Painting proteins to study cardiovascular pathology

Impact

This work is focused on the molecular and cellular pathways underlying cardiovascular pathology. Cardiovascular disease (CVD) is amongst the leading causes of morbidity and mortality worldwide and as consequences are often debilitating, they have a vast societal and economic impact. Especially ischemic diseases like stroke, myocardial infarction, and pulmonary embolism lead to long-lasting disabilities leaving some patients partly paralyzed, with permanent heart failure or lung damage. Increasing aging and an unhealthy lifestyle with sedentary behavior, smoking, and a diet revolving around the consumption of alcohol, processed foods, refined carbohydrates, red meat, and an abundance of saturated fatty acids (not coincidentally named Western-type diet) can lead to typical diseases of affluence. Despite an increase in knowledge about the risk factors of CVD and better healthcare, these diseases, also called lifestyle or prosperity diseases, are on the rise. Although lifestyle is a modifiable risk factor, aging is the major non-modifiable risk factor. In a worldwide aging landscape, the global socio-economic and public healthcare concerns are increasing, calling to unravel aging and age-related disease, to suppress the burden on the public. Since the etiology of CVD is multifactorial and complex, and takes years or even decades to develop, the understanding of molecular mechanisms underlying the development of CVD is crucial to dissect pathways leading to disease, to optimize diagnostic procedures, primary and secondary prevention, and treatment of CVD. Earlier diagnosis will increase the chance of successful treatment, will aid in determining which treatment should be followed, and will ultimately save the spending of the healthcare industry and society.

The current work is composed of diagnostic (*chapters two and seven*), methodologic (*chapter four*), and fundamental (cell-)biologic research (*chapters three, five, and six*) underlying CVD. The primary goal of curiosity-driven fundamental research is to gain knowledge on molecular pathways, which is extremely important as almost all translational and clinical research emanates from fundamental, basic research. The impact can have large and sometimes unexpected overlap with other pathologies, for example, cancer or even communicable diseases like viruses, as I will touch upon below.

Diagnostic research

To advance early diagnosis in CVD, extracellular vesicles (EVs) can potentially be used as diagnostic and prognostic biomarkers. EVs are circulating in the blood and membrane composition of EVs and their content is determined by the parental cell. When the parental cell is damaged, the number of EVs will increase. By measuring EV content, relative or absolute numbers will indicate any underlying pathology. In *chapter two*, we have written a review on using EVs as biomarkers for CVD. The biggest challenge in the implementation of measuring EVs is their small size. We have listed different isolation and detection methods, markers to determine the cellular origin, and several studies that use information in and on the vesicles, as biomarkers for disease. In several trials, a rise in EV numbers and certain nucleic acids was predictive for later CVD development. However, these are all retrospective, single-center studies, and the small size of EVs makes handling and standardization difficult. We envision a significant role for EVs as biomarkers in the future, which can aid in the prognosis and diagnosis of CVD, and help identify which individual will benefit from primary or secondary prevention. The other main medical field looking at EVs as biomarkers is cancer research. What we call 'liquid biopsies' is the characterization of plasma for circulating tumor DNA, tumor-educated platelets, or EVs from cancer cells or tumor-educated platelets. Using sequencing, a multitude of cancers can already be identified using liquid biopsies, and both cardiovascular and oncology scientists work towards optimization. Compared to traditional biopsies, a blood sample is, is less invasive, quicker, and much more comfortable to the patient, with less chance of side-effects like intratumoral bleeding.

Molecular imaging is a very valuable tool for early diagnosis of CVD. Imaging thrombosis is important when symptoms appear, to localize the culprit, better yet, to predict treatment outcomes. We have therefore developed and evaluated an imaging tracer in a mouse model, that can potentially discriminate young thrombi with enzymatic activity versus older thrombi. The gold standard non-invasive treatment for ischemic disease, an intravenous (IV) injection with tissue plasminogen activator (tPA), can only be used on young, active thrombi. The imaging tracer we have developed and tested in *chapter seven* will only bind to these active thrombi, and can thereby predict whether tPA treatment will work, or whether more invasive endovascular procedures should be followed immediately. This tracer may aid in diagnosis and shorten time-to-treatment, which should reduce permanent disabilities due to ischemic stroke, heart attacks, or pulmonary embolisms.

Methodologic research

Fundamental research underlying vascular diseases is mainly based on cellular and molecular interactions, specifically under flow conditions. It has become increasingly clear in the last decades, that hemodynamic forces in the vasculature have a high impact on molecular interactions. The absence of flow or shear by itself, altered mechanistic stimuli, is a sign for endothelial cells to upregulate proteins that promote atherogenesis and attract leukocytes. To advance research under flowing conditions, we have described a method that can be adapted to study interactions of many different cell types in the blood or the vasculature. By using this laminar flow assay, mechanistic roles of leukocyte interaction with endothelial cells or platelets can be investigated, as well as the role of different pharmaceuticals that can potentially influence these interactions. Studying these interactions in an *in vitro* setup is relevant for both fundamental and applied medical research. In parallel, it will reduce the number of animal experiments, one of the three R's that the Experiments on Animals Act is based upon, as explained in greater detail in the discussion. The method we present is easily adaptable and will aid in the probability that *in vitro* observed interactions correctly predict the *in vivo* situation.

Fundamental research

As said, the primary aim of fundamental research is to improve knowledge of, in our case, molecular and cellular interactions underlying vascular diseases. Although very much studied, underlying pathways are highly complex and intertwined, and not all signaling pathways are completely characterized. This complexity also adds to the importance of deciphering pathways, as unwanted side effects of treatment are often due to unforeseen interactions of underlying pathways. Since the major hemostatic pathway to fibrin formation, also known as the coagulation cascade, is full of positive and negative feedback loops, it is evident that a small change can lead to a big impact.

One of the main proteins this fundamental work is based upon is platelet factor 4 (PF4, CXCL4). This is a chemokine present in large numbers in platelets and is released when platelets are activated. A chemokine has the function to attract circulating leukocytes and direct them to sites of inflammation, where the immune cells respond to disturbance. PF4 is an atypical chemokine, with many different described functions. The underlying receptors, pathways, and interactions are incompletely understood, and even (sub)cellular localization in different cell types remains ambiguous. In the current work, we describe the localization in platelets relative to RANTES in *chapter five*, as PF4 can form heterodimers with RANTES which exacerbates inflammation and amplifies the attraction of monocytes, driving atherosclerosis. It is important to study where this

heterodimerization occurs, as it is a potential pharmacologic target. When the platelet is activated and releases its granule constituents, the endothelial cell is the first vascular cell that is encountered. We showed that PF4 and RANTES are internalized independently in the endothelial cells in *chapter three*, although the function remains to be elucidated. We have used a laminar flow-based assay, described in *chapter four* and under 'methodologic research' to study monocyte recruitment to endothelial cells after internalization of PF4 and RANTES, which was not altered after the internalization. We also showed a positive feedback loop where PF4 by itself can activate platelets, a pathway distinct from the protein galectin-1, in *chapter six*. Recent work based on these complementary platelet responses shows that there is a specific cross-linking of PF4 with galectin-1, which does not seem to alter platelet activation.

Fundamental research, or curiosity-driven research, has become more and more vulnerable, as the application is not always evident. The abstraction of the work is difficult for the public to understand, whilst public opinion or patient advocates have been getting a more prominent role in granting research in recent years. Of course, patients can give a new perspective in the evaluation of clinical research, but we have to be careful not to rate solution-driven research with a straightforward goal and immediate benefits over curiosity-driven research, which can drive innovation over the longer run, not seldom in an unrelated context. Basic researchers and their funders even take risk of being ridiculed by the public and even their peers. For example, a US-government-sponsored project by Knipling and Bushland, to investigate the sex life of the screwworm fly between the 1930s-1950s has long been a target of Members of Congress to illustrate "Washington Waste". It took until 2016 for the work to be granted the Golden Goose Award, for previously called ridiculous research which resulted in significant benefits to society, as insight in mating habits of the screwworm fly resulted in its eradication, saving America's livestock industry billions of dollars.

COVID-19

When this study was started, never could we have imagined the work to be concluded in 2021, in the midst of the SARS-CoV2 pandemic that has been challenging the resourcefulness of our national health service, politics, and the public for over a year and a half. First described as 'pneumonia of unknown cause' emerging in December 2019 in Wuhan, China, was later identified to be caused by a beta coronavirus. The first patient in The Netherlands was confirmed late February 2020, and the official pandemic status was declared on 12 March 2020 by the World Health Organization. An early Dutch study in April 2020, of 184 ICU admitted patients all receiving at least standard doses of thromboprophylaxis with low molecular weight heparin (LMWH), showed a remarkable 31% incidence of thrombotic complications. Not before long, endothelial involvement was apparent, and endothelial dysfunction was postulated essential for initiation and propagation of severe COVID-19, the term used for disease caused by SARS-CoV2. Two independent studies have shown high EV-TF^+ -levels in COVID-19 patients, where absolute numbers correlated with disease severity, D-dimer levels, inflammation markers, and mortality, whereas PAI-1 activity was not increased, as opposed to patients with septic shock. These studies indicated the use of EVs as prognostic biomarkers for disease progression, and the overlap of at first glance unrelated health problems, which share common molecular pathways and pathologic mechanisms to CVD.

It took SARS-CoV2 only 3 months from first recognition to pandemic properties, illustrating its extreme virulence, resulting in unprecedented global measurements, including lockdowns and travel restrictions, with huge economic consequences. This leads to collaborations of researchers and the pharmaceutic industry worldwide, to develop and test several vaccines in record times. As of today, four different vaccines are approved by the EMA, three of which by the FDA. Two of these are mRNA vaccines, the other two are vector vaccines. When vaccinating entire nations, there will be incidences of extremely rare side effects that were not discovered during clinical testing. In this case, the vector vaccines can cause vaccine-induced immune thrombotic thrombocytopenia (VITT), while mRNA vaccines are not linked to these excess venous thrombosis episodes. VITT seems to be caused by (auto-)antibodies to PF4, activating platelets, causing thrombosis, and clearing by the immune system, resulting in thrombocytopenia. This syndrome is canonically described as Heparin-Induced Thrombocytopenia (HIT), caused by autoantibodies against PF4-heparin (ultra-large) complexes. And indeed, anti-PF4 antibodies were found in patients suffering from VITT, without past exposure to heparin, which has occasionally been shown after bacterial or viral infections. We currently do not know whether these antibodies are auto-antibodies formed due to an excessive immune response inflicted by the vaccine, or whether in

some cases antibodies against the coronavirus spike protein can cross-react with PF4. Another explanation that has been postulated is that cross-reacting antibodies are formed against impurities in batches of vaccines, although this possible explanation is highly under debate and speculative to the development of VITT, and the research article was published as a pre-print without peer-review.

The publication of pre-prints is on the rise. Although pre-print servers as BioRχiv and medRχiv have existed for years, the SARS-CoV2 pandemic has revolutionized the use of pre-print servers. In the present era, where fast-paced and open science has helped scientists around the globe to develop several successful vaccines within a year, these benefits come with a price. Scientific information, even when not peer-reviewed, is made available to everyone, misunderstandings by scientific illiteracy can easily fuel fake news. These misunderstandings, whether unintentional or deliberately used to propagate conspiracy, spread over social media, with the same pace as SARS-CoV2 spread over the globe. In 2021, the public has been more opinionated and scientists and their science are scrutinized as never before, as quotes from scientists are blown out of proportion, and scraps and pieces of text without their context are used as proof to back up preconceived opinions. As scientists, we should remain critical and possibly reconsider the way science is communicated and shared with the world.