Proteomics Group of Utrecht University) has been awarded the Joseph Black Award 2014 by the Royal Society of Chemistry. The Joseph Black Award is for a young scientist in any field covering the practice and teaching of analytical science and is sponsored by the Analytical Chemistry Trust Fund.

The Royal Netherlands Academy of Arts and Sciences (KNAW) has awarded Celia Berkers the 2014 Heineken Young Scientists Award for Biochemistry and Biophysics. Berkers (33) is receiving the award for her research into the workings of the proteasome, a structure that breaks down proteins in biological cells. The Heineken Young Scientists Awards are meant as an encouragement for young and talented researchers. The winners are promising young researchers whose achievements set an example for other young scientists. The Young Scientists Award consists of a piece of sculpture by Jeroen Henneman, an artist based in Amsterdam, and € 10,000.

The 2014 Heineken Young Scientists Awards will be presented on Thursday 2 October 2014 during a special KNAW meeting at the Beurs van Berlage Building in Amsterdam. The Heineken Prizes will be presented there as well.

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**Recent NPC PhD theses**

The NPC congratulates the following NPC affiliated researchers with successfully defending their thesis:

- **Tale Sliedrecht**, UMC Utrecht, 3 December 2013
  Probing Mps1 function in mitosis
- **Andras Kiss**, FOM-AMOLF, 8 January 2014
  New Ionization and detection technologies for Mass Spectrometry Imaging
- **Erik de Graaf**, Utrecht University, 12 February 2014
  Identification and structural characterization of novel a-kinase anchoring proteins
- **Pepijn Burgers**, Utrecht University, 14 March 2014
  Proteomics enabled Vaccinology. Probing Antigen and Epitope Repertoires
- **Geert Mommen**, Utrecht University, 17 March 2014
  Methodological aspects in the proteomics workflow

**IP Management Horizon2020**

With Horizon2020, the European Union aims at strengthening the European scientific and technological base and fostering benefits for society as well as better exploitation of the economic and industrial potential of policies of innovation, research and technological development. In fact, it is essential that the public resources and efforts used in research are converted into socio-economic benefits to the EU. For this reason Horizon 2020 establishes commitments from the participants in terms of dissemination and exploitation of the projects’ results, including their protection through intellectual property. At www.iprhelpdesk.eu a factsheet is published which intends to assist applicants with the management of intellectual property in the proposal stage of their project.

**NPC Poster Prizes 2014**

On 10&11 February the NPC Progress Meeting 2014 took place at the Media Plaza, Utrecht. Over 230 people from inside and outside the NPC participated.

Poster prizes were awarded to:
- **Sara Rosati** (Utrecht University)
- **Marco Benevento** (Utrecht University)
- **Karen Sap** (Erasmus Medical Centre Rotterdam)

The winners can use their prize of € 1000 for a journey abroad to attend a conference, summer school or other activity that is directly related to their research.

The poster prizes were made possible by Advion.

**Thousands of unknown antigens identified**

Researchers of Utrecht University, Intravacc and the National Institute for Public Health and the Environment (RIVM) used a new method to identify hitherto unknown peptide antigens. This type of antigen had long been searched for, as they may be the starting points for new vaccines and cancer immunotherapy. “With this new method, we can identify more than twelve thousand peptide antigens, whereas before, we could only see the tip of the iceberg,” explains immunologist Dr Cécile van Els of the RIVM. The new method is not only much more sensitive, but also makes more routine research possible.

The new method is also a very powerful technique for demonstrating the subtle differences between healthy cells and damaged cells, according to Geert Mommen of Intravacc. Of the twelve thousand peptide antigens, there are maybe a few dozen damaged ones that actually deviate from or are completely different from healthy cells. Researchers have found more proof that these deviating or unique peptide antigens are best equipped to prepare our immune system for the fight against cancer.


**AbLab: your partner for native protein MS services**

AbLab is the service unit that operates within the Hecklab, providing state of the art Native MS services. Due to this unique position AbLab has access to the latest insights, innovations, methodology and instrumentation for providing mass spectrometry services.

Native MS is emerging as a powerful technique for the characterization of antibodies, especially antibody mixtures, and in principle all therapeutic proteins. It provides the identity and accurate relative quantitative data of species in a mixture and complements, in many cases supersedes, conventional techniques such as cation exchange chromatography. As service unit within an academic group, AbLab offers commercial services to R&D driven companies in the pharmaceutical and biotechnology sector.

For more information: www.hecklab.com/ablab
Rewarded with a €27.6 million NWO Gravity grant, the new Institute for Chemical Immunology can take off. Coordinating researcher Sjaak Neefjes unfolds the plans and explains why this initiative can bring new life to the drug development field and beyond.

ICI collaborating partners
- Sjaak Neefjes (Netherlands Cancer Institute)
- Hermen Overkleeft (Leiden University)
- Carl Figdor (UMC St Radboud)
- Piet Gros (Utrecht University)
- Albert Heck (Utrecht University)
- Ton Schumacher (Netherlands Cancer Institute)

Where chemistry and immunology meet

Former NPC project leader Sjaak Neefjes is a man on a mission. He aims to bring the largely separate worlds of immunology and chemistry together in a new research endeavor: the Institute for Chemical Immunology (ICI). For Neefjes (1959), it is a perfect match with his multifaceted scientific profile. He is a chemist who studies cell biology, with a particular focus on immunology. “To me, immunology is a specialisation within cell biology, although not all immunologists will agree,” he laughs.

Tackle the threat
“My personal interest is to understand movement within a cell and the whole dynamics of the cellular machinery. And I want to study how we can use chemistry to manipulate those dynamic cellular processes.” As Head of the division of Cell Biology II at the Netherlands Cancer Institute, the biological context of his research is the oncology field. At the Leiden University Medical Centre, he holds a chair in ‘Cell biology of antigen presentation and processing’. A truly immunological topic indeed. The link between oncology and immunology is not as far-fetched as it may seem. It is the responsibility of the immune system to tackle any threat, whether it is an external pathogen like a virus or an internal aberration such as a tumor. Boosting the body’s immune response to more effectively attack tumor cells has become a major line of interest in cancer drug development. Within the ICI, cancer is a leading theme, but it is not the sole focus, says Neefjes. “Rheumatoid arthritis is the other core disease within our programme. It is a deliberate choice to focus on an autoimmune disease like rheumatoid arthritis, in which an out-of-control immune response is the problem and on a disease that requires a much stronger immune response, in our case cancer.” But the relevance and applicability of the ICI’s research and technology development will be much broader. “Within ICI, we combine chemistry, immunology, biology and a range of analytical technologies.” These of course include proteomics, on which ICI will collaborate with NPC/Proteins At Work. “We expect the technologies and concepts that will be developed to be of use to a wide range of topics. It is fair to state that at least 50% of all diseases are in one way or another connected to the immune system.”

Cellular puzzles
The medical context is clear, but where does the chemistry come in? Let’s take a few steps back in time, to around 1990 when the chemist Neefjes was a PhD student in the lab of Hidde Ploegh at the Netherlands Cancer Institute. “That is where I learned about protein chemistry and cell biology.”
“We combine chemistry, immunology, biology and a range of analytical technologies.”

Shortly thereafter, he was one of the world’s first to use GFP (green fluorescent protein) as a tool to track the intracellular movement of molecules and vesicles. With this new possibility, studying the dynamics of cellular processes entered a whole new avenue. But solving the cellular puzzle remained, and still remains, a tough challenge. Which is exactly why Neefjes enjoys his research so much. “With every step we take, the complexity increases, again. We really are in the Golden Age of biology now, especially when it comes to molecular and cell biology. With all the new techniques we have, I keep on going from one surprising observation to the next. There is always something new and unexpected to see in a cell.” Despite the overwhelming experience of watching a living cell at work, Neefjes never lost his interest in chemistry.

Drivers behind ICI
Combining chemical and genetic approaches is the key to Neefjes’ view of chemical immunology. He provides an example of one of his first experiments along this new line of thinking. “Together with the Bioorganic Synthesis group of Hermen Overkleeft at Leiden University, who is also one of the ICI applicants, we ran a project to construct an antibiotic against the host cell instead of against the pathogen. Gradually, they expanded their ‘chemical genetics’ approach into the scientific driver behind the ICI, says Neefjes. The fact that funding bodies are increasingly pushing for translational research, was the other driver. “The problem is that it is very hard to come up with translational research that is original from a scientific point of view. Testing compounds that have been developed by others is not particularly appealing. But to cover the whole track from target and lead discovery to preclinical and even the first clinical phase, that is a very attractive challenge. And that is exactly what we envision to do within ICI.”

Fill the gap
Although deeply rooted in science, the ultimate goal of the ICI is to operate like a mini-pharmaceutical enterprise. That may sound megalomaniac, but it is the only logical response to the changes in the drug development industry, says Neefjes. “Increasingly, Big Pharma is withdrawing from research, especially from the first stages of drug development. These companies prefer to let others, mostly start-up companies or public research organisations, perform everything from target discovery to lead optimization, medicinal chemistry and preclinical work. Sometimes even phase I clinical trials. Only when compounds have passed all these stages is Big Pharma willing to invest. They basically shift the risks to society.” This is the gap that ICI aims to fill. “We will provide a platform for this whole discovery and development track. In essence, we will take over part of the work that used to be performed within the pharmaceutical industry.” A big leap for such a relatively small group? Neefjes is of course optimistic: “Look at the BRAF-inhibitors; very successful cancer drugs. They were in essence developed by a small company of around 40 people. The problem is that it is very hard to come up with translational research that is original from a scientific point of view. Testing compounds that have been developed by others is not particularly appealing. But to cover the whole track from target and lead discovery to preclinical and even the first clinical phase, that is a very attractive challenge. And that is exactly what we envision to do within ICI.”

Career

Sjaak Neefjes
2009 Head Division of Cell Biology II, Netherlands Cancer Institute
2007 Deputy Scientific Director, Netherlands Cancer Institute
1999 Professor at LUMC, chair of ‘Antigen Presentation and Processing’
1998 Head Division of Tumor Biology, Netherlands Cancer Institute
1995 Staff member, Cellular Biochemistry, Netherlands Cancer Institute

Awards
2014 Elected member of the Norwegian Academy of Science and Letters (Det Norske Videnskaps-Akademi)
2013 Elected member Academia Europaea
2010 Elected member European Academy of Cancer Sciences
2009 ERC Advanced Grant
2007 Elected member EMBO

Recent key publications
Comprehensive mass spectrometry analysis of post translational modifications is a challenging job. Much progress could be made by using a modified Orbitrap Exactive Plus Instrument combined with native electrospray ionisation mass spectrometry. High-mass resolving power and high-mass accuracy proved to allow for elucidating micro-heterogeneity in complex proteins under native conditions. In this article we describe the successful use of the new MS technique for analysing co-occurring proteoforms in therapeutic antibodies and in chicken ovalbumin.

Protein micro-heterogeneity resulting from genetic variants, RNA editing, cellular processing or post-translational modifications (PTMs) affects protein activity and stability, including in the case of recombinant therapeutic proteins. In particular, PTMs diversify and extend protein function beyond what is regulated by gene transcripts. They reversibly or irreversibly alter the structure and properties of proteins through biochemical reactions. An accurate and reliable characterisation of PTMs not only facilitates the understanding of protein complexity, it is also crucial to understand biological function studies.

Currently, chromatographic-based techniques (SCX, HILIC), gel electrophoresis (SDS-PAGE, 2D gel) and capillary electrophoresis are widely used for analysing protein micro-heterogeneities particularly raised by extensive glycosylation. Yet the comprehensive analysis of PTMs remains challenging since it requires high-resolution separation techniques. Mass spectrometry (MS) has gained increasing importance in this area based on its inherent strengths: speed, sensitivity, resolution and accuracy. We recently described a modified version of the Orbitrap Exactive Plus instrument (ThermoFisher Scientific) adapted to be amenable for the native MS analysis of large biomolecules and molecular assemblies [1, 2]. The key feature of this mass spectrometer is that it allows high mass accuracy and high resolution measurements at m/z values up to 20000, facilitating the analysis of protein PTMs at the intact protein level.

**Antibody glycosylation** Controlling and understanding the protein micro-heterogeneity of monoclonal antibodies (mAbs) both in a qualitative and quantitative manner represents one of the main focuses in the development and manufacture of this class of therapeutics. By taking advantage of the new
Orbitrap instrument, we developed a method to minutely characterise micro-heterogeneities originating from complex N-glycosylation on mAbs [3](see Figure 1) at the intact protein level.

Several δhingeIgG4 single-point mutants (Y407E, Y407A, Y407Q and Y407K) that are known to display very complex glycosylation profiles were analysed under native conditions. Besides the complex glycosylation profiles, these specific point mutations situated in the CH3 domain also affect the CH3-CH3 interaction strength, so that these mAbs show high predominance of the half-antibody. The high resolving power of the new instrument allows the discrimination of a plethora of peaks arising from the different glycoforms that are quite close in mass, so that the mass and structural assignment become rather straightforward, even for the lower abundant species in the very congested spectra. More than 20 different complex-type N-glycan compositions could be assigned within a single spectrum. Our data reveals a considerable increase in
mAb halfbody, which has a peptide backbone mass of 73,143 Da. PNGaseF. We used for this a zoomed-in analysis of the [M+13H]13+ ions of the neuraminidase (sialidase), treated with analysed in its fully glycosylated form; it was subsequently also exhibits a rather complex and extended glycosylation benchmarked this approach using an IgG1Y407E mutant which deglycosylation enzymes for the specific cleavage of particular glycans. For a more detailed validation of the glycan assignments, we proposed a slightly different approach which makes use of pathways typical of human (HEK) cells.

For a more detailed validation of the glycan assignments, we proposed a slightly different approach which makes use of deglycosylation enzymes for the specific cleavage of particular carbohydrate residues from the native intact mAbs [5]. We benchmarked this approach using an IgG1Y407E mutant which also exhibits a rather complex and extended glycosylation profile and is mainly present as half antibody. The sample was analysed in its fully glycosylated form; it was subsequently treated with β1,4-galactosidase and/or neuraminidase (sialidase) and finally with PNGaseF. By comparing the resulting spectra, one can observe the progressive simplification of the glycosylation profiles when the mAbs are incubated with different enzymes [see Figure 2].

Chicken ovalbumin As a subsequent example to benchmark the potential of the high-resolution native MS for the analysis of protein PTMs, we selected chicken ovalbumin (45 kDa) as we believe it exhibits a typical molecular heterogeneity for eukaryotic proteins. In this case we also took advantage of specific enzymes, namely a glycosidase or phosphatase, to selectively remove PTMs from the intact protein under native conditions. Enzymatic removal of PTMs potentially simplifies the number of N-glycan species compared to previous studies [4]. Most notably, next to the glycan structures frequently observed on mAbs expressed in human cell lines, we were able to identify lower abundant glycans that occur less frequently, but that are still allowed considering the N-glycan biosynthetic pathways typical of human (HEK) cells.

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We analysed the natural unprocessed, deglycosylated, dephosphorylated, deglycosylated and dephosphorylated ovalbumin by native MS using the modified Orbitrap Exactive Plus instrument (see Figure 3). We were able to profile 59 different proteoforms in the unprocessed chicken ovalbumin, including 45 proteoforms with different glycans, 13 mono-phosphorylated proteoforms, a few non-phosphorylated proteoforms and a few proteoforms in which the N-acetylation was missing. Remarkably, only 19 out of the 45 glycan compositions assigned in our work had been previously annotated in chicken ovalbumin, meaning that approximately 20 new possible glycosylated proteoforms were identified.

**Strengths and weaknesses** Ease of sample preparation and analysis speed are indeed clear advantages in performing protein micro-heterogeneity analysis by using native MS. In addition, a few more technical advantages can make the native MS approach desirable over other mass spectrometric approaches. Firstly, in contrast to classical glycan structure MS studies where the released glycans or glycopeptides are analysed in the mass spectrometer, here we electrospray the intact protein measuring mass shifts between different glycoforms on the intact polypeptide backbone. In this way we significantly reduce any bias induced by differences in the ionisation efficiency towards different sugar residues (for example sialic acid). Secondly, incomplete digestion due to glycosidase specificity does not represent a problem for the glycan assignment. In this way it is possible to preserve comprehensive information even for lower abundant glycoforms. Finally, our approach gives a panoramic view of all co-appearing species including low abundant ones, while excluding possible interferences from co-purified contaminant proteins.

The analysis at the intact protein level is powerful, yet it still has some limitations such as the inability to deduce monosaccharide stereoisomers, linkages, anomeric configurations, and glycan branching and the inability to directly locate the identified PTMs on the protein backbone, which would require efficient top-down fragmentation.

**Future perspectives** We foresee that native ESI-MS using the modified Orbitrap Exactive Plus Instrument will make a significant contribution to the field. One single native ESI-MS spectrum reveals the masses and relative abundances of each co-occurring proteoform, providing a qualitative and quantitative fingerprint spectrum. This will allow the analysis of most PTMs (phosphorylation, glycosylation, lysine-acetylation, etc.) at the intact protein level. These analyses are applicable to any class of proteins and can complement typical top-down proteomics experiments. Kinases, oncogenes and chromatin-related proteins in particular are known to be decorated by a plethora of functionally important PTMs, leading to rather complex protein micro-heterogeneity.

The achieved high-mass resolving power and high-mass accuracy allows for comprehensive, in-depth and detailed parallel characterisation of various modifications, contributing to our understanding of protein functioning in general. Moreover, we believe that biotechnology and biopharmaceutical companies
working on therapeutic antibodies or other types of biotherapeutics can benefit substantially from the accuracy and the speed of this method. This kind of analysis can, in fact, also be seen as a high-resolution fingerprint that can be used, for instance, for batch-to-batch comparisons or for comparability studies between biosimilar antibodies and their reference products.

In this article we show new strategies to characterise protein micro-heterogeneity raised by PTMs, particularly glycosylation, at the intact protein level using high resolution native MS. We describe a fast, easy-to-use and sensitive method to profile intricate N-glycosylation profiles of monoclonal antibodies. We show that our method can handle highly complex glycosylation profiles, identifying more than 20 different glycoforms per mAb preparation on a single highly purified antibody. The method presented here will aid in the comprehensive analytical and functional characterisation of protein micro-heterogeneity, which is crucial for successful development and manufacture of therapeutic antibodies.

Furthermore, we dissect the micro-heterogeneity of chicken ovalbumin in its full qualitative and quantitative glory, whereby we are able to detect, identify and semi-quantify at least 59 different proteoforms. This variety is largely induced by the presence of multiple phosphorylation sites, and a glycosylation site that we find to be occupied by at least 45 different glycan structures, nearly half of which had not been reported previously.

Mass analysis of the intact protein in its native state is straightforward and fast. It requires very little sample preparation and provides a direct view on the stoichiometry of all different co-appearing modifications that are distinguishable in mass. As such, this proof-of-principal analysis shows that native electrospray ionisation MS in combination with an Orbitrap mass analyser offers a means to characterise proteins in a manner highly complementary to standard bottom-up shot-gun proteome analysis.

References
3 Rosati, S., et al., In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. mAbs, 2013. 5(6).

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summary
In this article we show new strategies to characterise protein micro-heterogeneity raised by PTMs, particularly glycosylation, at the intact protein level using high resolution native MS. We describe a fast, easy-to-use and sensitive method to profile intricate N-glycosylation profiles of monoclonal antibodies. We show that our method can handle highly complex glycosylation profiles, identifying more than 20 different glycoforms per mAb preparation on a single highly purified antibody. The method presented here will aid in the comprehensive analytical and functional characterisation of protein micro-heterogeneity, which is crucial for successful development and manufacture of therapeutic antibodies.
Christine Mummery

Human pluripotent stem cells: linking patients to therapy

Pluripotent stem cells are receiving much attention because of their unique capacity to form all cells of the adult body. Control of this property in vitro would offer tremendous opportunities in medical science because it would allow access to cells of the human body that are usually difficult to reach. There is particular interest in using human stem cells and their differentiated derivatives in drug and toxicology screening. Our research focuses on the heart and the vascular system. In this article we describe how stem cell approaches can be used to address problems of chronic and genetic cardiovascular diseases.

The emerging use of human pluripotent stem cells (hPSCs) for disease modelling and drug development rather than simply for therapeutics has been among the most significant conceptual changes in stem cell biology of the past decade. Following the discovery that normal cells could be reprogrammed to a pluripotent state using just a few crucial transcription factors, it quickly became clear that even the most inaccessible cells of the body could be generated quickly and easily from any individual.

We can now produce cells of human heart, brain and blood vessels not only for genomic research but also in the relatively large numbers required for proteomics. We are particular interested in the cardiovascular system and have made more than 50 of these so-called hiPSC lines from patients with genetic cardiac and vascular disease. Using a variety of electrophysiological, biophysical and imaging techniques, we have characterised these cells and demonstrated that they mimic the salient features of the disease evident in patients. We have not only obtained new insights into the mechanisms underlying several cardiac and vascular diseases, but have identified unexpected toxicogenetic sensitivities to drugs associated with some genotypes. The stage is set to move this technology into the drug discovery platforms of the future.

The heart and vascular system An essential prerequisite for exploiting opportunities offered by human pluripotent stem cells in full is that the specialised cell types of interest for research can be derived effectively in reasonable numbers and that they have properties closely resembling their counterparts in the human body. Our focus has been on cells of the heart and vascular system, which have many specialised subtypes. These concern atrial, ventricular and pacemaker cells in the heart, for example, and smooth muscle and endothelial cells in the vasculature. We can now derive these cells as pure populations using differentiation and selection protocols that are based largely on defined culture reagents, so that there is minimal variability.
between experiments and we fully understand exactly what we are doing during optimisation. Much of our early research to reach this point, however, was not based on stem cells but on a profound knowledge of what controls differentiation and growth of these cells in the developing embryo and how their phenotype changes over time as they progress from the foetal stage to adulthood and even aging.

All differentiated derivatives of stem cells at this point have an immature phenotype, that is, they resemble foetal cells. However, for some of the cardiac diseases we study, like those causing abnormal heart rhythm for example, this does not particularly matter because the heart functions early in development and most ion channels present in the adult human heart are also present in the foetus. If these genes have mutations, they can cause ‘arrhythmia’, which needs to be treated by drugs or a pacemaker if serious since it can lead to sudden cardiac death.

**Two ways to derive diseased cells** We presently have two ways we use to derive diseased heart cells, not only for these arrhythmia conditions called channelopathies but also for
make the vessels more robust, as we have already seen with tube and the smooth muscle cells surrounding it. This should

We hope to find a new drug that enhances the interaction between the vascular endothelial cells lining the vascular tube and the smooth muscle cells surrounding it. This should make the vessels more robust, as we have already seen with Thalidomide treatment. Unfortunately, although Thalidomide works fairly well, it has major side effects, so we are looking for an alternative.

Steady progress Advances in research are often the sum total of incremental changes, no less so in this area of stem cells research. Our most important findings have concerned in depth analysis of isogenic pairs of hPSC lines with mutations causing channelopathies. These were the first isogenic pairs for any cardiac disease. The results showed that the phenotypic differences between genetically match cardiomyocytes were actually quite small, but that was exactly what revealed a new mechanism underlying the disease we were studying. The mutation caused a trafficking problem whereby too little protein of the ion channel subunit reached the cell surface and the ion channel functioned inadequately.

In our study of vascular disease using hiPSC, we have shown we can recreate tumour vasculature as a plexus in co-cultures of the right types of endothelial cells and smooth muscle cells and that in this synthetic vascular plexus we see responses to drugs to inhibit tumour angiogenesis and reduce tumour growth already in clinical trial. We thus have a robust and renewable screening platform for vascular drug discovery. We have also identified a new mechanism underlying HHT, which may provide new targets for therapy. The pharmaceutical industry is showing growing interest, not only for modelling diseases for which they have no human assays and therefore no new drugs in the pipeline, but also assessing drug safety. Our work has been the first to show direct relationships between genotype and toxic responses of the human heart to Thalidomide. Unfortunately, although Thalidomide works fairly well, it has major side effects, so we are looking for an alternative.

Explosion of new discoveries With human pluripotent stem cell biology now emerging as a robust field of research along the lines outlined above, we now expect an explosion of new discoveries, not only in the area of cardiovascular disease but also in neurodegenerative, liver and lung diseases. It may even be possible to capture neural conditions that we never dreamed might be possible to model in culture in the lab. For instance, diseases like ADHD, autism spectrum diseases, depression, schizophrenia and the like. Over a decade of research investing in reagents, cells and methodologies
resulted in an array of commercial products of excellent quality available to the researcher just starting out so that, whilst not quite at the level of buying a ‘cake mix’, the extreme levels of expertise required just 5 years ago for stem cell culture and differentiation are no longer required. The opportunities are almost too numerous to mention. Among the most important, however, may be the possibility of directly linking data from Genome Wide Association Studies, which show correlations between specific variants of DNA or SNPs and disease traits or predisposition, to direct proof of cause and effect, based on hPSC engineered to express the relevant genome variants. We may also expect the repertoire of human models of disease to expand significantly and the use of these models to lead to both new drugs and to repurposing of exiting drug for novel therapies based understanding underlying mechanisms of disease.

References

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summary
The ability to generate pluripotent stem cells (called iPSC) by reprogramming somatic tissues is arguably the greatest breakthrough in biomedical science of the last decade. The most inaccessible cells of the body can now be derived repeatedly from any individual. This could have a huge impact on understanding disease and the development of new therapeutic drugs, but it will require a new level of sophistication in bioassays to create disease models and realise the value of hPSC for acute and chronic, genetic and somatic diseases. The work described has developed technologies to use human stem cells in this way to understand and eventually treat cardiac and vascular diseases. The generation of cell types of the heart and blood vessels not normally accessible for research is based on principles of developmental biology and how the embryo induces the formation of these cells types. How these hPSC and their derivatives can be used now and in the future to address problems of chronic and genetic cardiovascular maladies is discussed.
Epigenetics is the study of heritable changes in gene activity that are not caused by changes in the DNA sequence. The process of cellular differentiation, for example, is caused by changes in gene expression that are driven by epigenetic processes. Complex interactions between DNA and proteins are important to regulate gene expression mechanisms. We have developed advanced proteomics techniques to study these DNA-protein interactions and to identify the involved proteins, the so-called chromatin readers.

The development of an adult organism from a single fertilised egg is accompanied by the generation of some 300 different cell types. Each of these cell types expresses a specific subset of genes in a highly regulated manner. During cellular differentiation, the genome in every cell type remains unchanged, which raises one key question: How does a single genotype give rise to such a large diversity of phenotypes? At least part of the answer to this question is epigenetics. Epigenetics is defined as changes in gene expression and phenotype that are independent of the underlying DNA sequence. In higher eukaryotes this is mainly achieved through methylation of DNA on cytosines and by post-translational modifications of histones. These epigenetic modification patterns are dynamically being established, maintained and removed from the genome during differentiation and they help to create cell-type-specific gene expression profiles. Regulatory proteins can be recruited to these modifications and exert their function at the site of recruitment. The specific binding of these so-called chromatin ‘readers’ therefore contributes significantly to the biological function of each individual epigenetic modification.

Our lab uses state-of-the-art quantitative mass-spectrometry based proteomics technology to identify chromatin readers for epigenetic histone and DNA modifications (Figure 1). We characterise the (dynamic) complexes that these readers form, we study their biology in (differentiated) stem cells and in different model organisms, and we investigate their potential deregulation in cancer.

**Epigenetic modification** Methylation of cytosine residues in promoter regions of higher eukaryotes, which mostly occurs in the context of CpG dinucleotides, represents an important mechanism via which cells can shut down expression of genes. DNA methylation is thought to be very stable and can be transmitted from mother to daughter cells. DNA methylation is therefore considered to be an epigenetic modification, since
it has a profound impact on phenotype, but is inherited independent of the underlying DNA sequence. Several fundamental processes such as genomic imprinting, X-inactivation and retrotransposon silencing are regulated by DNA methylation. DNA methylation plays an essential role during development since knocking out DNA methyltransferases in mice results in embryonic lethality. Furthermore, aberrant DNA methylation patterns are a hallmark of cancer, which is why large efforts are currently being made to profile genome wide methylation patterns in cancer tissues.

Epigenetics controls cellular differentiation, which implies the process whereby a pluripotent cell becomes a more specialised cell type. This process of differentiation occurs in embryonic stem cells during the development of a multicellular organism. Cell differentiation, however, also takes place in adulthood as adult stem cells divide and create fully differentiated daughter cells during tissue repair and during normal cell turnover. Epigenetic processes are characterised by chemically modified DNA, which provides suitable marks to direct the expression. A well-known mark is DNA methylation. “Just marking the DNA, however, is not enough,” explains Vermeulen. “For actively influencing gene expression, specific proteins should be bound to the modified site. The biological effect of the gene expression (or gene suppression) depends on the nature of the protein bound to it. It is our goal to identify these so-called chromatin readers.”

Vermeulen’s research group succeeded in identifying several novel readers in mouse embryonic stem cells, neuronal precursor cells and adult mouse brain cells. “To achieve this goal we performed extensive mass-spectrometry based proteomics screening of nuclear extracts that we cultivated in our lab using DNA pull-down methods.” Future research will focus on expanding the proteomics approach to study the uncontrolled cell grow in cancer cells. “Aberrant DNA methylation patterns are a hallmark of cancer, which is why large efforts are currently being made to profile genome wide methylation patterns in cancer tissues,” concludes Vermeulen.

What this research is about:

**Search for the molecular basis of the epigenome**

Each cell in an individual human’s body contains the same nuclear DNA, and yet we have hundreds of different cell types such as skin cells, heart cells or bone cells. How such great diversity can arise from one single genetic source is explained by epigenetic mechanisms. Epigenetic changes lead to the expression of different genes in different cells. “We try to unravel the molecular basis of the epigenetic processes,” says Michiel Vermeulen, professor of Proteomics and Chromatin Biology at the Radboud Institute for Molecular Life Sciences. “We develop proteomics technology to study the epigenome.” Epigenetics controls cellular differentiation, which implies the process whereby a pluripotent cell becomes a more specialised cell type. This process of differentiation occurs in embryonic stem cells during the development of a multicellular organism. Cell differentiation, however, also takes place in adulthood as adult stem cells divide and create fully differentiated daughter cells during tissue repair and during normal cell turnover. Epigenetic processes are characterised by chemically modified DNA, which provides suitable marks to direct the expression. A well-known mark is DNA methylation. “Just marking the DNA, however, is not enough,” explains Vermeulen. “For actively influencing gene expression, specific proteins should be bound to the modified site. The biological effect of the gene expression (or gene suppression) depends on the nature of the protein bound to it. It is our goal to identify these so-called chromatin readers.”
and the role it has in the regulation of gene expression during development. We and other researchers have shown that in addition to the classic group of MBPs, a large number of other cellular proteins, including transcription factors, also possess (sequence specific) methyl-CpG binding activities [2,3]. In addition, nonmethylated CpGs were recently shown to recruit activator proteins, and this phenomenon may be as or even more relevant than the recruitment of MBPs by methylated CpGs [4]. Finally, to make things even more complicated, hydroxymethylcytosine (hmC) was discovered in 2009 as the so-called ‘6th base of life’ [5,6]. This modification is particularly abundant in embryonic stem (ES) cells and in neuronal tissues. The Ten-Eleven-Translocase (TET) family of oxygenases catalyses the conversion from mC to hmC. Significantly, deregulation of TET proteins is implicated in a variety of malignancies. Recent genome wide profiling studies have revealed that hmC is found in gene bodies of active genes as well as on developmentally regulated promoters in mouse ES cells. Until now, the exact function of hmC remains unclear, although it is thought that hmC forms an intermediate in an active DNA demethylation pathway involving the TET enzymes and a glycosylase (TDG) to revert mC back to C.

**Novel chromatin readers** Several interesting observations were made in these affinity purification experiments from mouse embryonic stem cell nuclear extracts. First of all, we identified a number of novel mCpG interacting proteins such as the pluripotency transcription factor Klf4. This protein was not previously known to bind to methylated DNA. We validated these findings using recombinant protein as well as genome wide profiling, which revealed a correlation between Klf4 binding and methylated DNA. Furthermore, mC and hmC containing DNA each recruit a distinct and only partially overlapping set of proteins in mouse ES cells. Interestingly, hmC-containing DNA recruits a number of DNA repair proteins and glycosylases, further strengthening its association with base excision repair and active DNA demethylation. Finally, we also identified a large number of novel nonmethyl-CpG binding proteins in mouse ES cells, including many transcription factors and Polycomb group proteins.

Next, we profiled interactions with mC and hmC in neuronal precursor cells (NPC) and adult mouse brain tissue. In this case, we did not use SILAC labelling but performed triplicate pull-downs combined with label free quantification to determine modification-specific interactions. Interestingly, readers for mCpG and hmCpG are highly dynamic in these different cell types and show only a limited overlap. For example, in neuronal precursor cells and the adult mouse brain, the transcriptionally repressive protein complex MBD2/NuRD is a prominent reader for mCpG, whereas this protein complex does not interact with mCpG in the pluripotent embryonic stem cells. Finally, some NPC and brain specific readers for mCpG, such as certain Hox proteins and Dlx proteins, are known to be involved in organogenesis, indicating that ‘reading’ DNA methylation is essential for normal development. A selection of identified ‘readers’ in mouse ES cells, neuronal precursor cells and adult mouse brain cells are shown in Figure 3.

**Further studies needed** Although DNA methylation is generally known to be associated with repression of transcription, our data force us to reconsider this textbook model. In pluripotent cells, an uncoupling between DNA methylation and repression of transcription appears to exist. This is evident from a lack

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**Schematic overview** of the SILAC-based DNA pull-down approach. Please refer to the main text for more information.
of transcriptionally repressive readers for methylated DNA in mouse ES cell extracts. Interestingly, this lack of correlation between DNA methylation and repression of transcription in pluripotent cells is highly conserved in vertebrates such as Xenopus [8] and Danio rerio (unpublished observations). Further experiments are required to determine whether the functional readout of CpG methylation is indeed strongly affected by the repertoire and abundance of different DNA methylation 'readers' acting at any given time in a cell or a developing organism. For more information please refer to Spruijt et al., Cell 2013 (see ref. 7).

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**Summary**
In this study, we have used quantitative mass spectrometry-based proteomics to identify readers for mC and hmC in mouse embryonic stem cells, neuronal precursor cells and adult mouse brain. Readers for individual modifications were found to be highly dynamic throughout the three cell types and tissues that we investigated. Furthermore, readers for distinct cytosine modifications show limited overlap. This developmentally dynamic binding is in contrast to interactions with histone modifications, such as trimethylated lysines on histone H3. In this case, the majority of interactors are constant between different cell types or developmental stages [unpublished observations and 9]. Our findings suggest that, at least from a biochemical perspective, mC and hmC behave quite differently. A number of hmC readers are associated with DNA repair, indicating that hmC forms an intermediate in active DNA demethylation pathways. However, several transcription factors not known to be associated with DNA repair also interact with hmC in neuronal precursor cells and adult mouse brain. This suggests that hmC additionally has a DNA demethylation independent function in particular cell types or on certain target genes.

**References**
One single data set — be it genomic, epigenomic, proteomic, or metabolomic — will not provide a complete answer to researches’ questions. It is basically just one snapshot from one point of view,” says Edwin Cuppen, professor of human genetics, who spoke first at the last NPC Progress Meeting. Because of his specialism in the field of genomics and genetics, he was introduced as an ‘outsider’. Nevertheless, his lecture fits perfectly with the theme of the meeting: next generation proteomics.

Cuppen began by referring to the memorable milestone that has been reached: the 1,000 dollar genome. He showed a graph illustrating the rapidly decreasing costs for genome sequencing. “An app to have your complete DNA sequence available at any moment and at any place will soon be available,” he predicts. But what is the use of that? Not so much, his answer implies. “Understanding such personal genomes and the biological consequences of the enclosed genetic variation remains a major challenge,” Cuppen explains.

The Cuppen group studies the general mechanisms underlying genotype-phenotype relationships using systematic genetical-genomics approaches in rat model systems and patient cases. A diversity of high-throughput techniques and bioinformatic tools, including next-generation sequencing approaches, are employed to systematically discover and study genetic variation and functional genomic elements. In a recent study with Albert Heck’s group, they also integrated proteomics research. “Matching proteomic data to a database enhanced by strain-specific genome and transcriptome data, for example, enabled us to identify thousands of peptides we would otherwise have missed.”

Proof-of concept
In a proof-of-concept study, the researchers demonstrated the value of combining genomic and proteomic data. They analysed liver tissue from two inbred rat strains, one of which involved the spontaneous hypertensive rat. The genomes of both strains had previously been sequenced. “To analyse protein mass spec data, scientists usually map the spectra back to a peptide database derived from the reference genome of the organism they are studying,” Cuppen explains. “However, that database does not include strain-specific genetic variation, so peptides possessing amino acid changes due to non-synonymous variants will not be matched.” Therefore the researchers enhanced the existing rat peptide database by incorporating strain-specific non-synonymous genetic variants. Furthermore, they added transcript splice events from their RNA-seq data. Finally, the team compared gene expression levels at the RNA and protein level. In this manner they identified a genetic variant in the promoter of that gene that likely alters its expression in the hypertension rat strain and might contribute to their phenotype. “If you look critically at the data, you could have picked it up from just RNA-sequencing or just proteomics,” Cuppen admits, “but then, it would have been one of many events in a cloud of outliers. By combining these techniques, this protein stood out very clearly as the most prominent candidate that is differentially regulated, both at the RNA level and at the protein level.”

Breaking down barriers
Going forward, Cuppen and his colleagues want to apply the integration of genome, transcriptome, and proteome data to experimental cancer systems, with the goal of better understanding tumour induction, the effects of treatment, and drug resistance mechanisms. “A number of cancer research studies have combined DNA-based data, such as genome or exome sequences, and RNA-seq, but few have incorporated protein mass spec data as well.” While Cuppen believes the approach to be powerful, and likely to be used more often in the future, he cautioned that it is not easy. “It is not just measuring two things and putting them together in a computer and then better solutions come out. There is still work that needs to be done. Barriers between disciplines should be broken down, which first of all requires learning to speak each other’s language,” Cuppen concludes.
Proteomics is challenged by unravelling the mechanisms that generate protein diversity. Protein biosynthesis at transcription and translation level proves to be an important source. Today, next generation proteomics provides advanced techniques to unravel the underlying mechanisms. It is the research area Petra van Damme has her heart set on, since “it allows breakthroughs in our understanding of protein N-terminal biology.”

Petra van Damme, professor at University of Ghent’s department of Medical Protein Research, is especially interested in the biological importance of N-terminal protein diversity by applying so-called N-terminomics. In her scientific career, she has gained expertise in the field of gel-free proteomics at the VIB Proteomics Expertise Centre. She was involved in the development of a non-gel technique to isolate N-terminal peptides. “Such an approach is particularly useful because it is a good strategy to reduce complexity in proteome analysis by mass spectrometry,” she explains.

For the isolation of proteome-representative peptides, like amino and carboxyl terminal peptides, the researchers developed dedicated mass spectrometry driven proteomics techniques, based on the so-called COFRADIC principle, an acronym that stands for COmbined FRActional DIagonal Chromatography. As a pioneering technology, COFRADIC has been used in numerous studies in which N-terminal protein modifications were investigated. Van Damme is especially interested in protein N-terminal acetylation. “Nt-acetylation occurs on the majority of eukaryotic proteins and is increasingly recognised as a vital modification with functional implications ranging from protein degradation to protein localisation.”

**N-terminal protein isoforms**

Proteins can be modified either during their synthesis (cotranslational) or after synthesis has been completed (posttranslational). Nt-acetylation occurs during protein synthesis. “To develop a clear functional understanding of this modification, it is necessary to gain insight into cotranslational processes at a molecular level,” says Van Damme. Such studies need proteomics and genomics methods suitable to analyse the protein modifications occurring when mRNA is being translated on polyribosomes.” The development of ribosome profiling methods proved to be a perfect godsend to this goal. This method, also called RIBO-seq, provides a powerful tool for dissecting the molecular mechanism of translation in vivo. It is shown that protein-coding genes can give rise to multiple translation products of which expression is regulated at multiple levels. In that manner functional protein isoforms emerge due to e.g. alternative splicing, usage of alternative promotors or alternative translation initiating sites. “Such studies reveal the sources of proteome diversity in higher eukaryotes and help explain the development of complex organisms from genomes carrying only a few tens of thousands of protein-coding genes.” Van Damme’s research focuses primarily on alternative translation initiation sites (TIS) within a single transcript. “Employing our knowledge on Nt-acetylated protein N-termini serving as proxies of translation initiation, we found, for instance, no fewer than 1,700 alternative start sites in mice and humans giving rise to N-terminal proteinisoforms.” Interestingly, starting translation at an alternative site may have been evolutionarily selected for. For instance, in genes where the database annotated start codon (dbTIS) resides within a suboptimal nucleotide context, it has been found that the downstream TIS (dTIS) are significantly more conserved between species than in genes where the dBdTIS resides within the optimal context.

**Biological significance**

Next generation proteomics and genomics research provides a wealth of data. Van Damme is particularly interested in the biological significance from that data. To gain biological knowledge, N-terminomics and ribosome profiling should be combined. “Both techniques prove to be complementary, and integration leads to new insights.” This approach has proven to be successful, for instance, in demonstrating for the first time at the proteome-wide level, translation initiation at near-cognate start codons besides the existence of N-terminal extended protein variants. The N-terminal isoforms generated by alternative translation initiation may furthermore display altered localisation, stability and functionalities, thereby diversifying proteome functionalities even further.
Real time identification of tissues

Of all the speakers during the Progress Meeting, Zoltán Takáts definitely presents the coolest gadget. It is called the iKnife and provides surgeons with real-time MS-based in vivo tissue analysis data. The key question in cancer surgery is where to cut.

“What we need is in situ, real time identification of tissues,” says Zoltán Takáts. “With the iKnife, live sampling is possible. By comparing the profiles of the sample’s vapor to a large dataset of MS profiles of healthy and cancerous tissue, the iKnife offers instantaneous feedback on whether the surgeon should cut or leave the tissue intact.”

He seems surprised with the invitation to speak at a proteomics meeting. “I’m by far the odd one out, I have never done proteomics in my life.” But when it comes to MS, he surely fits in. During a postdoc at Purdue University, the Mecca of MS according to Takáts, he moved towards fundamental MS research. “Technology development, building new MS equipment, that type of work. This was also when I started to develop ways to avoid sample preparation and to enable real-time MS.” This is still a key theme in his research. “Current analytical chemistry methods rely on preparing the sample. There is grinding involved and dissolution in solvents. The sample therefore is not representative anymore of the intact organism,” he explains. “You lose most of the information along the way.”

Absolute

During his chemistry undergraduate years in Budapest, his interest in analytical chemistry and MS was sparked more or less by coincidence. “In the Hungarian university system, students can apply to perform extra research work next to the regular curriculum and I wanted to take up a topic in either green chemistry or environmental analytics. But I came across the work of a professor in analytical chemistry who worked with gas chromatography and that got my interest.” It was in his lab that Takáts first encountered MS. “A corner in the lab was reserved for the holiest of all equipment, a GC/MS,” he jokes. And he got hooked. “What I liked about MS was that it gave absolute information. Information that can be interpreted directly in a qualitative manner.”

Useful

As a researcher working on fundamental technology development, what is his main objective? “To make something that is useful.” A perfect match with the opening of his keynote where he stated that being an analytical chemist means that you try to answer other people’s questions. But Takáts wants to reach outside the academic world with his answers. “The use has to apply to a much broader context than just science.” That is why he has founded already three companies to work on getting new technologies applied in the real world. “Without start-up companies, nothing would happen,” he says. “This is the only road to translating scientific results into tangible applications.” He would like to see more scientists involved in bringing their results a step further. “We have a huge pile of technologies on one side and an even bigger pile of needs on the other. Bridging the gap is what matters to me. Even in cancer research, where the greater good is obvious, I am still amazed that a large part of the researchers is not interested in getting their results applied to develop for example a new drug or diagnostic. As a scientist, I am interested in the private life of ions flying back and forth, but I wouldn’t sleep very well without exploring the potential applications of my work.”
**Her research that includes work on glycan folding and misfolding in systemic amyloid disease, on the potential biological warfare threat Francisella tularensis, on isomeric lipids associated with prions and on peptides in the synovial tissue of arthritis patients. And those are just a pick from her most recent activities! In addition to all this, she has held positions on the boards of various scientific associations and is currently Vice President of the International Mass Spectrometry Foundation and a board member of US-HUPO. With a resume like that, surely she was hooked on MS from the moment she first set foot in a lab? But no. It actually took nothing less than NATO involvement to get MS on her radar.**

**Glasgow**

“As a graduate student, I worked on low temperature NMR to study organic reactions. We are talking the 1960s here,” Costello begins. “I wanted to travel, but being on a limited budget I had to be creative and look for funded opportunities.” One of those was presented by the NATO Summer Conferences, an initiative to bring scientists from different countries together fuelled by the idea that talking science across borders is a lot easier than talking politics. “I applied to participate and was selected to attend a conference in Glasgow on MS. That was how my interest started. Particularly the greater sensitivity of this technology compared to NMR and the possibility to analyse mixtures intrigued me.” The interest didn’t fade and after graduating she applied for a postdoc position in the group of MS pioneer Klaus Biemann at MIT. While proteins became the center of everyone’s attention, Costello decided to focus on other molecules, such as glycans. “It was a great place, full of innovations. The first computer-assisted MS was built there, as well as a literally huge MS-MS in 1985.”

When Biemann retired in the early 1990s, Costello succeeded him and decided to move to a medical school. “That is the best place to learn about the needs in the field, because here are the people who have to deal with real problems,” she explains the transition to the Boston University Medical School.

**Femtograms**

Moving to the present, she is still fascinated by MS. “I never got bored with the technology and there is still a great need for new methods. For example to study glycan structure. These structures are template-free, have a huge potential for branching and the different glycoforms vary over time. That’s a challenge, but these glycans are extremely important. They are crucial in determining the fate and characteristics of a cell.” When asked about the main driver of her research, she answers without hesitation: “To do stuff that hasn’t been done before and for which there is a clear need.”

Looking back, she feels the increase in sensitivity and the size of the molecules that can be studied to be the major breakthrough in MS. “When I started out, we needed hundreds of micrograms or even milligrams to measure molecules with a molecular weight of only 700. Now, we only need femtograms to measure molecules with MWs that are in the 100,000 range.” But looking back is not really her thing. “No, thank you. I don’t want to miss out on all the new things.”

“**I never got bored with the technology and there is still a great need for new methods.**”
Arzu Umar is an assistant professor at the ErasmusMC Cancer Institute. Her research focuses on applying proteomics to identify biomarkers for breast cancer. Among other things, that has resulted in a prognostic protein profile for triple negative (TN) breast cancer. Using this profile, physicians can determine whether chemotherapy is necessary or whether the patient’s good prognosis fails to justify such a harsh treatment. With John Foekens of ErasmusMC, Umar filed for a patent on this protein profile. “I have gained some experience in this area by now. I have filed a number of patent applications, but this one for the TN prognostic profile is the first we will actively develop further,” says Umar. “About a year ago we contacted several pharmaceutical companies, investors and entrepreneurs to discuss the possibilities.”

Asia

Frans Trouwen, an experienced life sciences consultant and entrepreneur, was included in that group. After initial agreements were reached, Trouwen put things in motion. Umar explains: “He founded a new company called ProBC, which will take on the commercial development and marketing of the TN prognostic profile. The first target is the Asian market. The national health care systems in many emerging economies are still very much under development. Our profile allows for serious cost reductions because it prevents unnecessary, and costly, treatments. ProBC expects that this cost savings aspect will be very attractive to these markets.” Only to these markets? Umar explains: “Here in Europe the situation is different because TN breast cancer patients are always treated, that is the standard of care. Many oncologists are therefore hesitant to consider that not treating a patient may be the better option. So convincing European physicians to use our prognostic profile is much more difficult. You need pioneering clinicians who embrace this new line of thinking. And there is so much to gain using prognostic profiles. Chemotherapy has a serious impact on the quality of life of a patient, not to mention that it is also very expensive.”

New perspective

Umar acts as scientific advisor to ProBC, but has no formal attachments to the company. She remains dedicated to her research. “There is so much left to be done. It would be nice if revenues from ProBC could be used to fund further research, but I don’t see anything coming in on short notice,” she laughs. Money isn’t her motivation anyway. “What I would like to achieve is a new perspective on treating cancer. We need to approach the problem from a different angle.” When it comes to ProBC, she has no outspoken strategy in mind. “I’m not knowledgeable on business development, that is exactly why we teamed up with Frans.” Her preference, however, would be for ProBC to develop and market its own products. Umar: “Even though my research is rather translational already, it also is still basic work. If I don’t do anything further with the outcomes, it will just stay where it is and never reach the patient.”

“The first target is the Asian market.”
A viral wake-up call

With the help of an NPC Valorisation Voucher, Tokameh Mahmoudi is building a strong patent position on a promising new class of compounds that can improve HIV-eradication therapies. “Protecting your findings as best as you can is a prerequisite to get a drug to the market and thus, to patients.”

To date, collaborating with Big Pharma has been a rewarding experience for biochemist Tokameh Mahmoudi, in spite of her earlier reluctance. “My view of the pharmaceutical industry was filled with all the stereotypes about losing your academic freedom and having your research dictated and so on,” Mahmoudi admits. She is an associate professor at the Biochemistry Department of ErasmusMC, and along with US pharma giant Merck, she is working on new therapies to eradicate latent HIV viruses, literally a persisting problem. “Current antiviral combinatorial therapies are very effective in suppressing HIV replication, but they are unable to really wipe out all infected cells in the patient. The problem is that a small number of viruses get incorporated in the immune system’s memory T-cells and there they form a latent reservoir. Awakening and then eradicating this reservoir is the final step towards a real curative therapy for HIV. We don’t need better antivirals. What we need are combinatorial therapies that can activate the latent viruses so that the antiviral drugs together with a charged immune system can wipe them out.”

Lithium
The transcription process in the dormant viruses, which the viruses need to replicate, is blocked. But how? That is just the sort of puzzle for Mahmoudi. “The expertise of my lab is in basic transcriptional research. One of our topics is the regulation of the HIV promoter, and that brought us to the problem of the latent viruses. We found that the Wnt-signaling pathway plays a role in awakening the reservoir and that this pathway can be activated by lithium.” It was this discovery that set Mahmoudi on the track to contact the pharma industry. “Merck already holds IP on a candidate compound for re-activation, but this compound does not target the whole range of pathways that need to be activated for purging of the full reservoir. That is why we contacted them regarding our findings and they were very interested. We initiated several collaborative research agreements, which are still running.” More or less simultaneously, Mahmoudi’s group discovered a completely new class of compounds that proved to be effective in activating latent HIV. It was a discovery worth pursuing, and she was awarded an NPC Valorisation Voucher at the end of 2012 to do so. The voucher enables her to fund further work on these compounds in house, even though the industry is already interested. “We are not sharing the data yet. We first want to build a strong patent. Protecting your findings as best as you can is a prerequisite to get a drug to the market and thus, to patients.”

Opportunities
Working with pharma also offers new possibilities for scientists, says Mahmoudi. “It gives you opportunities you would never get otherwise, such as access to clinical trial data and samples from responders and non-responders.” She has a clear strategy on how to operate in such a collaboration. “For me, it is about really being involved throughout the development track. That requires a strong and long-term vision on what you want and what you can do. That way, you stay in control of your research. In my experience, that is absolutely possible.”
Top publications with NPC contribution

In this NPC HighLights we provide a short list of papers that appeared recently in some of the top journals and to which NPC participants contributed. With the guarantee of being by far not comprehensive, this overview shows some elegant ground-breaking research.

Control of epithelial cell migration and invasion by the IKKβ- and CK1α-mediated degradation of RAPGEF2

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Epithelial cell migration is crucial for the development and regeneration of epithelial tissues. Aberrant regulation of epithelial cell migration has a major role in pathological processes such as the development of cancer metastasis and tissue fibrosis. Here, we report that in response to factors that promote cell motility, the Rap guanine exchange factor RAPGEF2 is rapidly phosphorylated by I-kappa-B-kinase-β and casein kinase-1α and consequently degraded by the proteasome via the SCF(βTrCP) ubiquitin ligase. Failure to degrade RAPGEF2 in epithelial cells results in sustained activity of Rap1 and inhibition of cell migration induced by HGF, a potent metastatic factor. Furthermore, expression of a degradation-resistant RAPGEF2 mutant greatly suppresses dissemination and metastasis of human breast cancer cells. These findings reveal a molecular mechanism regulating migration and invasion of epithelial cells and establish a key direct link between IKKβ and cell motility controlled by Rap-integrin signaling.

Arrayed BUB recruitment modules in the kinetochore scaffold KNL1 promote accurate chromosome segregation

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Fidelity of chromosome segregation relies on coordination of chromosome biorientation and the spindle checkpoint. Central to this is the kinetochore scaffold KNL1 that integrates the functions of various mitotic regulators including BUB1 and BUBR1. We show that KNL1 contains an extensive array of short linear sequence modules that encompass TxxΩ and MELT motifs and that can independently localize BUB1. Engineered KNL1 variants with few modules recruit low levels of BUB1 to kinetochores but support a robust checkpoint. Increasing numbers of modules concomitantly increase kinetochore BUB1 levels and progressively enhance efficiency of chromosome biorientation. Remarkably, normal KNL1 function is maintained by replacing all modules with a short array of naturally occurring or identical, artificially designed ones. A minimal array of generic BUB recruitment modules in KNL1 thus suffices for accurate chromosome segregation. Widespread divergence in the amount and sequence of these modules in KNL1 homologues may represent flexibility in adapting regulation of mitotic processes to altered requirements for chromosome segregation during evolution.

Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome

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Patient-specific induced pluripotent stem cells (iPSCs) will assist research on genetic cardiac maladies if the disease phenotype is recapitulated in vitro. However, genetic background variations may confound disease traits, especially for disorders with incomplete penetrance, such as long-QT syndromes (LQTs). To study the LQT2-associated c.A2987T (N996I) KCNH2 mutation under genetically defined conditions, we derived iPSCs from a patient carrying this mutation and corrected it. Furthermore, we introduced the same point mutation in human embryonic stem cells (hESCs), generating two genetically distinct isogenic pairs of LQT2 and control lines. Correction of the mutation normalized the current (IKr) conducted by the HERG channel and the action potential (AP) duration in iPSC-derived cardiomyocytes (CMs). Introduction of the same mutation reduced IKr and prolonged the AP duration in hESC-derived cardiomyocytes (CMs). Further characterization of N996I-HERG pathogenesis revealed a trafficking defect. Our results demonstrated that the c.A2987T KCNH2 mutation is the primary cause of the LQT2 phenotype. Precise genetic modification of pluripotent stem cells provided a physiologically and functionally relevant human cellular context to reveal the pathogenic mechanism underlying this specific disease phenotype.
Quantitative and qualitative proteome characteristics extracted from in-depth integrated genomics and proteomics analysis


Quantitative and qualitative protein characteristics are regulated at genomic, transcriptomic, and posttranscriptional levels. Here, we integrated in-depth transcriptome and proteome analyses of liver tissues from two rat strains to unravel the interactions within and between these layers. We obtained peptide evidence for 26,463 rat liver proteins. We validated 1,195 gene predictions, 83 splice events, 126 proteins with nonsynonymous variants, and 20 isoforms with nonsynonymous RNA editing. Quantitative RNA sequencing and proteomics data correlate highly between strains but poorly among each other, indicating extensive nongenetic regulation. Our multilevel analysis identified a genomic variant in the promoter of the most differentially expressed gene Cyp17a1, a previously reported top hit in genome-wide association studies for human hypertension, as a potential contributor to the hypertension phenotype in SHR rats. These results demonstrate the power of and need for integrative analysis for understanding genetic control of molecular dynamics and phenotypic diversity in a system-wide manner.

Expanding the detectable HLA peptide repertoire using electron-transfer/higher-energy collision dissociation (EThcD)


The identification of peptides presented by human leukocyte antigen (HLA) class I is tremendously important for the understanding of antigen presentation mechanisms under healthy or diseased conditions. Currently, mass spectrometry-based methods represent the best methodology for the identification of HLA class I-associated peptides. However, the HLA class I peptide repertoire remains largely unexplored because the variable nature of endogenous peptides represents difficulties in conventional peptide fragmentation technology. Here, we substantially enhanced (about threefold) the identification success rate of peptides presented by HLA class I using combined electron-transfer/higher-energy collision dissociation (EThcD), reporting over 12,000 high-confident (false discovery rate <1%) peptides from a single human B-cell line. The direct importance of such an unprecedented large dataset is highlighted by the discovery of unique features in antigen presentation. The observation that a substantial part of proteins is sampled across different HLA alleles, and the common occurrence of HLA class I nested sets, suggest that the constraints of HLA class I to comprehensively present the health states of cells are not as tight as previously thought. Our dataset contains a substantial set of peptides bearing a variety of posttranslational modifications presented with marked allele-specific differences. We propose that EThcD should become the method of choice in analyzing HLA class I-presented peptides.

Other highlighted publications


A decade of membrane proteomics

In 2004 the Membrane Proteomics Research Hotel of the NPC started in parallel a number of projects on the identification and full characterisation of membrane proteins in microbes, plants, and mammals. The study of membrane proteins offers great opportunities towards the development of new antibiotics, vaccines and antibodies. Research on membrane proteins is challenging, requiring ample experience in cell fractionation, protein isolation and analysis. Exactly to achieve that, a dedicated proteomics facility had to be established.

The Membrane Proteomics Research Hotel comprises a complete proteomics pipeline integrating cell cultivation, sample preparation, advanced mass spectrometry and bioinformatic tools underpinning protein identification and quantification in complex biological samples. Several academic and industrial research projects have been excellently supported by Dr. Fabrizia Fusetti and Dr. Hjalmar Permetier and their teams. The discovery of a protein factor that controls protein synthesis, the identification of new leukemic stem cell markers, the discovery of signalling proteins involved in chemotaxis, the characterisation of probiotic features in bacteria, and the development of new bacterial strains with improved capacity to produce recombinant proteins are just some of the highlights of this joint effort.

Ten years of proteomics research in the Netherlands has contributed to solve some parts of the puzzle of life. The impact of membrane proteomics is high and has resulted in a large number of publications over the last ten years, several of which appeared in top-ranked journals. Major breakthroughs have been acknowledged by the scientific and popular press, and resulted in invitations for plenary lectures at international conferences.

Additionally, the NPC hotels have contributed to gather an active network of excellent scientists, even far beyond the NPC consortium. In Groningen PhD students and guest researchers working on NPC projects have been trained in state-of-the-art proteomics, while many more profited from the facilities by having their samples analysed by expert researchers and technical assistants. The proteomics facilities have been used in courses and master classes to train BSc and MSc students. The advanced knowledge accumulated in the past decade will form a solid base to address future societal challenges, such as taming microorganisms relevant for the bio-based economy or the design of novel strategies and better drugs for treatment of diseases.