Sanquin
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9892
NL-1006 AN Amsterdam
The Netherlands
Telephone +31 20 512 30 00
Fax +31 20 512 33 03
info@sanquin.nl
www.sanquin.nl

Sanquin
Blood Supply

2010

Scientific Report

Blood and Beyond

2010

Scientific Report

Contents

Preface

Dear Reader,

It is a pleasure and honor to present to you the scientific report 2010 of Sanquin Blood Supply.

Covering the complete spectrum of blood and blood transfusion research we aim to perform original work on not only basic and clinical but also applied topics. As a consequence our research departments deal with a complementary range of subjects including fundamental biology and biochemistry of blood cells and plasma proteins, hematopoiesis, immunohematology, coagulation, immunopathology, blood-borne infections, blood transfusion technology, transfusion monitoring, transfusion medicine, and donor studies.

On 3 June 2010, Prof Ernest Briët, who in the last 5 years has led Sanquin's research department in an excellent manner, stepped down as our director of Research. On the occasion of his leave a farewell symposium was organized which was attended by over 200 friends and colleagues. Sanquin Research is indebted to Ernest Briët for his tremendous input.

In 2010 the department of Hematopoiesis, headed by dr Marieke van Lindern, started its work at Sanquin Research. The new department aims to combine basic research on various aspects of hematopoiesis with the development of novel cellular products. These novel activities are important since it is becoming clear that innovations on advanced cellular therapy products increasingly find their way to clinical applications and Sanquin Blood Supply wants to play an active role in this field.

Sanquin is proud that the LSBR (Landsteiner Foundation for Blood Transfusion Research) has awarded two prestigious fellowships to Sanquin researchers, namely Jaap van Buul (dept of Molecular Cell Biology) and Martijn Nolte (dept of Hematopoiesis). With these grants, these young researchers will be able to establish their independent groups within Sanquin Research.

We are very happy that Marieke van Ham was appointed 'Professor of Biological Immunology' at the science faculty (FNWI) of the University of Amsterdam (UvA). This appointment strengthens the long lasting relation Sanquin Blood Supply has with the UvA in the area of teaching immunology to the (medical) biology students. Education is an essential task of the research division as the students that we train represent the new PhD students that we want to interest in research on blood and blood transfusion in the near future.

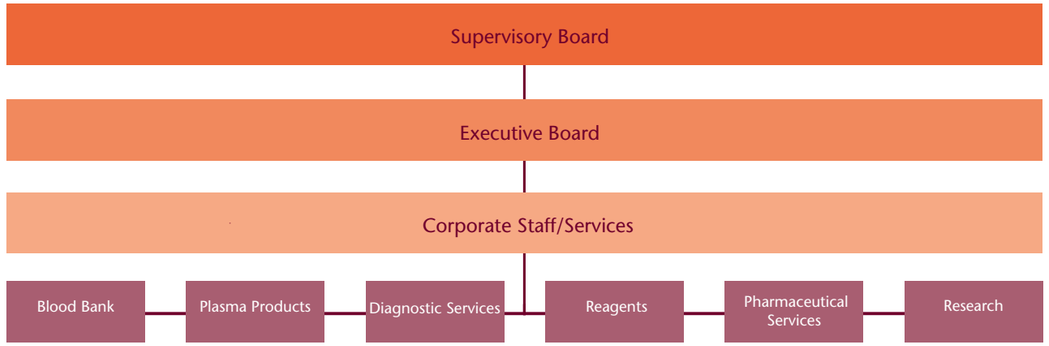
René van Lier
Director of Research

Introduction

Jan Willem Smeenk
Sanquin Corporate Staff
jw.smeenk@sanquin.nl

Sanquin Blood Supply comprises five divisions and a business unit. A three member Executive Board is responsible for the organization and reports to the Supervisory Board. A corporate staff office and a number of Corporate Services support the organization. Sanquin Blood Bank operates in four regions.

At the Amsterdam premises we find Sanquin Plasma Products, Sanquin Diagnostic Services, Sanquin Research, Sanquin Reagents and the business unit Sanquin Pharmaceutical Services. The CAF-DCF Product Development Division (former R&D) is located in Brussels. Besides Sanquin Research, R&D is performed in all other divisions, with emphasis on Product and Process Development.



Research Lines

Research performed at Sanquin ranges from basic, biological issues to clinical and applied issues. All research lines are headed by Principal Investigators (PI). In this scientific report you will find more information on the following research lines, ordered from basic to clinical and applied research:

Department	Research Lines	Principle Investigators
Molecular Cell Biology	Molecular cell biology	Peter Hordijk PhD
Blood Cell Research	Phagocyte laboratory	Timo van den Berg PhD
	Blood transfusion technology	Dirk de Korte PhD
Plasma Proteins	Plasma proteins	Prof Koen Mertens PhD
	Cellular hemostasis	Jan Voorberg PhD
Hematopoiesis	Hematopoiesis	Marieke von Lindern PhD
Experimental Immunohematology	Experimental immunohematology	Prof Ellen van der Schoot MD PhD
Immunopathology	Immunopathology	Prof Marieke van Ham PhD
	Autoimmune diseases	Prof Lucien Aarden PhD
Laboratory for Viral Immune Pathogenesis, AMC	Viral immune pathogenesis	Prof Hanneke Schuitemaker PhD
Blood-borne Infections	Blood-borne infections	Prof Hans Zaaijer MD PhD
Transfusion Technology Assessment	Transfusion technology assessment	Cees van der Poel MD PhD
Transfusion Monitoring	Transfusion monitoring	Janny de Wildt-Eggen PhD
Transfusion Medicine	Transfusion medicine	Prof Anneke Brand MD PhD
		Prof Dick van Rhenen MD PhD
Donor Studies	Donor studies	Wim de Kort MD PhD

Scientific Advisory Board

The Scientific Advisory Board supervises the quality system, advises the Sanquin Executive Board on all matters concerning strategy, (co-ordination of) research and research infrastructure, and checks annually whether Sanquin's research program meets the framework of the policy plans. Furthermore, the Scientific Advisory Board assesses the quality of Sanquin's research, based on bibliometric analyses and reports of site visits. On 31 December 2010 the Scientific Advisory Board consisted of:

- *Prof RAW van Lier MD PhD (Chairman, Sanquin Executive Board & University of Amsterdam)*
- *Prof A Brand MD PhD (Sanquin Blood Bank Leiden & Leiden University)*
- *Prof AF Cohen MD PhD (Center for Human Drug Research & Leiden University)*
- *Prof RRP de Vries MD PhD (Leiden University)*
- *Prof DE Grobbee MD PhD (University of Utrecht)*
- *Prof MM Levi MD PhD (University of Amsterdam)*
- *Prof DJ van Rhenen MD PhD (Sanquin Blood Bank Leiden & Erasmus University Rotterdam)*
- *JW Smeenk MSc (Executive secretary Sanquin Corporate Staff)*

Research Assessment

All research groups of Sanquin are visited by an international peer review committee once in every five years.
In 2010, the department of Donor Studies of Wim de Kort was reviewed. The preliminary findings of the peer review committees were positive on the quality of research. A number of recommendations were given and are being taken into account in 2011. As in earlier years, the Peer Review Commitee was supported by an executive secretary from the independent agency Quality Assurance Netherlands Universities (QANU).

Academic affiliations

Sanquin research departments attract many students who participate in scientific projects. Historically there is a strong collaboration with the Academic Medical Center (AMC) of the University of Amsterdam. This joint AMC – Sanquin Landsteiner Laboratory is mainly housed within Sanquin premises.
In 2010 the very long standing collaboration with Leiden University Medical Center was formalized in the joint Sanquin-LUMC Jon J van Rood Center for Clinical Transfusion Research.

At many Dutch universities, members of the staff from various Sanquin divisions are involved in theoretical and practical training programs for undergraduate and graduate students in (medical) biology, pharmacy, medicine, and health sciences as well as for laboratory technicians. Of course, Sanquin is also involved in training of specialists in blood transfusion medicine, other medical specialties, and training of nurses. Sanquin has established a recognized training program for medical doctors specializing in transfusion medicine and donor care.

Sanquin Consulting Services provides training on the job for colleagues from sister organizations in developing countries in Africa, South America, and Asia as well as the former East European Countries. With the University of Groningen Medical Center, Sanquin Blood Bank Groningen runs a postgraduate masters program, under the heading of the Academic Institute for International Development of Transfusion Medicine

(IDTM). Sanquin is a WHO Collaborating Organization for Transfusion Medicine.

Professorships Sanquin Staff

- *Prof Lucien Aarden PhD (Molecular immunology, Academic Medical Center, University of Amsterdam)*
- *Prof Anneke Brand MD PhD (Blood transfusion medicine, Leiden University Medical Center)*
- *Prof Taco Kuijpers MD PhD (Pediatric immunology, Emma Children's Hospital, University of Amsterdam)*
- *Prof Koen Mertens PhD (Pharmaceutical plasma proteins, Faculty of Pharmacy, Utrecht University)*
- *Prof Hanneke Schuitemaker PhD (Virology, especially viro-pathogenesis of AIDS, Academic Medical Center, University of Amsterdam)*
- *Prof Ellen van der Schoot MD PhD (Experimental immunohematology, Academic Medical Center, University of Amsterdam)*
- *Prof Marieke van Ham PhD (Biological immunology, Faculty of Science, University of Amsterdam)*
- *Prof René van Lier MD PhD (Experimental Immunology, Academic Medical Center, University of Amsterdam)*
- *Prof Dick van Rhenen MD PhD (Blood transfusion medicine, Erasmus University Medical Center, University of Rotterdam)*
- *Prof Hans Zaaijer MD PhD (Blood-borne infections, Academic Medical Center, University of Amsterdam)*

CAF-DCF professorships

- *Prof Michel Delforge MD PhD (CAF-DCF professor in Hematology and stem cell plasticity, Catholic University of Leuven)*
- *Prof Jacques Pirenne MD PhD (CAF-DCF professor in Abdominal transplant surgery, Catholic University of Leuven)*

Landsteiner Laboratory

As mentioned above, there is a long-standing collaboration with the University of Amsterdam in the joint AMC-Sanquin Landsteiner Laboratory. Through this collaboration Sanquin staff members participate in research programs and curricula of the AMC. Researchers of Sanquin contribute to the research programs of the Center for Immunology Amsterdam (CIA) and the Center for Infection and Immunity Amsterdam (CINIMA).

Jon J van Rood Center for Clinical Transfusion Research

On May 26, 2010 the Jon J van Rood Center for Clinical Transfusion Research officially opened. The Center is a joint collaboration between Sanquin and the Leiden University Medical Center. This already long standing collaboration is focused on scientific research and education in the field of blood transfusion medicine. The Center is involved in training for medical specialists on blood transfusion medicine.

Within the Jon J van Rood Center, the Department of Clinical Transfusion Medicine of Sanquin Research collaborates closely with the departments of Clinical Epidemiology and Immunohematology & Blood Bank of Leiden University Medical Center. Various clinical departments of this university hospital are involved in a number of clinically relevant studies and clinical trials in the field of blood transfusion medicine. The Cord Blood Bank is also part of the Center.

Accreditation and quality assurance

Code of conduct

In 2006 Sanquin Executive Board decided on a research code of conduct, that is based on various codes of conduct from Dutch Universities and the Royal Netherlands Academy of Arts and Sciences. Sanquin was awarded membership of LOWI – the national organization for scientific integrity – that acts as independent advisory body in case of a breach of scientific integrity by a Sanquin member of staff. An independent ombudsman was already appointed in 2006.

Accreditation

Jan Waas, j.waas@sanquin.nl

The department Blood Transfusion Technology was visited by the Dutch Accreditation Council (RvA) and the CCKL in March 2010. It prolonged its accreditation according to ISO 17025 and certification according to the CCKL 'Code of practice version four'. The laboratory for Stem Cell Transplantation held its certification to ISO 9001 and ISO 13485 as it was successfully visited by the Lloyds auditor. An audit team of JACIE (Joint Accreditation Committee ISCT & EBMT) and CCKL also inspected the laboratory for Stem Cell Transplantation and granted a certificate to the Standards for Haematopoietic Progenitor Cell Collection, Processing & Transplantation and the CCKL 'Code of practice version four'. The department for Cryobiology prolonged its ISO 9001:2008 certificate. In November 2010 Sanquin Research management asked the QA department to make a plan to build a dedicated Research Quality Management System.

Milestones

In November 2010 the internal science day took place and was again a great success. The biennial PhD award was given to Micha Nethe for his research leading to new insights in Rac1 signaling. The poster prize was awarded to PhD student Xiwen Zhao.

Personnel

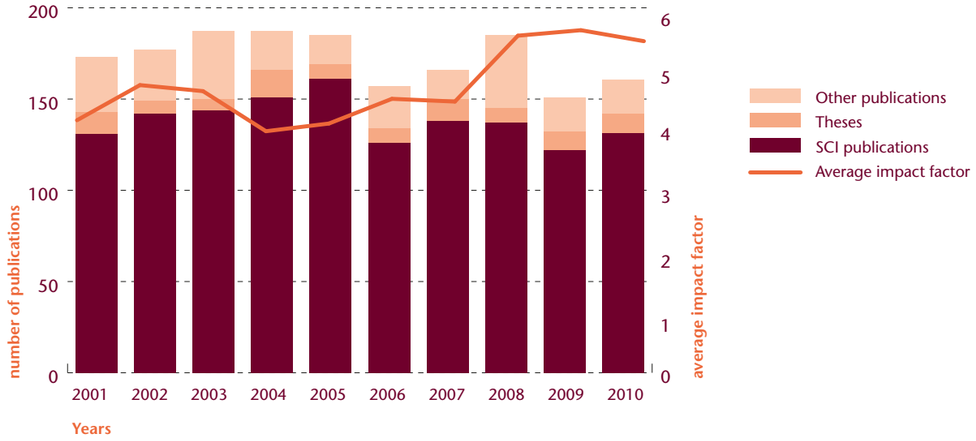
Director of Sanquin Research and member of the executive Board Prof Ernest Briët retired in 2010. He was succeeded by Prof René van Lier in both capacities. Prof van Lier took a number of staff with him from the Academic Medical Center (University of Amsterdam), among which Martijn Nolte, who will head the research group on adaptive immunology that will find its place within the new department of Hematopoiesis. Dr Marieke von Lindern and her group joined Sanquin from Erasmus University Medical Center to head this new department.

Dr Cees van der Poel of the Transfusion Technology Assessment Unit of Sanquin and Julius Center Utrecht University retired in 2010 from Sanquin. He will still be involved in TTA research as an honorary fellow of Utrecht University.

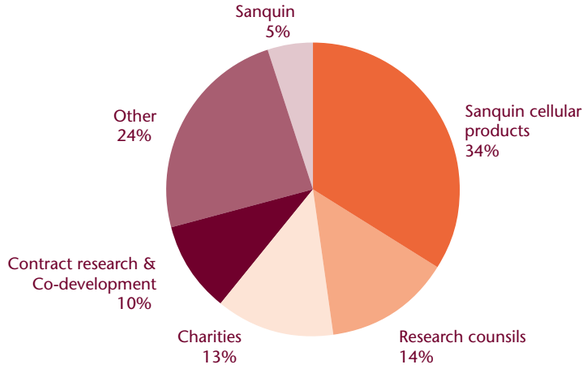
Publications

The number of papers in peer reviewed journals is higher than last year (131). The average impact factor is slightly lower, 5.5. The average number of citations in the five years after publishing (2005) was 18.6 citations per paper.

Scientific publications and average impact factor



Research project income 2010



Scientific publications and average impact factor

Articles* published in 1996 through 2005 annual reports cited** in five full years after publication

Publications from year	Average number of citations per publication
1996	15.0
1997	15.0
1998	20.6
1999	17.5
2000	19.7
2001	16.9
2002	21.4
2003	22.2
2004	18.3
2005	18.6

** Only SCI publications are included* *** Excluding self citations*

Funding

Miel Josemans
Manager Operations
Research
e.josemans@sanquin.nl

In 2010 Sanquin researchers were again successful in obtaining external funding (see page 112 for an overview of our sponsors). Several projects were applied for, ranging from European funding to Charity funds. Fourteen research projects out of 44 were funded from Sanquin resources for product and process development for cellular products, after a review on quality by external experts and relevance to Sanquin's mission by the Research Programming Committee. The available funds for product and process development within the organization are expected to grow slightly in the years to come.

Valorization

Theo Ariëns
Manager Business Development
t.ariens@sanquin.nl

Be it in the area of therapeutic or diagnostic product development, testing of devices or process innovation, Sanquin Research is a sought-for partner in co-development and contract research activities. Within Sanquin the generated know-how & innovations are shared with our stakeholders: Blood Bank, Plasma Products, Pharmaceutical Services, Diagnostic Services and Reagents to improve patient health care.

Biotech, pharmaceutical, diagnostic & devices companies value Sanquin's in-depth knowledge & expertise and translational mindset of our researchers in the areas of blood transfusion, immunology, infectious diseases, blood coagulation, hematology, hemostasis & thrombosis. Revenues generated by research collaborations, contract research and out-licensing of patents/hybridoma's provide additional funding for our research activities. On page 112 an overview of commercial parties with whom Sanquin Research collaborates through the years is shown. On page 111 an overview of out licensed and available published patents and cell lines is shown.

Research Lines

Peter L Hordijk PhD
p.hordijk@sanquin.nl

Molecular Cell Biology

Academic staff
PL Hordijk PhD (PI)
M Fernandez-Borja PhD
JD van Buul PhD
PB van Hennik PhD

Post docs
A Schaefer PhD
S van Helden PhD

PhD students
BJ de Kreuk
J Kroon
BD Lam
M Nethe
E Reinen
I Timmerman
TJ van Duijn
J van Rijssel
Y Zoughlami

Technical staff
EC Anthony
M Hoogenboezem
C Mollenaar
E Mul
J Ottenhof
FPJ van Alphen

Students
M Assimudin
H Belkasim
N Fontijn
E Kostadinova
G Isijk
E Riesebos
J Vermeer
L Vogelpoel

Secretariat
AEPT Engels
MLMW Lutkie

Address
Sanquin Research
Department of Molecular Cell Biology
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3377
F +31 20 512 3474
E secretariaatMCB@sanquin.nl
W mcb.sanquin.nl

Molecular Cell Biology

Within the department of Molecular Cell Biology, we study the molecular basis of leukocyte transendothelial migration from different perspectives. We are interested in the regulation of the actin and microtubule cytoskeleton as this translates into the dynamics of cell adhesion and motility. In addition, we are interested in the control of leukocyte-endothelium interactions by adhesion molecules such as integrins and their ligands, as well as regulatory, membrane-associate proteins such as the prion protein.

Rho GTPase signaling in cell adhesion and migration

Peter Hordijk PhD, p.hordijk@sanquin.nl

Cytoskeletal dynamics is regulated by the family of Rho-like small GTPases. We have been focusing on the Rac1 GTPase, a key member of this family, that is known for its induction of actin polymerization and regulation of integrin and cadherin-based cell adhesion. Over the past years we have identified a series of novel Rac1-interacting proteins and have identified the biology that accompanies these interactions. In the past year, we have published on recent findings of two of these new interactors, CD2AP and Caveolin-1, that bind to the Rac1 hypervariable C-terminal region.

CD2AP is an adapter protein which is critical for the formation and maintenance of a specialized cell-cell contact between kidney podocyte foot processes, the slit diaphragm. Here, CD2AP links the cell adhesion protein nephrin to the actin cytoskeleton. In addition, CMS/CD2AP binds actin-regulating proteins, such as CAPZ and cortactin, and has been implicated in the internalization of growth factor receptors. CD2AP interacts specifically with the C-terminal domain of Rac1 but not with that of other Rho family members. Efficient interaction between Rac1 and CD2AP requires both the proline-rich domain and the poly-basic region in the Rac1 C terminus, and at least two of the three N-terminal SH3 domains of CD2AP. CD2AP co-localizes with Rac1 to membrane ruffles, and small interfering RNA-based experiments showed that CD2AP links Rac1 to CAPZ and cortactin. Finally, expression of constitutive active Rac1 recruits CD2AP to cell-cell contacts and we found that CD2AP participates in the control of the intercellular barrier function.

In addition to CD2AP, Rac1 binds the membrane-associated adapter protein Caveolin-1. We found that Rac1 activity promotes Cav1 accumulation at Rac1-positive peripheral adhesions in adherent cells. Using Cav1-deficient mouse fibroblasts and depletion of Cav1 expression in human epithelial and endothelial cells, mediated by small interfering RNA and short hairpin RNA, we found that loss of Cav1 induces an increase in Rac1 protein and its activated, GTP-bound form. Cav1 controls Rac1 protein levels by regulating the poly-ubiquitylation and degradation of activated Rac1 in an adhesion-dependent fashion. Finally, we show that Rac1 ubiquitylation is not required for effector binding, but regulates the dynamics of Rac1 at the periphery of the cell. These data extend the canonical model of Rac1 inactivation and uncover Cav1-regulated poly-ubiquitylation as an additional mechanism to control Rac1 signalling.
The regulation of Rac1 signaling by ubiquitylation is a novel aspect that we will pursue further in the coming years. Identification of the Rac1 E3 ubiquitin ligase represents an essential yet very challenging aspect of this work. In addition, we plan to define the role for mono-ubiquitylated Rac1, that appears to function as a separate protein species, in endothelial cell function and leukocyte transendothelial migration.

Recently, we have also embarked on a research line focusing on special adhesion structures called podosomes (Figure 1). These are very dynamic circular adhesion structures that are well known for their formation in myeloid cells, but also occur in primary human endothelial cells. The podosome proteome as well as the regulation of these structures is currently subject of study.

Key publications

Nethe M, Anthony EC, Fernandez-Borja M, Dee R, Geerts D, Hensbergen PJ, Deelder AM, Schmidt G, Hordijk PL. Focal-adhesion targeting links caveolin-1 to a Rac1-degradation pathway. J Cell Sci 2010; 123:1948-58.

Nourshargh S, Hordijk PL, Sixt M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. Nat Rev Mol Cell Biol 2010; 11(5):366-78.

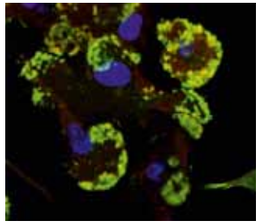
Endothelial cell signaling at the plasma membrane and at endosomes

Mar Fernandez-Borja PhD, m.fernandez@sanquin.nl

Role of the prion protein in cell adhesion and migration

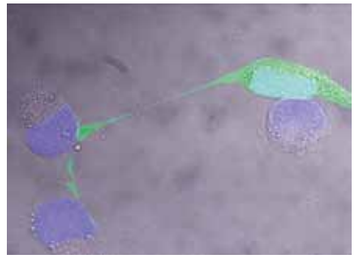
The cellular prion protein (PrP) is a highly conserved glycoprotein expressed primarily in the nervous and immune systems. PrP is clearly involved in the pathogenicity of prion diseases such as Creutzfeldt-Jakob disease. However, the physiological role of PrP remains elusive. The fact that PrP associates to several molecules involved in cell adhesion suggests that PrP may participate in the modulation of cell-cell and cell-substrate adhesion. We have studied the role of PrP in cell adhesion and migration in the context of our research theme on the molecular mechanisms of leukocyte transendothelial migration and endothelial permeability regulation. Antibody-mediated surface PrP ligation blocks

Figure 1



Podosomes in human dendritic cells (blue, nuclei; green, vinculin; red, F-actin)

Figure 2



Nanotube-mediated transfer of PrP between human endothelial cells. Endothelial cells transfected with GFP-tagged PrP were fixed and imaged by confocal microscopy (green: PrP; blue: nuclei). GFP-PrP was observed in long membrane tubes connecting two cells. In the image, GFP-PrP appears to be transferred from a transfected to a non-transfected cell.

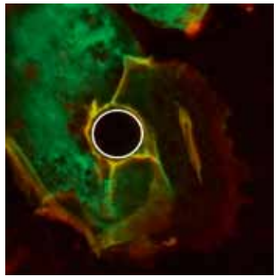
cell chemotaxis towards the chemokine CXCL-12 in the pro-monocytic cell line U937. In contrast, the silencing of PrP results in increased CXCL-12-driven migration. This suggests that PrP negatively regulates U937 cell migration. To understand the molecular mechanisms underlying PrP^C action, we followed a proteomic approach based on the identification by mass spectrometry of proteins binding to PrP. This analysis resulted in the identification of the integrin Mac-1 as a possible binding partner for PrP^C as well as several signaling molecules that could be involved in PrP-dependent signaling during cell migration.
In human endothelial cells, PrP accumulates at the intercellular junctions suggesting that it could be involved in the regulation of cell-cell adhesion. Furthermore, we have observed that PrP can be transferred from cell to cell via tunneling nanotubes (Figure 2). These thin membrane tubes have been observed in a variety of cell types and represent a novel form of long distance cell-to-cell communication.

In summary, our data support the hypothesis that PrP^C regulates cell adhesion to the substratum and to other cells. Studies to identify the mode of action of PrP are currently being performed in our group.

Signaling of the TNF receptor from endosomes

TNF is a pro-inflammatory cytokine that activates endothelial cells to express other inflammatory cytokines and receptors for leukocyte integrins facilitating the onset of inflammation. Binding of TNF to its receptor triggers intracellular signaling leading to gene expression. Recently, it was recognized that the TNF receptor signals not only from the plasma membrane but also from endosomes after being internalized. We found that TNF induces the synthesis of the endosomal small GTPase RhoB in endothelial cells and that RhoB regulates TNF receptor signaling. RhoB regulates intracellular TNF receptor traffic and MAP-kinase activation by TNF, however, RhoB has no effect on TNF receptor signaling from the plasma membrane. We propose that RhoB regulates TNF receptor inflammatory signaling from endosomes. Therefore, RhoB inhibition could be used as a strategy to selectively block TNF signaling from endosomes.

Figure 3



Trio (green) and ICAM-1 (red) accumulate around an ICAM-1-coated bead (dashed line).

RhoGTPase regulation and function in leukocyte transendothelial migration

Jaap van Buul PhD, j.vanbuul@sanquin.nl

Previously, our lab has shown that the endothelium activity participates together with the leukocytes in the process of TEM by forming dorsal membrane ruffles that surround the adherent leukocyte. We found that the RhoGTPases Rac1 and RhoG are responsible for this through their remodeling of the actin cytoskeleton. Activation of Rac1 and RhoG can be induced through so-called GEF (guanine-nucleotide exchange factor) proteins.

In the past year, our lab discovered a role for the GEF Trio in leukocyte transendothelial migration. Trio can activate Rac1 and RhoG, resulting in the formation of the dorsal protruding membranes that surround adhering leukocytes (Figure 3).

Moreover, we found that Trio protein and mRNA expression were upregulated by inflammatory stimuli such as TNF, IL-1 and LPS. Reduction of Trio expression by short hairpin RNA reduced migration of leukocytes across endothelial cells under physiological flow. In addition, a pharmacological inhibitor for Trio decreased leukocyte transendothelial migration.
Further characterization of Trio showed that this protein also mediates the spreading and migration of cells on a fibronectin-coated surface. Biochemical studies indicated that Trio activated the small GTPases Rac1 and RhoG through its N-terminal DH-PH domain, independent from its flanking SH3 domain. However, for Trio-mediated spreading and migration, no role for Trio-induced RhoG could be determined, suggesting that Trio-mediated spreading and migration is solely dependent on Rac1.

In the initial stages of transendothelial migration, leukocytes use the endothelial integrin ligands ICAM-1 and VCAM-1 for strong adhesion. Leukocyte binding is accompanied by the clustering of ICAM-1 or VCAM-1 on the cell surface. We found

that VCAM-1 localizes to sites of ICAM-1 clustering, induced by anti-ICAM-1 antibody-coated beads. Biochemical pull-down assays showed that ICAM-1 clustering induced its association to VCAM-1, suggesting a physical link between these two adhesion molecules. This association was partly dependent on lipid rafts, on F-actin and on the clustering of ICAM-1. These data show that VCAM-1 can be recruited, in an integrin-independent fashion, to clustered ICAM-1 which may serve to promote ICAM-1-mediated leukocyte adhesion. Related to protein-protein interactions within the plane of the apical endothelial cell membrane is the lateral mobility of ICAM-1. This is related to both its participation in specific membrane domains as well as its (integrin-induced) clustering. We therefore studied the dynamics of endothelial ICAM-1 under non-clustered and clustered conditions.
Detailed scanning electron and fluorescent microscopy showed that the apical surface of endothelial cells constitutively forms small filopodia-like protrusions that are positive for ICAM-1 and freely move within the lateral plane of the membrane. Clustering of ICAM-1, using anti-ICAM-1 antibody-coated beads, efficiently and rapidly recruits ICAM-1. Using fluorescence recovery after photo-bleaching (FRAP), we found that clustering increased the immobile fraction of ICAM-1, compared to non-clustered ICAM-1. This shift required the intracellular portion of ICAM-1. Moreover, biochemical assays showed that ICAM-1 clustering recruited beta-actin and filamin. Cytochalasin B, which interferes with actin polymerization, delayed the clustering of ICAM-1. In addition, we could show that cytochalasin B decreased the immobile fraction of clustered ICAM-1-GFP, but had no effect on non-clustered ICAM-1. Also, the motor protein myosin-II is recruited to ICAM-1 adhesion sites and its inhibition increased the immobile fraction of both non-clustered and clustered ICAM-1. Finally, blocking Rac1 activation, the formation of lipid rafts, myosin-II activity or actin polymerization, but not Src, reduced the adhesive function of ICAM-1, tested under physiological flow conditions. Together, these findings indicate that ICAM-1 clustering is regulated by the actin cytoskeleton in an inside-out fashion. Overall, these data indicate that signaling events within the endothelium are required for efficient ICAM-1-mediated leukocyte adhesion and transendothelial migration.

Key publications

Van Buul JD, van Rijssel J, van Alphen FP, Hoogenboezem M, Tol S, Hoeben KA, van Marle J, Mul EP, Hordijk PL. Inside-out regulation of ICAM-1 dynamics in TNF-alpha-activated endothelium. PLoS One 2010; 5:e11336.

Van Buul JD, van Rijssel J, van Alphen FP van Stalborch AM, Mul EP, Hordijk PL. ICAM-1 clustering on endothelial cells recruits VCAM-1. J Biomed Biotechnol 2010; 2010:120328.

Timo K van den Berg PhD
t.k.vandenberg@sanquin.nl

Phagocyte Laboratory

Academic staff
TK van den Berg PhD (PI)
Prof TW Kuijpers MD PhD

Post doc
AA Drewniak PhD

PhD students
J Alvarez Zarate
S Nagelkerke
K Szilagy
J van der Heijden
E van de Vijver
S Vendelbosch
X Zhao

Technical staff
M de Boer
J Geissler
R Gouw
J Hamme
K Schornagel
ATJ Tool
M van Houdt
K van Leeuwen
PJJH Verkuijlen

Guests
W Breunis
D Deng
J Heuvelmans
Prof D Roos PhD

Students
T Bloedjes
S Kenzel
B Koopmans

Secretariat
G Damhuis
W Winkel-Groeneveld

Address
Sanquin Research
Department of Blood Cell Research
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3317
F +31 20 512 3310
E secretariaatu2@sanquin.nl
W bcr.sanquin.nl

Phagocyte Laboratory

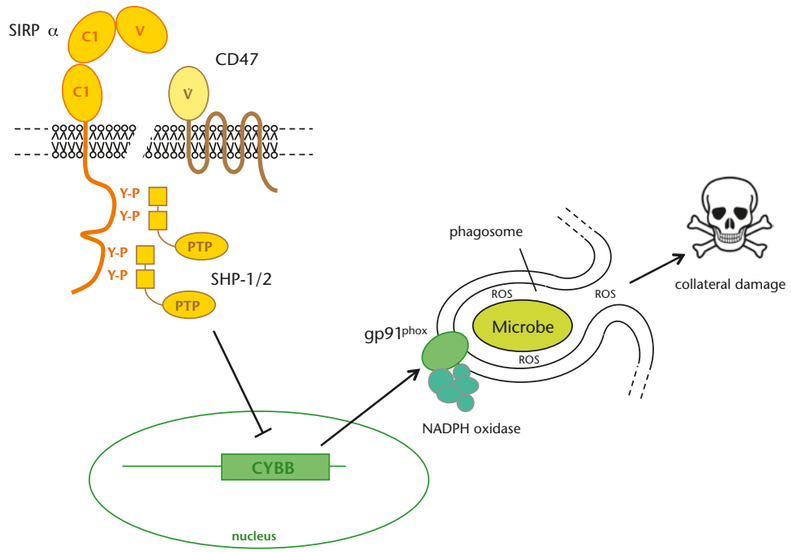
Phagocytes

Prof Taco Kuijpers MD PhD, t.w.kuijpers@amc.uva.nl

Neutrophils and macrophages recognize pathogens by means of a variety of surface receptors. These include non-opsonic pattern recognition receptors (PRR) as well as opsonic Fc-receptors and complement receptors. Among the various classes of PRR are the leucin-rich repeat-containing families of Toll-like receptors (TLR) and NOD-like receptors (NLR). While the pathogen-associated molecular patterns that act as ligands for the membrane-expressed TLR are relatively well defined, the activation mode of the intracellular NLR has remained enigmatic. The NLR member NLRP3 is part of a protein complex known as the inflammasome that mediates caspase-dependent activation of the inflammatory cytokine IL1b and related cytokines. While previous studies had suggested a role for reactive oxygen species (ROS) generated by a NADPH oxidase upstream of this inflammasome, we have shown, using cells from patients with chronic granulatomous disease (CGD), that in human phagocytes NLRP3-inflammasome function is independent of NADPH oxidases. Similarly, other functions like the formation of neutrophil extracellular traps, that is believed to be relevant in the context of bacterial clearance, also appeared NADPH oxidase independent, in contrary to earlier claims. We have also continued our efforts to identify and characterize novel mutations in the various subunits of the phagocyte NADPH oxidase that cause CGD. Among others we identified a patient with the insertion of a partially exonized retrotransposon of the TMF-1 gene into intron 1of the CYBB gene encoding the gp91^{phox} subunit of the oxidase. This represents a unique example of mRNA splicing ‘caught in action’ and causing a genetic disorder. The mutation analysis in CGD, performed by us and other laboratories, is also incorporated into the European CGD registry, a genetic database of mutations that includes information from about 500 patients, which is hosted by our laboratory.

We also identified a patient with a CARD9 deficiency allowing us to define the role of this protein in human phagocytes for the first time. CARD9 is expressed in myeloid cells and is known to function downstream of the β-glucan receptors dectin-1 and dectin-2, that play a critical role in the host defense against fungi. Strikingly, the patient suffers from a rare invasive Candida infection in the brain. Our further analyses indicate a prominent role of CARD9 in Candida-induced cytokine production by monocytes and in the induction of Th17 responses.

In addition to the pathways relevant for pathogen recognition and immune cell activation we are studying the function and genetics of various families of immunoreceptors expressed on phagocytes and other innate immune cells. These include Fc-receptors (FcR), signal regulatory proteins (SIRP), and killer-like immunoreceptors (KIR). SIRPa, the prototypic member of the SIRP family, is a typical inhibitory immunoreceptor expressed primarily on myeloid and neuronal cells. It acts as a receptor for the broadly expressed surface molecule CD47, and the ligation of SIRPa by CD47 results in the recruitment and activation of tyrosine phosphatases, such as SHP-1 and SHP-2, to immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tail of SIRPa. We have proposed that CD47 acts as a ‘self’ molecule to control phagocyte functions. Indeed, there is good evidence now that CD47-SIRPa interactions negatively regulate the clearance of host cells by macrophages *in vivo*. We are investigating whether other phagocyte activities are also controlled via SIRPa. We have demonstrated, for instance, that SIRPa is a negative regulator of the phagocyte NADPH oxidase both *in vitro* and *in vivo*. In fact, it appears that CD47-SIRPa interactions do this by modulating the developmentally controlled expression of gp91^{phox} in phagocytes and we are now characterizing the signaling pathway involved (see figure). This mechanism is anticipated to control the magnitude of the respiratory burst and thereby to reduce ‘collateral’ tissue damage of this toxic mechanism during infection. In addition, we have obtained evidence that interactions between CD47 on tumor cells and SIRPa on



Reactive oxygen species (ROS) generated by the phagocyte NADPH oxidase are critical in the host defense against bacterial and fungal infection, but may also cause collateral damage to the surrounding tissue when produced in excessive amounts. The various components of the NADPH oxidase are generated in a controlled fashion during phagocyte development. CD47-SIRPα interactions specifically limit the expression of the CYBB gene encoding the catalytic subunit of the NADPH oxidase gp91^{phox} during this process, in order to control the magnitude of the respiratory burst.

neutrophils form a critical limitation for antibody-dependent cellular cytotoxicity (ADCC) of phagocytes towards tumor cells. This is based on both *in vivo* experiments using SIRPa-mutant mice, as well as on *in vitro* evidence from ADCC experiments using a variety of human tumors and therapeutically relevant antibodies (e.g. trastuzumab and rituximab). We have also generated novel antibodies against SIRPa that have the capacity to improve ADCC and these may be instrumental for enhancing the efficacy of antibody therapy in cancer patients. Of interest, the various immunoreceptor families, like FcR, SIRP and KIR, are subject to an extraordinary genetic variation within the population. But which of these individual differences, that include polymorphisms and copy number variation (CNV), are the critical determinants of individual immunogenicity and disease susceptibility is unknown. We are investigating this by multi-ligation probe amplification (MLPA) and large scale sequencing technology.

Key publications

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Van Bruggen R, Koker MY, Jansen M, van Houdt M, Roos D, Kuijpers TW, van den Berg TK. Human NLRP3 inflammasome activation is Nox1-4 independent. Blood 2010; 115:5398-400.

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Erythrocytes

Robin van Bruggen PhD, r.vanbruggen@sanquin.nl
Rob van Zwieten, r.vanzwieten@sanquin.nl
Dirk de Korte PhD, d.dekorte@sanquin.nl

Our studies are focused on the function, aging and clearance of red blood cells. In particular we have been investigating the role of CD47 on red cells in these processes. CD47 is an established 'don't eat me' signal on red blood cells that prevents premature clearance by binding to the inhibitory immune receptor SIRPa on macrophages in the spleen and other relevant tissues. However, our findings suggest that CD47 does not only act as an inhibitory signal for phagocytosis, but may actually also promote phagocytosis directly by binding to SIRPa. Moreover, this role of CD47 as an 'eat me' signal appears most pronounced on aged red cells, suggesting that CD47 acts as a molecular switch from anti- to pro-phagocytic regulation. In addition, it seems that CD47 is somehow involved in red cell vesiculation, a specialized process aimed to shed red cell material that has become damaged, mostly as a result of oxidation, during the long life span of red cells. While the mechanistic basis for these phenomena is under further investigation these findings suggests that CD47-SIRPa interactions act as a critical regulator of erythrocyte survival and clearance, and therefore play a major role in red cell homeostasis.

In addition to this we are exploring a role of red cells as carriers of pathogens during bacterial infection. We have already observed that red cells can interact with opsonized bacteria and this is proposed to facilitate transport to and clearance by phagocytes present in the spleen and other tissues. This may also be of relevance in the context of sepsis, where erythrocyte transfusion may cause a relatively high frequency of transfusion-related acute lung injury (TRALI). The latter is being investigated in collaboration with dr Nicole Juffermans (Intensive Care Unit, Academic Medical Center).

Key publication

Burger P, Korsten H, de Korte D, Rombout E, van Bruggen R, Verhoeven AJ. An improved red blood cell additive solution maintains 2,3-diphosphoglycerate and adenosine triphosphate levels by an enhancing effect on phosphofructokinase activity during cold storage. Transfusion 2010; 50:2386-92.

Platelets

Laura Gutierrez PhD, l.gutierrez@sanquin.nl

During this year a new line of research dedicated to basic aspects of platelets was initiated. We developed a novel FACS-based assay for the analysis of platelet aggregation using a variety of agonists. In contrary to the standard aggregometer test this test is suitable for analyzing platelet function in thrombocytopenic patients. Our studies also show that this test allows for the relatively simple functional discrimination of patients with related integrin-based but nevertheless genetically distinct bleeding disorders, including Glanzmann disease and LAD-1/variant syndrome, the latter of which was originally described in our laboratory. Glanzmann patients have a genetic defect in the integrin-β3 chain, which selectively affects fibrinogen-mediated binding. On the other hand patients with LAD-1/variant syndrome, which, as we have recently shown for the first time, carry mutations in the kindling-3 protein that is involved in integrin inside-out-signaling. This leads to a more generalized defect in integrin activation, which affects both fibrinogen- as well as collagen-mediated aggregation, of which the latter is mediated via the α2β1-integrin. This difference in the adhesion to collagen, which is anticipated to be representative for the initial binding of platelets to the damaged blood vessel wall, was confirmed with adhesion assays performed under physiologic flow conditions. These findings also provide an explanation for the more severe bleeding tendency observed in LAD-1/variant syndrome as compared to Glanzmann disease. We are also continuing our investigations on the role of kindling-3 in phagocytes.

In addition to this we have investigated the role of the caprin-2 protein in platelet formation and function. Caprin-2-deficient mice were shown to be thrombocytopenic and this appears to be due to an intrinsic defect in the megakaryocyte/platelet lineage. This is supported by caprin-2 knock-down studies in human megakaryocytic cell lines and current efforts are focused on characterizing the role of caprin-2 in the different stages of platelet development. We are also optimizing the available flow-based assays for the analysis of platelet function.

Dirk de Korte PhD
d.dekorte@sanquin.nl

Blood Transfusion Technology

Dirk de Korte performs Research both at Product and Process Development Blood Transfusion Technology at Sanquin Blood Bank as well at Sanquin Research, Department Blood Cell Research.

Sanquin Blood Bank, Department of Product and Process Development Blood Transfusion Technology

Academic staff

D de Korte PhD (PI)
J Lagerberg PhD
PF van der Meer PhD
H Vrielink MD PhD

Technical staff

I Bontekoe
B Daal
LAE de Laleijne-Liefting
R Hoenderdaal
W Karssing

Other contributors

R Koopman MD PhD
R Lieshout-Krikke MD
PJM van den Burg MD PhD

Address

Sanquin Blood Bank
Department Product and Process Development Blood Transfusion Technology
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9137
NL-1006 AC Amsterdam
The Netherlands
T +31 20 512 3860
F +31 20 617 8080
E d.dekorte@sanquin.nl
W bcr.sanquin.nl

Sanquin Research, Department Blood Cell Research Laboratory for Blood Transfusion Technology

Academic staff

A Gerrits PhD
L Gutierrez PhD
R van Bruggen PhD
TK van den Berg PhD
R van Zwieten

PhD students

P Burger
E Kostova
M Meinders
IGH Rood
M Scheenstra

Technical staff

I De Cuyper
M Go
E Gouwerok
P Hilarius-Stokman
H Korsten
R Vlaar

Blood Transfusion Technology

Improving materials and methods for storage of blood components

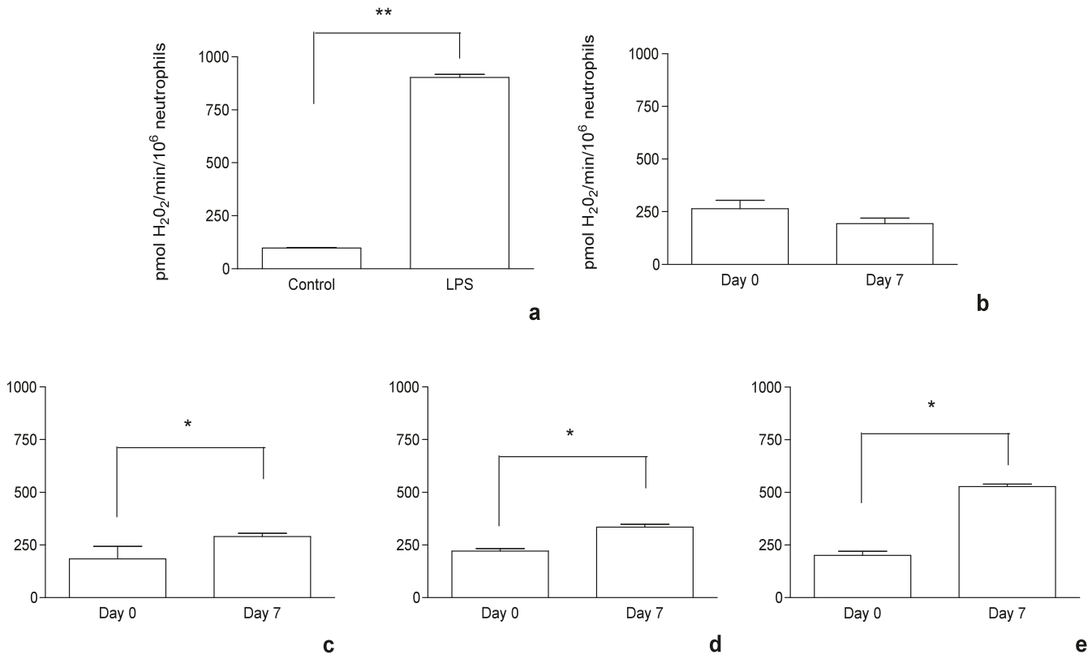
The blood bank is a producer of blood products under GMP conditions and needs to know the limits of the methods in use, for example with respect to temperature effects during collection and preparation. In 2010 some studies in this field were performed. Another aspect of this research line is the quality of blood components in relation to adverse effects of blood transfusion, like TRALI.

Active cooling of whole blood to room temperature improves blood component quality

Many countries use cooling plates to actively cool collected whole blood (WB) to room temperature. This was mainly introduced to standardize the temperature profiles and to allow component production during standard working hours. Until now, no paired comparison had been performed between active and not active cooling, and it was our aim to compare the effect of active versus no active cooling on the *in vitro* quality of WB and subsequently prepared blood components. For this study, two units of WB were pooled and divided shortly after donation. One unit was placed under a butane-1,4-diol plate to obtain active cooling; the other was placed in an insulated box with other warm units to mimic worst-case holding conditions. WB was held overnight and processed into a white blood cell (WBC)-reduced red blood cells (RBCs), buffy coat (BC), and plasma. The BCs were further processed into platelet (PLT) concentrates. After overnight storage, ATP content of the RBCs was 4.9 ± 0.3 mmol/g Hb for actively cooled WB versus 4.5 ± 0.4 mmol/g Hb for not actively cooled WB ($p < 0.001$). On Day 42 of storage, RBCs prepared from this WB contained 3.1 ± 0.3 mmol ATP/g Hb with active cooling versus 2.6 ± 0.3 mmol/g Hb without ($p < 0.001$). Hemolysis on Day 42 was 0.35 ± 0.08% with active cooling and 0.67 ± 0.21% without ($p < 0.001$). No effect was observed on the *in vitro* quality of plasma, BC, or PLT concentrates. Active cooling of WB results in improved ATP levels and less hemolysis in WBC reduced RBCs, although the clinical implications are unclear. It has no effect on the *in vitro* quality of plasma or PLT concentrates.

Accumulation of bio-active lipids during storage of blood products is not cell but plasma-derived and temperature dependent

Bio-active lipids (lysophosphatidylcholines, (lysoPCs)) accumulating during storage of blood products are thought causative in onset of TRALI through activation of neutrophils. LysoPCs are thought to be derived from cell-membrane degradation products such as phosphatidylcholines (PC) by partial hydrolysis of PC, a process that is catalyzed by phospholipase A_2 (PLA_2). We investigated the underlying mechanisms of lysoPC generation and its contribution to *in vitro* neutrophil-priming capacity during storage of red blood cells (RBCs), platelet concentrates (PLTs) and cell-free plasma (see figure). Storage of RBCs in SAGM did not result in accumulation of lysoPCs or neutrophil-priming capacity. Replacement of SAGM by plasma as RBC storage medium caused elevated lysoPC levels at day 0, which did not further increase during storage. Cell-free plasma stored at 22°C showed accumulation of lysoPCs during storage, which was not present at 4°C. Addition of a soluble (s)PLA_2 or cytosolic (c)PLA_2-inhibitor did not prevent accumulation of lysoPCs in plasma. In PLTs, lysoPC accumulation during storage was



*Panel A shows H_2O_2-release when neutrophils were incubated with buffer solution (negative control) or E.Coli LPS/LPB 20ng/mL (positive control). Panel B shows H_2O_2-release when neutrophils were incubated with supernatant of cell depleted plasma obtained directly from whole blood donation, stored at 22˚C of different storage time points (day 0 and day 7). Panel C shows H_2O_2-release when neutrophils were incubated with supernatant of platelet concentrates (PLTs) stored in 100 % plasma of different storage time points (day 0 and day 7). Panel D shows H_2O_2-release when neutrophils were incubated with supernatant of PLTs stored in 35% plasma: 65% SSP of different storage time points (day 0 and day 7). Panel E shows H_2O_2-release when neutrophils were incubated with supernatant of PLTs stored in 5% plasma: 95% SSP of different storage time points (day 0 and day 7). (n=3 batches for each measurement). *p<0.05, **p<0.01, paired t-test. Data are presented as Mean and SD.*

plasma dependent, but lysoPCs did not explain the observed neutrophil-priming effect as preventing accumulation of lysoPCs by removing the plasma fraction did not prevent the neutrophil-priming capacity. Based upon these results it was concluded that accumulation of lysoPCs during storage is not cell but plasma-derived and storage temperature-dependent and does not explain the neutrophil-priming effect of aged products. This research was performed in collaboration with Alexander Vlaar and Nicole Juffermans from Academic Medical Center.

Key publications

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Vlaar AP, Hofstra JJ, Kulik W, van Lenthe H, Nieuwland R, Schultz MJ, Levi MM, Roelofs JJ, Tool AT, de Korte D, Juffermans NP. Supernatant of stored platelets causes lung inflammation and coagulopathy in a novel in vivo transfusion model. Blood 2010; 116(8):1360-8.

Bacterial safety of blood products

Contamination of platelets with bacteria is the major microbiological risk of blood transfusion. This applies especially for platelet concentrates (PCs) because their storage conditions, at room temperature and under constant agitation, support bacterial growth. Therefore both the prevention of contamination and gathering knowledge on the type of contamination are subject of research within the blood bank. In 2010 a study on the frequency of false negatives was completed as well as a study on the origin of *Propionibacterium* species isolated from blood products.

Frequency of false negatives in the Dutch screening system

In the Netherlands, platelet concentrates (PCs) are screened with the BacT/ALERT culturing system and released as 'negative to date'. From all whole blood derived PCs in the Netherlands, approximately 0.37% are tested positive for bacterial growth with the BacT/ALERT culture system. Recently there were several reports showing the risk of false negatives to be around 1 in 1000 PC. For the Netherlands, no accurate numbers were available. To determine the frequency of false negative culture results for the screening system as used in the Netherlands, an 'outdated' study was initiated, in which five (in PASII) or seven day old (in plasma; outdated) PCs were re-cultured with the BacT/ALERT. Over the period of April 2009 to October 2010, 4082 outdated buffy coat PC units were re-tested with the BacT/ALERT. The 16S rRNA gene was sequenced for species confirmation and strains from the same species were analyzed by amplified fragment length polymorphism (AFLP) analysis to determine if the strains found in the PCs and the accompanying culture bottles were identical. During the study period 4 false negative PCs were found leading to a frequency of false negative BacT/ALERT results of 0.10%. Three PCs were contaminated with *Staphylococcus epidermidis* and one with *S. hominis*. AFLP analysis showed that all strains that were cultured from the PC and its accompanying culture bottles were identical but the *S. epidermidis* strains from the different PC bags did not show identical patterns. The bacterial concentration of the false negative PCs on the day of re-testing was estimated to be at least 10^5 CFU/ml. It was concluded that, although screening of PCs with the BacT/ALERT prevents the transfusion of PC contaminated with bacteria, not all contaminated PCs are detected. These false negative PCs can be a risk for patients receiving transfusion, but it has to be kept in mind that bacterial transmission by BacT/Alert tested PC's is a very rare event in the Netherlands; during the last 3 years only 2 cases were reported.

Molecular relatedness of *Propionibacterium* species isolated from blood products and on the skin of blood donors

From all the bacteria that are found with the BacT/ALERT more than half are *Propionibacterium acnes*, gram positive, anaerobic bacteria that are part of the normal resident skin flora. This bacterium is rarely implicated in transfusion related reactions. However, several studies suggested its involvement in various clinical conditions and infections such as spondylodiscitis, endocarditis, and it is the second most frequent bacterium colonizing catheter tips. It is generally assumed that the bacteria found in blood products originate from the skin of the donor but this has not been examined properly. The aim of this study was to investigate whether the *P. acnes* strains present in the PCs and related RBCs, originate from the skin of the donor. Therefore, strains that were found throughout 2007 and 2008 in PCs and RBCs with the BacT/ALERT and strains that were cultured in 2010 from the phlebotomy site (antecubital fossa) of the donors of these blood products were analyzed. The strains were determined to species level by sequencing of the 16S rRNA and *recA* genes and typed by AFLP. A part of the strains was also determined to species level by sequencing of the 16S rRNA and *recA* genes. Based on this sequencing, three different phylogenetic groups of *P. acnes* were found. All strains that were found in PCs and their accompanying RBCs were identical, which indicates that the strain is already present in the whole blood donation. *P. acnes* could be found on the skin of almost all screened donors. In eight out of twenty-two cases (36.4%), one of the strains from the donor skin was identical to the strains found in PCs and their accompanying RBCs. In two other cases the strains belonged to the same phylogenetic group. These results support the theory that the source of *P. acnes* contamination is in many cases the skin of the donor. However, further study is necessary to rule out other sources of contamination. Because it is difficult to prevent bacterial contamination by *P. acnes* completely, it is necessary to further investigate the clinical significance of blood products contaminated with *P. acnes*. This work was performed in collaboration with Paul Savelkoul and Annika Peterson from the VU University Medical Center.

Key publications

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Rood IG, Pettersson A, Savelkoul PH, de Korte D. Development of a reverse transcription-polymerase chain reaction assay for eubacterial RNA detection in platelet concentrates. Transfusion 2010; 50(6):1352-8.

Pathogen reduction of cellular blood products

One of the main side effects of pathogen reduction in cellular blood products is the negative effect on the quality of the blood products. In order to allow *in vitro* judgment of new or modified methods, an *in vitro* rating system was proposed for platelet concentrates. In 2011 this rating system will be applied in one or more clinical trials with platelet concentrates.

Prof Koen Mertens PhD
k.mertens@sanquin.nl

Plasma Proteins

Academic staff
Prof K Mertens PhD (PI)
AB Meijer PhD
JJ Voorberg PhD

Post docs
HJM Brinkman PhD
HB de Laat PhD
J Martin Ramirez PhD
MG Rondaij PhD
M van den Biggelaar PhD

PhD students
E Bloem
EAM Bouwens
L Castro
I Dienava
B Dragt
H Meems
F Stavenuiter PhD
C Zappelli

Technical staff
RC Boertjes
MG Boon-Spijker
M Hofman
JWTM Klein Gebbinck
C van der Zwaan
MG Zuurveld

Guest
JA van Mourik

Secretariat
G Damhuis
MJ Vergeer

Address
Sanquin Research
Department of Plasma Proteins
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3151
F +31 20 512 3310
E secretariaatu2@sanquin.nl
W pe.sanquin.nl

Plasma Proteins

Structure and function of coagulation factors

The coagulation cascade comprises several serine proteases that act in combination with a non-enzymatic cofactor on phospholipid-containing membranes. During the past decade we have been focusing on the mechanism by which activated factor IX assembles with its cofactor factor VIII. These proteins are indispensable for proper functioning of the coagulation cascade as functional absence of factor VIII (FVIII) and factor IX is associated with the bleeding disorder hemophilia A and hemophilia B. FVIII is composed of a series of repeated domains which appear in the order A1-a1-A2-a2-B-a3-A3-C1-C2. The A-domains of FVIII mediate the binding to activated factor IX and factor X whereas the C2 domain has been implicated in binding to phospholipids. We recently identified a human antibody (designated KM33) that inhibits FVIII cofactor activity. We further established that this antibody blocked endocytosis of FVIII by various cellular systems which may contribute to the regulation of the FVIII plasma level. Binding studies revealed that KM33 is directed towards the C1-domain of FVIII and that KM33 blocked the binding of FVIII to phospholipid membranes suggesting involvement of C1 domain in this process. We next established that FVIII-YFP variants in which the residues Lys2092 and Phe2093 at the tip of the C1 domain are replaced by alanines exhibit a reduced binding to KM33. FVIII-YFP-2092A/2093A also did not interact with phospholipid membranes containing a low percentage of phosphatidyl-L-serine. The binding of this variant was, however, indistinguishable from the corresponding wild type FVIII when membranes containing higher concentrations of phosphatidyl-L-serine were used. In agreement with these findings phospholipid membranes containing relatively high concentrations of phosphatidyl-L-serine were needed to develop full cofactor activity of FVIII-YFP-2092A/2093A. Together these findings provide evidence for the involvement of Lys2092/Phe2093 in binding to phosphatidyl- L-serine containing phospholipids. We next investigated the role of region 2992/2093 on the endocytic uptake of FVIII by cells that express FVIII's catabolic receptor LDL receptor-related protein (LRP). Confocal microscopy studies and flow cytometry studies showed that there was a marked defect in the uptake of FVIII-YFP-2092A/2093A relative to FVIII-YFP. These studies further demonstrated that there is a cell surface binding event that precedes the LRP dependent uptake of FVIII. Cell surface binding proved to be less effective for the FVIII-YFP variants K2092A, F2093A and K2092A/F2093A. This finding implies that the amino acid residues of region 2092-2093 comprise a binding site for the cell surface. Surface plasmon resonance analysis showed that these substitutions affect the direct binding of FVIII to LRP as well. This dual role of region 2092-2093 for both cell surface and LRP binding explained the major reduction of endocytic uptake of the FVIII-YFP variants. Our results demonstrate that C1 domain residues 2092-2093 are of major importance for FVIII function. This region not only contributes to cofactor function but is also involved in the cellular uptake of FVIII.

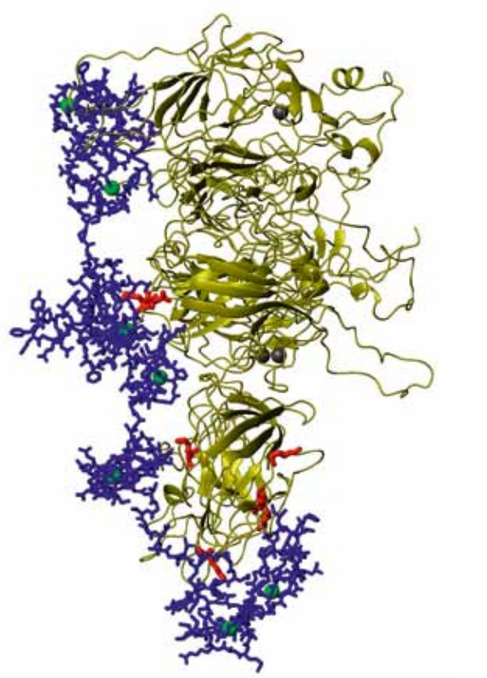
Key publications

Meems H, van den Biggelaar M, Rondaij M, van der Zwaan C, Mertens K, Meijer AB. C1 domain residues Lys 2092 and Phe 2093 are of major importance for the endocytic uptake of coagulation factor VIII. Int J Biochem Cell Biol 2011 Apr 8 [Epub ahead of print].

Cellular receptors involved in the uptake of coagulation factors

Alexander Meijer PhD, s.meijer@sanquin.nl

LDL receptor-related protein (LRP) contributes to the clearance of coagulation factor VIII (FVIII) from the circulation. Ligand binding of the low-density lipoprotein (LDL) receptor family is mediated by clusters of small complement-type repeats (CR). It has been proposed that at least two CRs are required for high-affinity interaction by utilizing two spatially distinct lysine residues on the ligand surface. LDL receptor-related protein (LRP) mediates the cellular uptake of a multitude of ligands, some of which bind LRP with a relatively low affinity suggesting a suboptimal positioning of the two critical lysines. We have recently addressed the role of the two critical lysines not only for LRP binding but also for endocytosis initially by employing Receptor Associated Protein (RAP) as a model ligand. Variants of the third domain (D3) of RAP were constructed in which lysines were replaced by alanine or arginine at the putative contact residues K253, K256 and K270. Surface Plasmon Resonance revealed that replacement of K253 has no effect on high-affinity LRP binding at all whereas replacement of either K256 or K270 markedly reduced the binding affinity. The interaction was completely abolished when both lysines were replaced. Substitution by either alanine or arginine exerted an almost identical effect on LRP binding suggesting arginine residues do not support receptor binding. Confocal microscopy and flow cytometry studies surprisingly revealed that the single mutants were still internalized by cells. We therefore propose that the presence of only one critical lysine is sufficient to drive endocytosis. We next set out to identify the contribution of individual lysines in the interaction between FVIII and LRP. To systematically address this issue, we



LDL receptor (blue) in interaction with factor VIII (yellow). Indicated in red are lysine residues of factor VIII that interact with the CR domains of LDL receptor. The calcium ions critical for correct folding of a CR domain are shown in green.

constructed a library of more than 40 FVIII variants carrying lysine to arginine or alanine substitutions. The interaction of the variants with LRP cluster II was evaluated using Surface Plasmon Resonance (SPR) analysis. Our SPR analysis showed that multiple lysines in the A3C1 domains contributed to the interaction. However, none of the substitutions completely abolished LRP cluster II binding. Of the various lysines within the A3C1 domains, the C1 residues K2065 and K2092 proved the most important. The finding that all variants still displayed substantial residual binding suggests that also other surface exposed residues in the FVIII light chain contribute to LRP interaction. Crystal structure analysis and docking studies revealed that all the putative 'hot spot' lysines may spatially align with the acidic binding pockets of the LDL receptor. We therefore propose that LRP cluster II interacts with the FVIII light chain via an extended surface that starts at the bottom of the C1 domain and extends to the top of the A3 domain.

Key publication

Van den Biggelaar M, Sellink E, Klein Gebbinck JW, Mertens K, Meijer AB. A single lysine of the two-lysine recognition motif of the D3 domain of receptor-associated protein is sufficient to mediate endocytosis by low-density lipoprotein receptor-related protein. Int J Biochem Cell Biol 2011; 43:431-40.

Proteomics and biomolecular mass spectrometry of hemostatic processes

Alexander Meijer PhD, s.meijer@sanquin.nl

The binding between individual proteins that are involved in a hemostatic process, like e.g. blood coagulation, is typically part of a complex protein-protein interaction network. Taking maximum advantage of the versatility of the nano-LC LTQ Orbitrap XL ETD mass spectrometer, several studies have been initiated to unravel complex mechanisms involved in protein-protein interactions, storage of hemostatic proteins in the secretory organelles, and intracellular processing of proteins. An example of the last study involves immune tolerance induction against coagulation factor VIII (FVIII). This study required the identification of FVIII peptides that are presented on dendritic cells via the MHC class II proteins. For this purpose, immature monocyte-derived dendritic cells were incubated with FVIII to allow for its uptake and intracellular processing. After extraction of the MHC class II molecules from the cells, the FVIII peptides bound to these molecules were identified employing mass spectrometry. This study has now provided an unbiased identification of donor dependent T-cell epitopes of FVIII. Another example involves a so-called chemical foot printing approach in which we scan for solvent exposed regions on the coagulations factors VIII and IX. This method provides insight into the functional regions of the coagulation factors as well as changes therein during their activations. We will further implement mass spectrometry technologies that will allow for the identification of differentially expressed proteins, or changes in modifications thereof in treated versus non-treated cells. These technologies will be particularly suitable to unravel protein signaling networks in cellular systems.

Key publication

Van Haren SD, Herczenik E, ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide-repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. Mol Cell Proteomics 2011 Apr 5 [Epub ahead of print].

Innovation of diagnostics of thrombotic and hemostatic disorders

Jan Voorberg PhD, j.voorberg@sanquin.nl

Innovation of diagnostic assays for hemostatic and thrombotic disorders is a major current focus within the department of Plasma Proteins. In the past year efforts have been made to improvement our understanding of the antiphospholipid syndrome, an acquired autoimmune disorder that can present with thrombosis or pregnancy morbidity. Patients with antiphospholipid syndrome (APS) display a heterogeneous population of antibodies that are frequently directed towards beta2-glycoproteinI. In a collaborative effort with Ronald Derksen, Flip de Groot and Bas de Laat from the Utrecht Medical Center, we have now for the first time isolated a series of human monoclonal antibodies directed towards different domains of beta2-glycoproteinI. All monoclonal antibodies display functional activity and it is expected that these antibodies will help to increase our insight into the pathogenesis of APS. It also anticipated that these reagents will facilitate standardization of diagnostic tests for this complex disorder. In parallel with current work focusing on refinement of current assays related to blood coagulation efforts are being undertaken to identify novel biomarkers for cardiovascular disorders. To facilitate this approach Sanquin participates in CTMM project INCOAG in which several and industrial academic partners collaborate towards innovation in this field. Current focus is on microRNA as potential novel biomarkers for cardiovascular disease. This approach is being pursued in close collaboration with Joost Meijers from the Academic Medical Center. Within the framework of this project Sanquin works on novel biomarkers derived from platelets, circulating blood cells as well as endothelial cells.

Key publication

Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, Brinkman HJ, Voorberg J, Mertens K, Derksen RH, de Laat B. Patient-derived monoclonal antibodies directed towards beta2-glycoprotein I display LAC activity. J Thromb Haemost 2011; 9:738-47.

Jan J Voorberg PhD
j.voorberg@sanquin.nl

Cellular Hemostasis

Academic staff
JJ Voorberg PhD (PI)

Post docs
E Herczenik PhD

PhD students
B Dragt
W Pos PhD
N Sorvillo
EL van Agtmaal
D van Breevoort
SD van Haren
K van Hooren
A Wroblewska

Technical staff
PHP Kaijen

Students
H Peltenburg
A Rusiecka

Secretariat
G Damhuis
MJ Vergeer

Address
Sanquin Research
Department of Plasma Proteins
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3151
F +31 20 512 3310
E secretariaat: secretariaatu2@sanquin.nl
W pe.sanquin.nl

Cellular Hemostasis

Research in the Department of Plasma Proteins is performed by two Principal Investigators, Prof Koen Mertens PhD and Jan Voorberg PhD. Both PI's are mutually involved within the overall focus Hemostasis and Thrombosis on the different research lines. The research lines of Jan Voorberg are described here, while research lines by Koen Mertens involving Jan Voorberg as well can be found with Plasma Proteins.

Biosynthesis of von Willebrand factor

Biosynthesis of VWF occurs in vascular endothelial cells and megakaryocytes. In endothelial cells VWF is stored in rod-shaped endothelial cell-specific storage organelles, the Weibel-Palade bodies. Besides VWF, these Weibel-Palade bodies contain a number of other proteins, including P-selectin, angiopoietin-2, osteoprotegerin and a number of other components. Upon stimulation of endothelial cells by agonist such as thrombin or epinephrine, Weibel-Palade bodies undergo exocytosis, resulting in release or surface expression of their contents. The elongated shape of Weibel-Palade bodies has been attributed to the packaging of VWF multimers into helical structures. Analysis by electron microscopy reveals tubular-like structures that most likely are composed of tightly packed helically organized VWF multimers. Following their release Weibel-Palade bodies, VWF tubules are rapidly converted into ultra-large VWF strings that are anchored to the surface of endothelial cells. These ultra-large VWF strings provide multiple attachment sites for blood platelets (figure 1b).

We have recently investigated the effect of the shear stress-induced transcription factor KLF2 on clustering and composition of WPBs using peripheral blood derived endothelial cells. Lentiviral over-expression of KLF2 resulted in a 4.5 fold increase in number of WPBs per cell when compared to mock-transduced endothelial cells (figure 1a). Unexpectedly, the average length of WPBs was significantly reduced. In mock-transduced endothelial cells WPBs had an average length of 1.8 µm whereas in KLF2 overexpressing cells WPBs had a average length of 1.4 µm. Overexpression of KLF2 abolished the perinuclear clustering of WPBs observed following stimulation with cAMP-raising agonists such as epinephrine. We previously hypothesized that perinuclear clustering of WPBs provides a means to limit excessive release of bioactive components from these organelles. We subsequently explored the pro-inflammatory P-selectin and angiopoietin-2 (Ang-2). Ang-2 has been shown to promote vascular leakage and endothelial cell migration thereby contributing to vascular remodeling. P-selectin was readily visualized in both KLF2 and mock-transduced endothelial cells. In contrast, confocal microscopy revealed that WPBs in KLF2-transduced cells did not contain Ang-2. Together our findings suggest show that KLF2 not only regulates the dynamics of WPBs but also regulates the size and contents of this highly versatile storage pool in endothelial cells.

Key publications

Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J. Functional architecture of Weibel-Palade bodies. Blood 2011 Jan 25 [Epub ahead of print].

Figure 1a

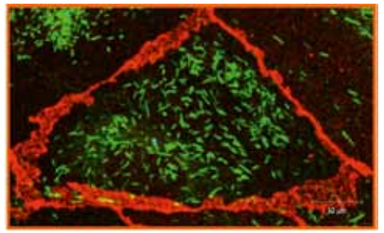


Figure 1a: *Increased number of Weibel-Palade bodies (green) in KLF2 transduced endothelial cells. The periphery of the cell is shown by staining for ß-catenin (red).*

Figure 1b

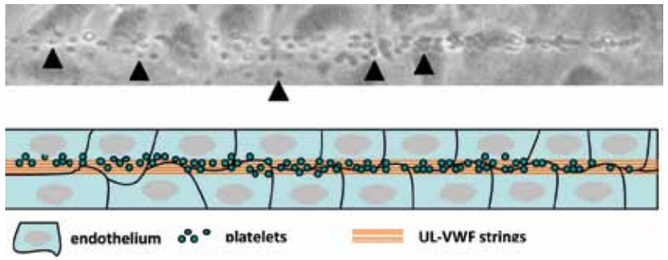


Figure 1b: *Ultra-large VWF strings on the surface of endothelial cells. Stings are visualized by adhering blood platelets (arrowheads).*

Immune response to hemostatic proteins

Hemophilia

Hemophilia is an X-linked bleeding disorder that is caused by a deficiency of factor VIII (hemophilia A) or factor IX (hemophilia B). Coagulation factor replacement therapy of hemophilia may be complicated by the formation of inhibitory or neutralizing antibodies (inhibitors). This side-effect occurs in approximately 25% of the patients with severe hemophilia A, and in about 5% of the patients with mild hemophilia A.

Increased risk of inhibitor formation in mild hemophilia A has been linked to FVIII missense mutations which include R593C in the A2 domain of FVIII. We have analyzed FVIII-specific T cell responses in two unrelated patients heterozygous for HLA-DRB1*1101 using DR1101-HLA class II tetramers. CD4+ T cells reactive with a peptide including the R593 that was modified in the FVIII protein of both patients were observed (figure 2). Our findings support the concept that presentation of peptides specifically derived from wild type and not endogenously expressed factor VIII promote the formation of inhibitors in

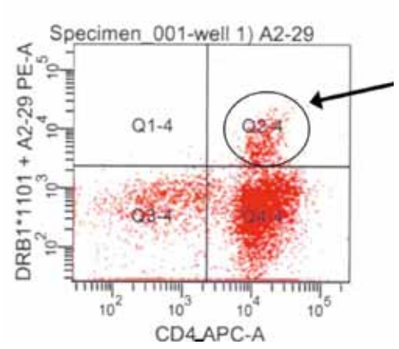


Figure 2: *HLA-DR11 tetramer guided selection of FVIII-specific CD4+ T cells in a patient with mild hemophilia A. Arrow indicates the population of cells that is stained by HLA-DR11 tetramers loaded with FVIII peptide containing R593.*

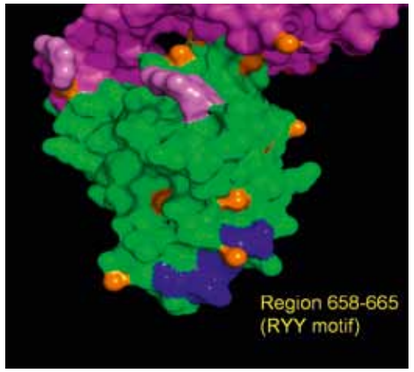


Figure 3: *Exposed surface containing residues R660, Y661 and Y665 (indicated in blue) in the spacer domain (indicated in green) of ADAMTS13 that is crucially involved in the binding of anti-ADAMTS13 antibodies. Water molecules are displayed in orange.*

patients with mild hemophilia A carrying the appropriate HLA-class II alleles. These findings contribute to our knowledge as to why inhibitors develop in a subset of patients with mild hemophilia A.

Thrombotic thrombocytopenic purpura

Thrombotic thrombocytopenic purpura (TTP) is a micro-angiopathy that is related to an acquired or congenital deficiency of the von Willebrand Factor (VWF) cleaving protease ADAMTS13. In the absence of ADAMTS13, ultra large VWF (UL-VWF) polymers, originating from endothelial cell specific organelles, designated Weibel-Palade bodies, accumulate in the circulation. These UL-VWF polymers mediate the formation of platelet-rich thrombi in the microcirculation that give rise to hemolytic anemia and thrombocytopenia. In plasma of the majority of patients with acquired TTP, antibodies directed towards ADAMTS13 are present. The majority of patients develop antibodies directed towards the spacer domain of ADAMTS13. In a recent study we have shown that in exposed surface in the spacer domain comprising residues R660, Y661 and Y665 contribute to the binding of anti-ADAMTS13 antibodies (Figure 3). Replacement of these residues also affected the ability of ADAMTS13 to process small VWF substrates. Also the processing of full length VWF under denaturing conditions or under flow on the surface of endothelial cells was greatly impaired by replacement of R660, Y661 and Y665 by an alanine. Using complementary VWF variants we were able to show that the exposed surface formed by R660, Y661 and Y665 interacts with a conserved alpha-helix in the A2 domain of VWF. This part of the A2 domain unfolds under the influence of shear stress allowing it to interact with ADAMTS13. Our findings suggest that human antibodies directed towards residues R660, Y661 and Y665 in the spacer domain interfere with productive assembly of the VWF-ADAMTS13 complex thereby interfering with cleavage of ultra large VWF multimers on the surface of endothelial cells (figure 1b).

Key publications

Pos W, Crawley JT, Fijnheer R, Voorberg J, Lane DA, Luken BM. An autoantibody epitope comprising residues R660, Y661 and Y665 in the ADAMTS13 spacer domain identifies a binding site for the A2 domain of VWF. Blood 2010; 115:1640-9.

James EA, van Haren SD, Ettinger RA, Fijnvandraat K, Liberman JA, Kwok WW, Voorberg J, Pratt KP. T cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. J Thromb Haemost 2011; 9(4):689-99.

Marieke von Lindern PhD
m.vonlindern@sanquin.nl

Hematopoiesis

Academic staff
M von Lindern PhD (PI)
DC Thijssen-Timmer PhD
PB van Hennik PhD
C Voermans PhD
M Wolkers PhD

Post docs
E van den Akker PhD

PhD students
M Balzarolo
K Brussen
SE Klamer
MW Maijenburg
F Salerno
S Zeddies
Y Zoughlami

Technical staff
M Kleijer
AD van Stalborch
K Vermeul
N Yagci

Students
S Jansen
L Vogelpoel

Secretariat
AEPT Engels
MLMW Lutkie

Address
Sanquin Research
Department of Hematopoiesis
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 1394/3377
F +31 20 512 3474
E secretariaatIHE@sanquin.nl
W www.hep.sanquin.nl

Hematopoiesis

Initiated mid 2010, our department aims to combine basic research on various aspects of hematopoiesis with the development of novel cellular products.

Mesenchymal stromal cells in the bone marrow environment

Carlijn Voermans PhD, c.voermans@sanquin.nl

Mesenchymal stromal cells (MSC) are an important constituent of the bone marrow (BM) microenvironment where they support hematopoiesis via direct cell-cell interactions with hematopoietic cells and by releasing soluble factors. Transplantation of MSC is a promising therapeutic modality used to suppress graft-versus-host reactions and to sustain tissue regeneration upon injury. However, optimal use is hampered by a lack of knowledge on MSC identity and –function and on the migratory capacity of MSC.

Migration of MSC

Culture-expanded MSC from various tissues contain only a small percentage of migrating cells *in vitro* and the optimal stimulus differs among sources. To study the molecular signature of migratory MSC, we established the genome wide expression profile of migrating and non-migrating MSC. The nuclear orphan receptor family members Nurr1 and Nur77 were the most differentially expressed genes. Overexpression of these genes increased MSC migration, while it did not affect the production of cytokines involved in immune suppression. Co-cultures of MSC overexpressing Nurr1 or Nur77 with peripheral blood mononuclear cells confirmed that these cells maintained their immune suppressive activity. Thus, modulation of Nur77 and Nurr1 activity may offer perspectives to enhance the migratory potential of MSC to regulate a local immune response.

MSC identity

Although the phenotype of (culture expanded) MSC has been defined, these MSC may still harbor heterogeneous populations since they are derived from different MSC subsets. Based on expression of CD271 and CD146, we identified three new BM subpopulations within the 'classical' MSC population; $CD271^+/CD146^-$, $CD271^+/CD146^+$ and $CD271^-/CD146^+$ populations. Both $CD271^+$ populations co-expressed MSC markers CD105 and CD90, contained colony forming units-fibroblasts (CFU-F), and showed differentiation capacity towards adipocytes, osteoblasts and chondrocytes. The $CD271^-/CD146^+$ cell fraction, however, could not be expanded. Interestingly, the distribution of MSC subsets differs significantly between young and adult bone marrow donors. In children, the dominant fraction was $CD271^+/CD146^+$, whereas in adults this was reversed. We are currently investigating whether these subpopulations are distinct in their hematopoietic support.

The extracellulair matrix (ECM) protein βig-h3

Tgfβ-induced gene h3 is produced by MSC as well as by hematopoietic stem- and progenitor cells (HSPC). We previously demonstrated that βig-h3 is strongly upregulated in regenerating mouse hematopoietic stem cells (HSC). Increased expression of βig-h3 accelerated differentiation of HSPCs and resulted in a rapid exhaustion of murine, primitive progenitors *in vivo* as well as *in vitro*. We now investigated the role of βig-h3 in human HSPC differentiation and self-renewal by lentiviral mediated overexpression. Overexpression of βig-h3 in HSPC decreased the number of colony-forming-unit-granulocyte-monocyte (CFU-GM), while it increased the percentage of mature megakaryocytic cells in cultures supporting megakaryopoiesis. Additionally, knock-down of βig-h3 in HSPC decreased proliferation and increased apoptosis in HSPC maintenance cultures. By consequence, this resulted in a dramatic drop of CFU-GM and colony-forming-unit-erythrocyte (CFU-E) formation. In conclusion, enhanced expression of βig-h3 in HSPC accelerated differentiation towards megakaryocytes and decreased CFU-GM formation, suggesting that βig-h3 might drive lineage commitment of HSC. Moreover, reduced expression of βig-h3 induced apoptosis, indicating βig-h3 also as an essential survival factor for HSC.

Key publications

Maijenburg MW, Gilissen C, Melief SM, Kleijer M, Weijer K, ten Brinke A, Roelofs H, van Tiel CM, Veltman JA, de Vries CJM, van der Schoot CE, Voermans C. Nuclear receptors Nur77 and Nurr1 modulate mesenchymal stromal cell migration. Stem Cells Dev 2011 Apr 11 [Epub ahead of print].

Homing and engraftment of hematopoietic stem- and progenitor cells

Paula van Hennik PhD, p.vanhennik@sanquin.nl

The production and application of cellular products depends on the ability to mobilize HSPC as well as on the capacity of (modulated) cells to home to and engraft the target tissue. Stromal Derived Factor-1 (SDF1/CXCL12) expressed in the BM environment, and its receptor CXCR4 expressed on hematopoietic cells are important factors to target hematopoietic cells to their niche. The aim of our studies is to find mechanisms that control homing and engraftment of hematopoietic cells. Specifically, we studied how Rac1 controls the conformation and function of CXCR4, and how signaling of Slit factors through their Robo receptor alter their migratory capacity.

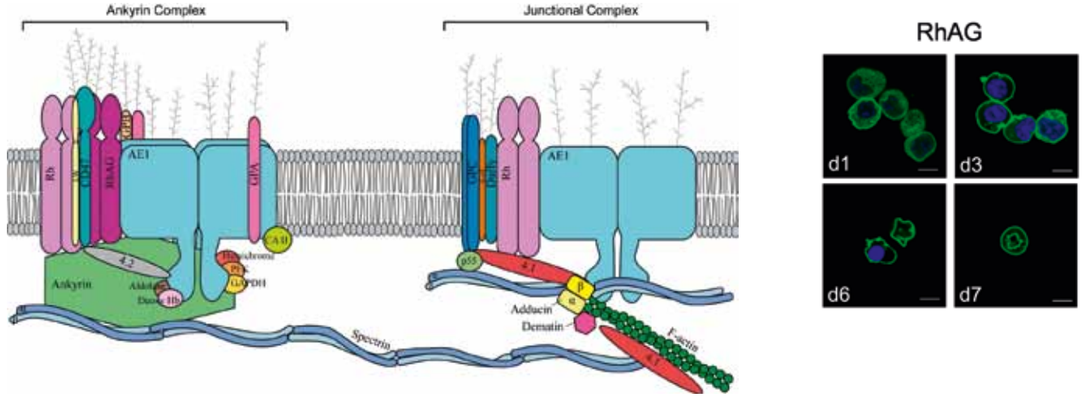
Rac1 controls CXCR4 conformation

The chemokine receptor CXCR4 is a critical regulator of cell migration and HIV-1 infection. The chemokine Stromal Cell Derived Factor-1, also known as CXCL12 binds to CXCR4 and exerts its biological functions partly through the small GTPase Rac1. We observed that Rac1 inhibition decreased CXCR4 surface signal as detected by conformation-dependent antibodies in various cell types. Inhibition of Rac1 did not affect receptor internalization or degradation, but induced a conformational change of the receptor. This conformational change of CXCR4 impaired ligand binding and ligand-induced receptor internalization. Importantly, we found that the conformation adopted by CXCR4 after Rac1 inhibition blocks HIV-1 infection into host cells. In conclusion, our data show that Rac1 activity is required to maintain CXCR4 in the responsive conformation that allows ligand binding and facilitates HIV-1 infection. This implies that Rac1 can also act upstream of CXCR4 and identifies Rac1 as a new target for preventing HIV-1 infection.

Slit/Robo signaling controls homing and engraftment of HSPC

Slit and its cognate receptor Robo are required for proper directional migration and for the regulation of proliferation and differentiation of various cell types during embryogenesis. The human genome encodes four Robo genes of which we found Robo1 to be expressed by $CD34^+$ HSPC, and three Slit

Protein complexes in the red cell membrane



RhAG, a protein associated with the rhesus polypeptides is initially expressed as a cytoplasmic protein and increasingly integrates into the red cel membrane as erythroblasts mature to reticulocytes.

homologues of which Slit2 and -3 were expressed in primary BM stroma and BM-derived endothelial and stromal cell lines. Previously we showed that Slit differentially affected *in vitro* and *in vivo* migration of distinct hematopoietic cell types. Analysis of Slit/Robo signaling at the cellular level suggests that Slit regulates cell polarity. This is predicted to affect two major aspects of stem cell biology: cell migration which is important for homing, and asymmetric cell division which controls lineage commitment and differentiation. We now showed that Slit3 increases the formation of erythroid but not of myeloid colonies, indicating that Slit3 may act as a growth or survival factor on early progenitors able to differentiate along the erythroid lineage.

Key publications

Geutskens SB, Hordijk PL, van Hennik PB. The chemorepellent Slit3 promotes monocyte migration. J Immunol 2010; 185:7691-8.

Assembly of the red cell membrane

Emile van den Akker PhD, e.vandenakker@sanquin.nl

Red cells are unique in their capacity to extrude the nucleus and adopt a membrane bound cytoskeleton that endows erythrocytes with the appropriate flexibility to pass through the capillaries of tissues. We study the assembly and functionality of specific red cell membrane protein complexes during maturation of erythroid progenitors to erythrocytes (see figure). The aim is to understand the cellular and molecular mechanisms involved in hemolytic diseases caused by mutations within proteins comprising these membrane protein complexes. Furthermore, we aim to establish *in vitro* conditions that allow for large scale *in vitro* production of erythrocytes from (hematopoietic) stem cells for transfusion purposes.

Key publications

Van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye AM The majority of the in vitro erythroid expansion potential resides in CD34- cells, outweighing the contribution of CD34+ cells and significantly increasing the erythroblast yield from peripheral blood samples. Haematologica 2010; 95(9):1594-98.

Van den Akker E, Satchwell TJ, Pellegrin S, Flatt JF, Maigre M, Daniels G, Delaunay J, Bruce LJ, Toye AM. Investigating the key membrane protein changes during in vitro erythropoiesis of protein 4.2 (-) cells. Haematologica 2010; 95(8):1278-86.

Selective mRNA translation controls erythropoiesis

Marieke von Lindern PhD, m.vonlindern@sanquin.nl

We aim to unravel the mechanism underlying rare congenital anemias. Specifically we investigate the mechanism by which ribosomal haploinsufficiency as it occurs in Diamond Blackfan Anemia (DBA) selectively affects erythropoiesis. We identified several transcripts that were selectively lost from polyribosomes in an *in vitro* DBA model and in erythroblasts cultured from peripheral blood of DBA patients. These transcripts were highly expressed in erythroblasts and appeared essential for erythroid proliferation and differentiation. Thus we established the first erythroid specific mechanism that explains severe anemia in DBA.

Platelet formation

Daphne Thijssen PhD, d.thijssen@sanquin.nl

We aim to understand the transcriptional program underlying the production of megakaryocytes and platelets from hematopoietic stem cells. Expression of the transcription factor MEIS1 is high in stem cells, it decreased during lineage commitment and differentiation, but increased again in megakaryocytes. We established that MEIS1 expression levels modulate lineage choice and expansion of megakaryocytes. We now study the role of MEIS1 splice variants in megakaryopoiesis and platelet activation.

T-cell activation

Monika Wolkers PhD, m.wolkers@sanquin.nl

$CD8^+$ T cells can protect us from recurring infections. When they encounter a pathogen again, memory $CD8^+$ T cells get reactivated and rapidly produce cytokines that help to kill the pathogen. Paradoxically, while memory $CD8^+$ T cells already carry high amounts of mRNA for cytokines, they do not produce proteins unless they are reactivated. In the past year, we have developed an *in vitro* T cell reactivation system. Using this model system, we have identified the sequence within the Interferon-γ gene that is responsible for the block of protein production. This model system will allow us to study which molecular mechanisms are involved in this regulation of protein generation.

Prof C Ellen van der Schoot MD PhD
e.vanderschoot@sanquin.nl

Experimental Immunohematology

Academic staff
Prof CE van der Schoot MD PhD (PI)
M de Haas MD PhD
DC Thijssen-Timmer PhD
G Vidarsson PhD
C Voermans PhD
JJ Zwaginga MD PhD

Post docs
J Berbee PhD
B Veldhuisen PhD

PhD students
L Della Valle
H Einarsdottir
R Kapur
SE Klamer
A Laarhoven
MW Maijenburg
PG Scheffer
J Stutterheim
L Wigman
S Zeddies

Technical staff
A Ait Soussan
IW de Jong
A de Vries-van Rossen
FM di Summa
T Grijsen-den Bleker
JJ Janssen
M Kleijer
E Sellink
M Valk
JHM Verhagen
K Vermeul
R Visser
AHV Vos
A Zadurian

Students
F Ait Ichou
R Baalhuis
A de Jong
S Ferman
A Huisman
S Jansen
C Kuijk
R Jonkers
R Louiszoon

Visiting scientists
A Reinhardt
N Stapleton
A van der Laan
A Vestrheim
J Yanli

Secretariat
AEPT Engels
MLMW Lutkie

Address
Sanquin Research
Department of Experimental
Immunohematology
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3377/3976
F +31 20 512 3474
E secretariaatIHE@sanquin.nl
W ihe.sanquin.nl

Experimental Immunohematology

Immune response to blood group antigens

The aim of this research line is to develop new diagnostic and preferentially also therapeutic options to further prevent and/or treat allo- or autoimmunization against blood cells. We are studying both the antigens, which are the targets of the immune response, and the humoral immune response that leads to cell destruction.

Blood group antigens

Masja de Haas MD PhD, m.dehaas@sanquin.nl
Barbera Veldhuisen PhD, b.veldhuisen@sanquin.nl

In the past this research line has been focused on the biochemical and molecular characterization of platelet antigens and in later years on the molecular characterization of red blood cell antigens, especially Rh. We now focus on new techniques for mass scale red cell genotyping and aim to unravel the molecular background of high-frequency red cell antigen systems. The ultimate goal of our research in this field is to change transfusion policy. At present, blood transfusion is only matched for ABO and RhD and the donor is screened for the presence of red cell antibodies. By making available: 1) cost effective *genotyping* of both donors and recipients and 2) insight in *risk factors for alloimmunization* (genetic factors as well as disease related), new algorithms for transfusion can be developed. Selected patient groups can be transfused with matched blood cell products. This will cause shifting from lab based selection of blood products to electronic matching.

Cost effective genotyping

In 2010 we have developed in collaboration with MRC-Holland a genotyping assay based on the Multiplex Ligase Probe Amplification (MLPA) for 52 blood group antigens of 18 blood group systems (MNS, RH, LU, KEL, LE, FY, JK, DI, YT, SC, DO, CO, LW, GE, CROM, KN, IN and OK) and two platelet systems (HPA1 and HPA2). This assay is particularly useful for full genotyping of recipients, since also null alleles and variants (including >50 Rh variants) can be detected. For donor typing the fully automated ID-Core assay of Progenika which is based on our previously developed BloodChip is more suitable. The composition of this latter assay will be adapted to the requirements for Dutch blood banks.

Risk factors for alloimmunization

To identify genetic risk factors for alloimmunization we are continuously banking DNA samples of red cell alloimmunized pregnant women for a genome wide association study (GWAS). In 2010 we collected in collaboration with the blood bank in Amsterdam and investigators of the Netherlands Cancer Institute DNA from a control cohort of Dutch female blood donors.

In previous years we have developed several blood group genotyping assays (D, c, E, K) on cell free fetal (cff) DNA, and these assays are offered as a routine service since 7 years. In 2010 we evaluated their performance by comparing our test results with cord blood serology. Complete concordance was observed.

Key publication

Veldhuisen B, Ligthart PC, Vidarsson G, Roels I, Folman CC, van der Schoot CE, de Haas M. Molecular analysis of the York antigen of the Knops blood group system. Transfusion 2011 Jan 7 [Epub ahead of print].

Humoral immune response

Gestur Vidarsson PhD, g.vidarsson@sanquin.nl

In most immune mediated blood cell diseases and in all fetal/neonatal alloimmune cytopenias the destruction of blood cells is mediated by antibodies. We are therefore investigating *1)* the *B cells* producing these antibodies, *2)* the characteristics of these *antibodies* and 3) the interaction of antibodies with the *FcRn*, the receptor responsible for placental transport.

B cells

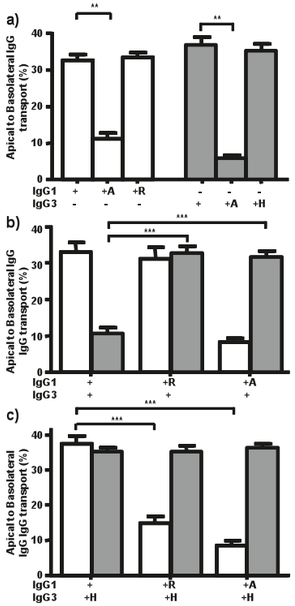
We have previously developed a culture method in which B cells can be cultured and stimulated to Ig production at the single cell level. Using this method we found that in hyperimmune anti-D donors the majority of antigen-specific memory cells resides in the IgM-positive B cells. Upon antigen challenge the number of IgG-positive cells increase, whereas the IgM positive cells remained stable. We found that the BCL6 mutational status of the IgM memory cells was lower than of their $CD27^{+}IgG^{+}$ counterparts. In analogy with recent studies in mice, we postulate that the IgM memory cells will not class switch in the presence of IgG in the serum, but can replenish the memory pool once the titer has dropped and in case of infectious agents the antigenic make-up of the pathogen might have changed.

Antibodies

By analyzing the Fc-glycosylation of the pathogenic, affinity-purified IgG1 alloantibodies formed during pregnancy against antigens of the fetus (HPA-1 or RhD) at the glycopeptide level using mass spectrometry, we found markedly decreased levels of core-fucosylation as well as increased levels of galactosylation and sialylation as compared to glycosylation patterns of total serum IgG1 of the same patients. Because IgG1 Fc-core-fucosylation is known to influence ADCC activity, modulation of core-fucosylation may have a profound effect on disease severity and prognosis. To correlate Fc-glycosylation with biological activity we have developed an assay to determine the induction of FcRIII mediated respiratory burst by anti-HPA opsonized platelets. Remarkably, this assay was found to be dependent on the presence of C-reactive protein. This serum protein was found to be increased in cord blood samples of FNAIT (Fetal Neonatal Alloimmune Thrombocytopenia) affected neonates.

FcRn

Human IgG3 displays the strongest effector functions of all human IgG subclasses but has a short half-life, suggesting FcRn-mediated IgG salvage to be defective for IgG3. We have previously observed that human IgG1 inhibited FcRn-mediated transport of IgG3 at the level of receptor binding. The observed inhibition was due to a single amino acid difference at position 435, where IgG3 has an arginine instead of the histidine found in all other known IgG species, as H435-engrafted IgG3 inhibited FcRn-mediated transport and rescue of R435-IgG1. An arginine in position 435 in IgG may theoretically affect (see figure) binding to FcRn in two ways – either through less sensitivity to deprotonation and enhanced binding at pH 7.4



Inhibition of IgG3 transport by IgG1 is due to R435 in IgG3. *A) Mutating the amino acid at position 435 in IgG1 (H435) and in IgG3 (R435) to an alanine reduces transport, while exchanging the histidine native to IgG1 and the arginine native to IgG3 on each others backbone had no effect on their transport rate when offered separately to FcRn-transfected A375 cells. B) While transport of IgG3-WT was inhibited in the presence of IgG1-WT, IgG1 bearing an alanine or an arginine at position 435 had no effect on IgG3 transport. C) Transport of IgG3 with a histidine at position 435 was not inhibited by WT IgG1. When the amino acids found at position 435 in IgG1 and IgG3 were swapped, IgG1-H435R transport was inhibited by IgG3-R435H. A-C) The +/- indicate the presence or absence of IgG (10 µg/ml/subclass), IgG1 is represented by open bars, IgG3 by hatched bars. The presence of mutated variants (435H, 435A, 435R) is indicated by the corresponding letter. The data represent mean and standard deviation from 3 independent experiments. Transport of WT IgG was compared to transport of mutant IgG by one-way ANOVA with Dunnett's multiple comparison test and significance is indicated as described in the Materials and Methods section.*

and/or through steric hindrance because of the longer side-chain. We found indeed that both factors may play a role. Both IgG1 and IgG3 bound FcRn in a pH dependent manner, with similar high affinity at pH 6.0 but residual binding at pH 7.4. However, R435-containing IgG bound better to FcRn at pH 7.4 than H435-containing IgG1, an advantage lost at pH 6.0 as seen by ELISA. Using an FcRn-coated biosensor we observed H435-containing IgG to bind FcRn slightly better at pH6.0, confirming previous findings showing inferior binding of IgG3 to FcRn and hinting at a possible allotypic variation affecting the binding affinity for IgG3. Our observation of the increased binding of IgG3 at pH 7.4 but decreased binding at pH 6.0 compared to IgG1, suggests a decreased capacity of IgG3 to release FcRn at neutral pH and decreased competitiveness for FcRn binding at pH 6.0. This is in line with the functional evidence presented here on the decreased recycling rate, and short biological half life of IgG3. Importantly we showed that the half lives of H435-containing IgG3 allotypes in humans are comparable to IgG1. This H435-IgG3 also proved better suited for protection against pneumococcal challenge in mice, demonstrating that H435-IgG3 is a formidable candidate for monoclonal antibody therapies in patients.

Key publication

Sesarman A, Vidarsson G, Sitaru C. The neonatal Fc receptor as therapeutic target in IgG-mediated autoimmune diseases. Cell Mol Life Sci 2010; 67(15):2533-50.

Minimal residual disease detection in childhood cancers

The prognosis of cancers in childhood is often better than in adults, but still many children do not survive. To recognize children that might benefit from other therapeutic strategies, we develop and evaluate in collaboration with Lieve Tytgat and Huib Caron (AMC/EKZ, Amsterdam), SKION (V de Haas), Erasmus MC (JJM van Dongen and VHJ van der Velden) assays for the detection of minimal residual disease in childhood cancers.

Key publication

Waanders E, van der Velden VH*, van der Schoot CE*, van Leeuwen FN, van Reijmersdal SV, de Haas V, Veerman AJ, van Kessel AG, Hoogerbrugge PM, Kuiper RP, van Dongen JJ. Integrated use of minimal residual disease classification and IKZF1 alteration status accurately predicts 79% of relapses in pediatric acute lymphoblastic leukemia. Leukemia 2011; 25(2):254-8.*
**These authors contributed equally to this work.*

Prof S Marieke van Ham PhD
m.vanham@sanquin.nl

Immunopathology

Academic staff
Prof SM van Ham PhD (PI)
JA ten Brinke PhD

PhD students
MA Boks
J de Wit
STHM Kolanowski
MD Makuch
M van Luijn

Technical staff
M Aalbers
A Baghat
B Bottelier
MC Dieker-Meijer
M Holsheimer
T Jorritsma
JR Kager-Groenland
DI Roem-Haagsma
GMW van Schijndel
AM Wolbink-Kamp

Students
H Edelijn
MSP Haasjes
G Marsman
A Zaal

Secretariat
K Boukdid
F Muntar-den Ouden

Address
Sanquin Research
Department of Immunopathology
Plesmanlaan 125
NL 1066 CX Amsterdam
P.O. Box 9190
NL 1006 AD Amsterdam
The Netherlands
T +31 20 512 3171/58
F +31 20 512 3170
E secretariaatimmunopathologie@sanquin.nl
W www.ip.sanquin.nl

Immunopathology

Immune regulation by B cells

The regulation of acquired immunity by antigen-specific B cells

This research line addresses the question how the humoral and cellular immune responses are regulated by MHC-mediated antigen presentation in B cells.
B cells behave as professional phagocytes of bacteria and particles when recognition is triggered via the specific B cell receptor (BCR). Phagocytosis of *Salmonella*, our model pathogen, leads to survival of the bacteria in a latent state in the B cells. This is subsequently followed by extracytosis of the bacteria and reinfection in other tissues followed by local multiplication. This year, we demonstrated in *in vivo* mouse models that B cells indeed contribute to dissemination of *Salmonella in vivo* in collaboration with Maria Rescigno (Milan, Italy) Thus, B cells can serve as a niche for survival of *Salmonella* from the innate immune system and a transport vehicle for systemic dissemination. In addition, we published our data showing that phagocytosis of *Salmonella* does lead to efficient antigen presentation of bacterial antigens to $CD4^+$ T helper cells and the induction of an efficient humoral immune response is mounted against *Salmonella*. In addition, we continued our research on the activation of $CD8^+$ cells by B cells that had taken up *Salmonella*. Research in animal models had demonstrated that B cells contribute to the cellular immune response against *Salmonella*, but the mechanism behind remained unclear. We demonstrated that efficient cross-presentation of *Salmonella* antigens by B cells leads to activation of $CD8^+$ memory T cells, but not naive $CD8^+$ T cells. The activation of the recall response induces $CD8^+$ cells that are specifically cytotoxic to *Salmonella*-infected target cells. Thus, B cells are able to reactivate *Salmonella*-specific cytotoxic T cells and therefore may contribute to the clearance of *Salmonella* during reinfection.

The regulation of the humoral immune response by antigen-specific B and $CD4^+$ T cells

We started to investigate how antigens that are taken up by the BCR lead to B cell differentiation, antibody formation and class switching and how this process is aided by activation of specific $CD4^+$ Thelper cell subsets. For this research line, we make use of our infections models and also of hyperimmunization protocols in which patients or donors come in contact with non-infectious antigens on a regular basis. We specifically focus on RA patients that are being treated with a therapeutic monoclonal antibody against TNFα, adalimumab and voluntary healthy donors that are hyperimmunized against tetanus. A large number of these patients and donors mount an antibody response against the therapeutic antibody or tetanus. For the patients this is detrimental as therapy becomes ineffective because of this inhibitory antibody formation.
In collaboration with Genmab (dr Paul Parren en dr Janine Schuurman) and Algonomics (dr Ignace Lasters en dr Jurgen Pletinckx), we have identified a number of T cell epitopes in the therapeutic antibody adalimumab that may play a role in antibody formation. This year, we have demonstrated actual binding of predicted antigenic peptides to MHC class II by mass spectrometry and have completed our analyses of T cell reactivity against these epitopes in adalumimab-experienced patients. As expected, T cell reactivity can also be observed in healthy donors that have not been exposed to adalumimab, but at much lower frequencies. For this project we carried on with the generation of MHC class II tetramers as tools to monitor antigen-specific $CD4^+$ T cells. We have expressed monomeric class II complexes associated to CLIP (Class II Associated Invariant Chain Peptide) in insect cells with the aim to generate large production batches of MHCII/CLIP that may be used for the versatile generation of arrays of different MHC II tetramers that each carry the desired antigenic peptide of interest. This year, we focused on the exchange of CLIP for antigenic peptides. The process works in principle, but is inefficient. Therefore, we are currently exploring the use of catalysts of reaction. In addition, we are investigating if we can create expression systems that yield higher levels of MHC/CLIP complexes.

MHC class II-mediated antigen presentation in leukemia

As effective MHC class II-Ag presentation is also required for effective cytotoxic T cell function against infected cells or malignant cells, we are studying the regulation of this pathway in leukemia. In B cell chronic lymphocytic leukemia (B-CLL) we demonstrated that aberrant MHC class II antigen presentation is correlated to chronic immune dysfunction of the T cell compartment. In collaboration with dr Arjan van de Loosdrecht (Dept of Haematology, VUmc), we demonstrated that in leukemic blasts an alternative Ii-independent antigen-processing pathway exists that involves TAP-dependent peptide loading of HLA class II complexes. This new route of antigen presentation may affect therapy strategies aimed to optimize antigen presentation in patients.

Key publications

De Wit J, Souwer Y, Jorritsma T, Klaasse-Bos H, ten Brinke A, Neefjes J, van Ham SM. Antigen-specific B cells reactivate an effective cytotoxic T cell response against phagocytosed Salmonella through cross-presentation. PLoS One 2010; 5:e13016.

Van Luijn MM, Chamuleau ME, Ressing ME, Wiertz EJ, Ostrand-Rosenberg S, Souwer Y, Zevenbergen A, Ossenkoppele GJ, van de Loosdrecht AA, van Ham SM. Alternative Ii-independent antigen-processing pathway in leukemic blasts involves TAP-dependent peptide loading of HLA class II complexes. Cancer Immunol Immunother 2010; 59:1825-38.

Immune modulation by Dendritic cells

Anja ten Brinke PhD, a.tenbrinke@sanquin.nl

Immune activation by dendritic cells

In this research line we are developing clinically approved, validated and cost-efficient monocyte-derived DC products. For the development of immuno-activatory DCs we extended our research on our DC maturation-cocktail, MPLA plus IFNγ. Previously, we demonstrated that these DCs can migrate, produce IL-12 and induce high percentage of specific and highly cytotoxic CTLs against tumor antigens. In addition, the DCs are also able to reactivate these tumor-specific CTLs in blood derived from melanoma patients. Furthermore we have shown that the MPLA plus IFNγ matured DCs produce a high level of the effector cell attracting chemokine CXCL10 and low levels of the Treg attracting chemokine CCL22. These data indicate that the MPLA/IFNγ DCs *in vivo* will preferentially activate inflammatory T cells and will likely not induce unwanted activation of Tregs during immunotherapy.
Together with dr Carlijn Voermans and dr Daphne Thijssen

(Stem Cell Laboratory), dr Hans Vrielink (Sanquin Blood Bank) and dr Sheila Krishnadath and dr Francesca Milano (Dept of Gastroenterology, AMC) and prof dr Martien Kapsenberg (Dept of Histology and Cell Biology, AMC) we have continued to set up a phase I/II trial to study the toxicity and use of MPLA plus IFNγ matured DCs in the treatment of patients suffering from esophageal or pancreatic cancer.
In addition, in a more basic research approach we are studying if and how the subcellular trafficking pathways of antigen and TLR regulate the DC effector function upon DC maturation. Furthermore, we are studying the role of complement activation products, such as C3a and C5a, on human DCs and the subsequent adaptive T cell response which is elicited. Studies in mice showed an important role for these factors in regulation of the adaptive immune response.

Immune tolerance and dendritic cells

Tolerogenic dendritic cells (tDC) are a promising tool as specific cellular therapy to maintain or restore immunological tolerance in transplantation and autoimmunity. In transplantation, tDCs need to confer donor-specific suppression, without affecting immune responsiveness to viruses that may be co-transferred with the transplant. Many described tDC types are not clinically applicable and lack systematic comparison of required functional characteristics, i.e. migratory capacity, stable immunosuppressive phenotype and regulatory T cell (Treg) induction. Previously, we generated an optimized assay to assess the suppressive capacity of induced Tregs. Now, we generated human clinical-grade tDC using different tolerance-inducing agents. For an optimal migratory and stable phenotype, co-maturation of tDC with immunoactivatory compounds was required. All tDC were shown to be highly stable in pro-inflammatory environments, but IL-10 DC show the most optimal tolerogenic properties with high IL-10 production, low T cell activation and strong Treg induction. Importantly, IL10 DC induce alloantigen-primed Treg that suppress allo-responsive T cells, but do not suppress CMV–specific T cell reactivity. Thus, clinically-applicable IL-10-generated tolerogenic dendritic cells induce allo-specific tolerance without affecting anti-viral responsiveness, which makes them suitable for tolerance inducing therapies in transplantation.

Key publications

Ten Brinke A, van Schijndel G, Visser R, de Gruijl TD, Zwaginga JJ, van Ham SM. Monophosphoryl lipid A plus IFNγ maturation of dendritic cells induces antigen-specific CD8⁺ cytotoxic T cells with high cytolytic potential. Cancer Immunol Immunother 2010; 59:1185-95.

Boks M, Zwaginga JJ, van Ham SM, ten Brinke A. An optimised CFSE-based T cell suppression assay to evaluate the suppressive capacity of regulatory T cells induced by clinically-applicable human tolerogenic dendritic cells. Scand J Immunol 2010; 72:158-68.

Immune regulation by Granzyme A and Granzyme escape mechanisms

Cytotoxic T cells and natural killer cells produce Granzyme A (GrzmA) and Granzyme B (GrzmB), effector molecules that cause apoptosis of target cells. Dendritic cells and many tumor cells are protected against GrzmB via expression of the GrzmB-inhibitor SerpinB9. Escape from GrzmA action is also likely to occur, but the mechanism is unknown. We have now identified the cellular mechanism that downmodulates the action of GrzmA in primary human cells and melanoma. In addition, we demonstrated that urinary GrzmA mRNA forms a biomarker for cellular rejection in kidney transplantation (van Ham et al., Kidney Int 2010; 78:1033).

Immune regulation by C1 esterase inhibitor

Sacha Zeerleder PhD, s.zeerleder@sanquin.nl
Diana Wouters PhD, d.wouters@sanquin.nl

C1 esterase inhibitor (C1-Inh) is an inhibitor of the complement system and the contact system of coagulation. Hereditary angio-edema (HAE), a disease characterized by recurrent attacks of edema, is caused by heterozygous C1-Inh deficiency. C1-Inh preparations purified from plasma have been used for HAE treatment. In collaboration with Sanquin Plasma Products and Viropharma we are exploring possible new fields of clinical application for C1-Inh and are investigating the function of C1-Inh in more detail. In collaboration with prof Hans Niessen (Dept of Pathology, VUmc) we demonstrated that in diseased aortic valves activated complement is more present than C1-Inh, which gives rise to the question if exogenous addition of C1-Inh might be useful for treatment of this disease (ter Weeme M et al., Eur J Clin Invest 2010; 40:4). In addition, we are investigating the role of C1-Inh in early vein graft remodeling.

Prof Lucien A Aarden PhD
l.aarden@sanquin.nl

Autoimmune diseases

Academic staff
Prof LA Aarden PhD (PI)
D Hamann PhD
GJ Wolbink MD PhD
D Wouters PhD
SS Zeerleder MD PhD

Post docs
R Engel PhD
F Ribeiro da Silva PhD
T Rispens PhD
H te Velthuis PhD

PhD students
M Keizer
I Kustiawan
L Lighaam
F Stephan
L van de Stadt
P van Schouwenburg

Technical staff
MC Brouwer
I Bulder
ER de Groot
N Derksen
MHL Hart
JM Klaasse Bos
P Ooijevaar-de Heer
HJAM Rensink
S Solati
J van Leeuwen
GJ van Mierlo
E Vermeulen

Students
S Bigler
A Ghabri
K Jairam
M Joling
S Lissenberg
J Ruinaard

Secretariat
K Boukdid
F Muntar-den Ouden

Address
Sanquin Research
Department of Immunopathology
Plesmanlaan 125
NL 1066 CX Amsterdam
P.O. Box 9190
NL 1006 AD Amsterdam
The Netherlands
T +31 20 512 3171/58
F +31 20 512 3170
E secretariaatimmunopathologie@sanquin.nl
W www.ip.sanquin.nl

Autoimmune Diseases

Inflammation

Diana Wouters PhD, d.wouters@sanquin.nl
Sasha Zeerleder MD PhD, s.zeerleder@sanquin.nl
Prof Marieke van Ham PhD, m.vanham@sanquin.nl

Complement activation

Complement is a major effector system in inflammation. We are interested in genetic variation and therapeutical application of proteins that regulate complement. In cooperation with prof Taco Kuijpers at the Academic Medical Center we study the molecular organization of the lectin pathway and investigate why complement activation is attenuated in pediatric oncology patients. We also developed diagnostic assays to analyze variations in complement regulatory protein factor H. C1-inhibitor (C1inh) is a major therapeutical product of Sanquin. We compare properties of recombinant C1inh with plasma-derived C1inh and investigate the effect of (glycosylated) C1inh on neutrophil/endothelial cell interaction. Moreover we investigate potential anti-inflammatory activity of C1inh in LPS stimulated whole blood and mononuclear cells.

Factor VII activating protein

Factor VII activating protein (FSAP) is a plasma serine protease. FSAP binds to dead cells and is activated. FSAP activity is further regulated by the serine protease inhibitors C1inh and alpha2-anti-plasmin which form covalent complexes with FSAP. We used these complex ELISAs to monitor *in vitro* as well as *in vivo* FSAP activation. Surprisingly coagulation does not activate FSAP. FSAP activation can be observed in sepsis patients and in patients undergoing major surgery. Our data suggest that FSAP acts as a plasma sensor for cell death.

Key publications

Ter Weeme M, Vonk AB, Kupreishvili K, van Ham M, Zeerleder S, Wouters D, Stooker W, Eijsman L, van Hinsbergh VW, Krijnen PA, Niessen HW. Activated complement is more extensively present in diseased aortic valves than naturally occurring complement inhibitors: a sign of ongoing inflammation. Eur J Clin Invest 2010; 40:4-10.

Stephan F, Hazelzet JA, Bulder I, Boermeester MA, van Till JO, van der Poll T, Wuillemin WA, Aarden LA, Zeerleder S. Activation of factor VII-activating protease in human inflammation: a sensor for cell death. Crit Care 2011; 15(2):R100 [Epub ahead of print].

Immunogenicity of biologicals

Theo Rispens PhD, t.rispens@sanquin.nl
Gertjan Wolbink MD PhD, gj.wolbink@sanquin.nl
Diana Wouters PhD, d.wouters@sanquin.nl
Prof Marieke van Ham PhD, m.vanham@sanquin.nl
Steven Stapel PhD, s.stapel@sanquin.nl

With over 20 therapeutic monoclonal antibodies (tmAb) registered and many more in development, an ever increasing number of patients is now exposed to these biologics, often for prolonged periods of time. Even in case the tmAb is fully human, anti-idiotypic responses are elicited in a considerable fraction of the patients. This leads to reduced efficacy due to blocking of the therapeutic drug by anti-drug antibodies (ADA). Moreover, patients may develop treatment related side effects due to the formation of immune complexes (IC).

We studied antibody formation in rheumatoid arthritis patients treated at 2-weekly intervals with the fully human anti-TNFa antibody adalimumab and found that within 6 months of treatment 70% of the patients produce anti-adalimumab antibodies, mostly of the IgG1 and IgG4 subclass. Small circulating IC can be detected in most patients even two weeks after administration of adalimumab. Therefore these patients are permanently exposed to high concentrations of small IC.

Key publications

Van Schouwenburg PA, Bartelds GM, Hart MH, Aarden L, Wolbink GJ, Wouters DA. Novel method for the detection of antibodies to adalimumab in the presence of drug reveals "hidden" immunogenicity in rheumatoid arthritis patients. J Immunol Methods 2010; 362:82-88.

Rispens T, van Leeuwen A, Vennegoor A, Killestein J, Aalberse RC, Wolbink GJ, Aarden LA. Measurement of serum levels of natalizumab, an immunoglobulin G4 therapeutic monoclonal antibody. Anal Biochem 2011; 411:271-76.

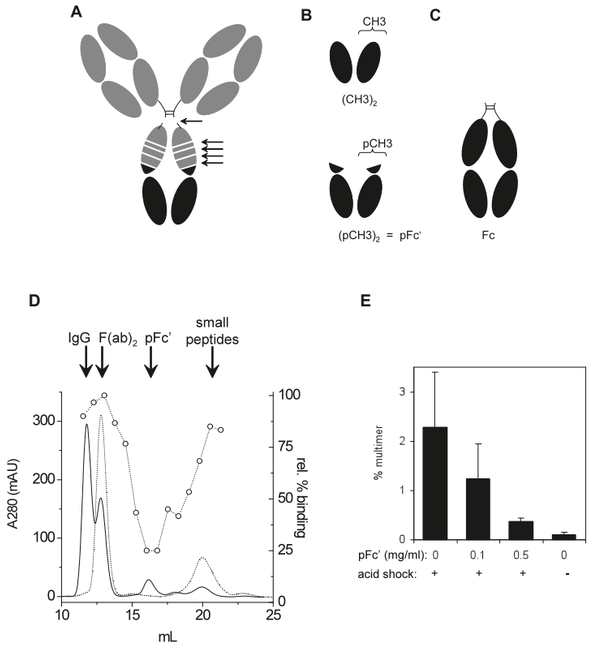
Bartelds GM, Wijbrandts CA, Nurmohamed MT, Stapel S, Lems WF, Aarden L, Dijkmans BA, Tak PP, Wolbink GJ. Anti-infliximab and anti-adalimumab antibodies in relation to response to adalimumab in infliximab switchers and anti-tumour necrosis factor naive patients: a cohort study. Ann Rheum Dis 2010; 69:817-21.

Immunochemistry

Theo Rispens PhD, t.rispens@sanquin.nl
Prof Rob Aalberse PhD, r.aalberse@sanquin.nl

Structural and functional properties of human IgG4

IgG4 is a unique antibody with characteristic structural properties that contribute to its status as 'blocking' antibody. We found that human IgG4 can exchange half-molecules with other IgG4 molecules in the blood. This usually results in antibodies with two different antigen-combining sites. The reaction is not observed upon mixing IgG4 antibodies in buffer, but is observed both *in vivo* (in a mouse model) and *in vitro* (in the presence of glutathione as catalyst). We identified the key structural features that are responsible for this phenomenon using a panel of IgG4/IgG1 mutants in collaboration with Genmab. Also, IgG4 was found to be able to bind to other IgG molecules, in particular, IgG4. This binding is related to the exchange process and could resemble an intermediate step. IgG4 binds to all human IgG subclasses if directly immobilized, but only to IgG4, bound to antigen. Mechanistic studies are undertaken to establish the individual steps of this process. A FRET assay has been developed to monitor the exchange reaction in real time.
IgG4 stands out also in its appearance during a Th2-driven immune response. Whereas IgG1 antibodies are readily formed upon antigenic challenge, IgG4 antibody titers rise only slowly upon persistent antigenic stimulation. However, the IgG4 response dominates in the end. The underlying mechanisms that control switch to and proliferation of IgG4-producing B cells are only partially understood. We initiated research aimed at unraveling these mechanisms. We quantified the number of IgG4-positive B cells in blood with FACS analysis and *in vitro* culture experiments. In line with the relatively



pFc' modulates Fc-Fc interactions of IgG and prevents aggregation.

Pepsin digests IgG into F(ab)2, pFc' (black) and a number of smaller fragments. B) Two CH3 or pCH3 domains form a non-covalent dimer in solution. C) Papain digestion results in formation of an Fc fragment. D) HP-SEC analysis of pepsin digests of IgG1 at 37oC after 90 minutes (solid line) and 1000 minutes (dotted line). Open circles indicate inhibition of Fc-Fc interactions by fractions of the pepsin digest after 90 minutes (right axis). E) Aggregation of IgG induced by acid shock in absence or presence of pFc'.

low serum levels of IgG4 (3-4% of total IgG), the number of IgG4 positive B cells is correspondingly low. Tools are currently being developed to investigate features that might explain why the immune regulation of IgG4 differs from other isotypes, 1) developmental stages of the IgG4 B cell; 2) how and when the isotype switch is induced; and 3) at which stage the cell becomes an IgG-secreting cell.

Key publications

Rispens T, den Bleker TH, Aalberse RC. Hybrid IgG4/IgG4 Fc antibodies form upon 'Fab-arm' exchange as demonstrated by SDS-PAGE or size-exclusion chromatography. Mol. Immunol 2010; 47:1592-94.

Pro- and anti-inflammatory fractions in intravenous immunoglobulin (IVIG)

Besides being used for replacement therapy in patients with antibody deficiency, intravenous immunoglobulin (IVIG) is used in conditions such as idiopathic thrombocytopenia purpura (ITP), Kawasaki syndrome and Guillain-Barré in high doses. In applications other than replacement therapy, the mechanisms of action are largely uncertain, and proposed mechanisms are a.o.: effects due to scavenging of complement activation products, blockade of Fc receptors, effects of IgG dimers and effects of specific antibodies (for example: cytokine neutralization).

We investigated properties such as stability of the IgG dimers present in IVIG under different physical conditions by a.o. size-exclusion chromatography and sodium dodecyl sulfate (SDS) electrophoresis. A substantial fraction of dimers dissociate rapidly under conditions mimicking those in patients after administering IVIG, but part of the dimers remain stable. Formation of dimers and larger aggregates may result in part from slightly denatured IgG. We found that aggregation of IgG may be counteracted by addition of fragments of IgG.

Treatment of conditions such as ITP require high doses of IVIG. It is reported that only the fraction of IgG molecules containing sialic acid is responsible for its anti-inflammatory action. These findings are based mainly on an arthritis mouse model. In cooperation with Sanquin Plasma Products, IVIG was enriched for sialic acid (SA). The SA-enriched and -depleted IVIG is currently being tested in a mouse model for ITP.

Furthermore, monomeric precursors for aggregation of IgG are difficult to detect. Usually, hydrophobic fluorescent probes such as 1-anilino-8-naphthalenesulfonate are used that may detect exposed hydrophobic surfaces as a result of partial unfolding. We extented this approach by detecting binding of such probes using isothermal titration calorimetry. In addition to fluorescent probes, non-fluorescent probes, including peptides, can be used to probe native or non-native configurations. Furthermore, IgG-derived peptides can be used to detect epitopes that are normally shielded.

Key publications

Rispens T, Himly M, Ooievaar-de Heer P, den Bleker PH, Aalberse RC. Traces of pFc' in IVIG interact with human IgG Fc domains and counteract aggregation. Eur J Pharm Sci 2010; 40:62-68.

Hawe A, Rispens T, Herron JN, Jiskoot W. Probing bis-ANS Binding Sites of Different Affinity on Aggregated IgG by Steady-State fluorescence, Time-Resolved Fluorescence and Isothermal Titration Calorimetry. J Pharm Sci 2011 Oct 18 [Epub ahead of print].

Prof Hanneke J Schuitemaker PhD
h.schuitemaker@amc.uva.nl

Laboratory of Viral Immune Pathogenesis (AMC)

As from mid December 2007, the research group of Professor Schuitemaker moved to the Academic Medical Center of the University of Amsterdam. The group is still part of the joint Sanquin - AMC Landsteiner Laboratory. Professor Schuitemaker also acts as advisor to Sanquin regarding virological issues.

Academic staff

Prof HJ Schuitemaker PhD (PI)
NA Kootstra PhD
AB van 't Wout PhD

PhD students

SM Bol
T Booiman
MJ Bunders
V Cobos-Jimenez
D Edo Matas
Z Euler
EF Gijsbers
A Rachinger PhD
TLGM van der Kerkhof
MJ van Gils
D van Manen

Post doc

EM Bunnik PhD

Technical staff

BDM Boeser-Nunnink
JA Burger
LC Setiawan
M Sieberer
KA van Dort
JL van Hamme
AC van Nuenen

Students

T Booiman
B Bruinsma
M Cosin Tomas
S de Taeyi
M Klein
B Kwayie
TLGM van den Kerkhof
D Verboom
N Verwer

Secretariat

E Kwak

Current address

Department of Experimental Immunology
Laboratory of Viral Immune Pathogenesis
M01-120
Academic Medical Center
Meibergdreef 15
NL-1105 AZ Amsterdam
The Netherlands
T +31 20 566 8298
E h.schuitemaker@amc.uva.nl
W www.amc.nl/index.cfm?sid=1645

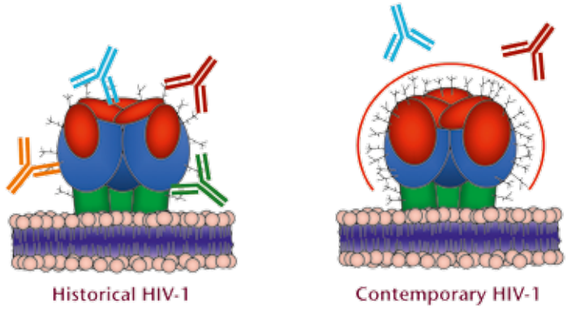
Laboratory for Viral Immune Pathogenesis

Neutralizing humoral immunity in HIV-1 infection

Broadly reactive neutralizing antibodies are the focus of human immunodeficiency virus (HIV) type 1 vaccine design. However, only little is known about their role in acquired immunodeficiency syndrome (AIDS) pathogenesis and the factors associated with their development. We used a multisubtype panel of 23 HIV-1 variants to determine the prevalence of cross-reactive neutralizing activity in serum samples obtained approximately 35 months after seroconversion from 82 HIV-1 subtype B-infected participants from the Amsterdam Cohort Studies on HIV Infection and AIDS. Of these patients, 33%, 48%, and 20%, respectively, had strong, moderate, or absent cross-reactive neutralizing activity in serum, with a strong correlation between neutralizing titer in serum and the breadth of neutralization. Another interesting observation was that the neutralizing activity in sera of these subtype B infected individuals was strongest against the subtype B variants in our virus panel, suggesting subtype-specific rather than strain-specific neutralizing activity, at least in these subtype B infected individuals. Viral RNA load at set point and AIDS-free survival were similar for the 3 patient groups.

We subsequently studied whether disease progression in the face of cross-reactive neutralizing serum activity is due to fading neutralizing humoral immunity over time or to viral escape. Viral escape was hypothesized to be complicated for the virus as cross-reactive neutralizing activity is assumed to be directed against conserved epitopes in which changes by definition likely coincide with decreased HIV-1 replication fitness. In three long-term non-progressors (LTNP) and three progressors, high-titer cross-reactive HIV-1-specific neutralizing activity in serum against a multiclade pseudo virus panel was preserved during the entire clinical course of infection, even after AIDS diagnosis in progressors. However, while early HIV-1 variants from all six individuals could be neutralized by autologous serum, the autologous neutralizing activity declined during chronic infection. This could be attributed to viral escape and the apparent inability of the host to elicit neutralizing antibodies to the newly emerging viral escape variants. Escape from autologous neutralizing activity was not associated with a reduction in the viral replication rate *in vitro* and coincided with an increase in the length of the variable loops and in the number of potential N-linked glycosylation sites in the viral envelope. Positive selection pressure was observed in the variable regions in envelope, suggesting that, at least in these individuals, these regions are involved in the escape from humoral immunity with cross-reactive potential. So rather than changes in the epitopes for cross-reactive neutralizing antibodies, escape from humoral immunity is mediated by changes in the viral envelope that occlude the conserved epitope, making it inaccessible to the antibody. These results may imply that the ability of HIV-1 to rapidly escape cross-reactive autologous neutralizing antibody responses without the loss of viral fitness is the underlying explanation for the absent effect of potent cross-reactive neutralizing humoral immunity on the clinical course of infection.

As described above, during the course of infection, HIV-1 can rapidly escape from neutralizing humoral immunity,



Longer variable loops with increased glycosylation in the viral envelope of HIV-1 coincide with increasing resistance to neutralizing humoral immunity over calendar time and at a population level. Shown is a cartoon of a single HIV-1 envelope spike that sticks through the lipid bilayer of the viral envelope (green is gp41, blue ovals is gp120 with in red ovals the variable loops; branches on top represent N-linked glycosylation; Y structure are antibodies that can or cannot access the neutralizing epitopes on HIV-1 variants from historical or contemporary seroconverters, respectively.

coinciding with changes in the envelope glycoprotein (Env) that include elongation of the variable loops in the gp120 subunit, and an increased number of potential N-linked glycosylation sites. In our subsequent study we analyzed whether these changes revert upon transmission to a new individual or that resistance of Env to antibody neutralization is accumulating over the course of the epidemic. For this purpose we tested whether the sensitivity of clonal HIV-1 variants isolated from patients presenting with primary HIV-1 infection in Amsterdam either in the period 1985-1988 (historical HIV) or 2003-2005 (contemporary HIV), to neutralizing antibodies and sera had decreased. Moreover, length and glycosylation characteristics, as well as immunogenicity of gp120 of historical and contemporary HIV-1 variants was analyzed. As compared to historical HIV-1, contemporary HIV-1 variants were more resistant to neutralization which coincided with an increased length of the variable loops in envelope, in particular the V1 loop, and an increased density of the viral glycan shield. In addition, historical viruses elicited broader NAb responses than more recently circulating viruses.

These finding suggest that over a period of 20 years, HIV-1 has evolved towards a neutralization resistant phenotype by enhancing the masking of epitopes on its envelope. As the increased neutralization resistance of contemporary HIV-1 seems to coincide with a blunted NAb response in recent seroconverters, these findings may be relevant for the choice of envelope in vaccine design.

Key publications

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HIV-1 superinfection

Angélique van 't Wout PhD, a.b.vantwout@amc.uva.nl

HIV-1 superinfection is the infection with a second HIV-1 variant after infection with a first variant has already been established. Incidence rates of HIV-1 superinfection differ among cohorts and, as yet, only 2 cohorts of homosexual men have been screened. We investigated the incidence of HIV-1 superinfection during the first year after infection among homosexual participants in the Amsterdam Cohort Studies on HIV Infection and AIDS who seroconverted between 1985 and 1997.

We analyzed diversity of the viral envelope C2-C4 region in viral RNA in the serum of therapy-naive participants, using a heteroduplex mobility assay. Heteroduplexes were considered to be indicators of potential dual infections, in which case env C2-C4 polymerase chain reaction (PCR) products were cloned and sequenced. Sequences were subjected to phylogenetic analysis. Data on the sexual behavior of participants were collected from 1 year before seroconversion until the end of the investigated period.

For 89 seroconverters with a detectable viral load (>1000 copies/mL), env PCR products were generated from serum samples obtained at seroconversion and 1 year later. Heteroduplexes were observed in 68 of the 89 patients; among these 68 patients, a median of 9 molecular clones per time point was sequenced. Phylogenetic analysis did not reveal evidence for superinfection; 1 patient was HIV-1 coinfected. Shortly after diagnosis of HIV infection, the number of sex partners decreased, the frequency of anal intercourse declined, and condom use increased.

From this study we concluded that the incidence of HIV-1 superinfection soon after seroconversion in the Amsterdam cohort on HIV-1 infection and AIDS is low. Risk reduction shortly after HIV-1 diagnosis early during the HIV-1 epidemic in the Netherlands may have contributed to the absence of HIV-1 superinfection observed in this study.

Key publication

Rachinger A, Stolte IG, van de Ven TD, Burger JA, Prins M, Schuitemaker H, van 't Wout AB. Absence of HIV-1 superinfection 1 year after infection between 1985 and 1997 coincides with a reduction in sexual risk behavior in the seroincident Amsterdam cohort of homosexual men. Clin Infect Dis 2010; 50(9):1309-15.

Prof Hans L Zaaijer MD PhD
h.zaaijer@sanquin.nl

Blood-borne Infections

Staff

Prof HL Zaaijer MD PhD (PI)
BM Hogema PhD

Post docs

M Molenaar-de Backer PhD
FJ Prinsze PhD

PhD students

E Slot MD
B Takkenberg
M van de Klundert

Student

K Kooistra

Technical staff

M Molier

guest

P van Swieten

Secretariat

AEPT Engels

Address

Sanquin Research
Department of Blood-borne Infections
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3377
F +31 20 512 3528
E h.zaaijer@sanquin.nl
W sanquinresearch.nl
W www.bbi.sanquin.nl

Blood-borne Infections

Q-fever among blood donors

In 2007, 2008 and 2009 outbreaks of Q-fever occurred in the Netherlands with increasing magnitude, caused by the release of large amounts of *Coxiella burnetii* spores in the environment. The 2009 outbreak with 2,354 reported cases is the largest human Q-fever outbreak ever recorded. To assess the extent of infection and the safety of donated blood, we tested local blood donations for presence of *Coxiella burnetii*-antibodies and -DNA. Starting May 2009, over 40,000 serum samples were collected from all consenting blood donors in the areas with high Q-fever incidence. A PCR for detection of *C. burnetii* DNA was developed. The 1,004 samples from the areas with the highest number of reported cases were tested for presence of *C. burnetii* DNA. Seroprevalence and incidence were determined using ELISA and immunofluorescence assays (IFAs) in the subset of 543 donors, of whom a follow-up sample was available. 6/1,004 donor samples tested reactive for *C. burnetii* DNA. Confirmatory testing (IFA) on the index and follow-up samples demonstrated seroconversion in 2 donors; high-level pre-existing antibodies in 1 donor and no seroconversion in 3 donors. Thus, 3/1,004 blood donations were proven to contain *C. burnetii* DNA.

IgG testing of the 543 serum pairs showed that 66 (12.2%) were reactive in the latest sample, of which 10 represented seroconversions. The ten seroconversions result in an incidence of 5.7% per year, which is more than 10-fold higher than the local number of reported clinical cases (0.47% per year).

HEV infection and blood safety

Recently, chronic infection with hepatitis E virus (HEV) was detected in several immunosuppressed, poly-transfused patients in the Netherlands. In the past acute hepatitis E predominantly occurred in persons returning from HEV-endemic countries, and the HEV seroprevalence in the Netherlands was low. More recently, the incidence of locally acquired HEV has increased in industrialized countries, which seems to be linked to HEV-genotype 3 infection among pigs. Reports show that many pig farms in the Netherlands are infected with HEV and that viral RNA can be detected in pig meat. Several studies showed that HEV can be transmitted by blood. The increased HEV incidence raises concern about the safety of blood and blood products. A large project, aiming to determine the incidence and seroprevalence of HEV infection among blood donors, has been proposed. To facilitate the study, sensitive HEV PCR and genotyping assays are being developed.

Residual risk

The transmission of classical blood-borne infections is largely prevented by donor selection, donor screening, and the removal and inactivation of infectious agents. A small residual risk remains because in some of these infections the viremia is below the limit of detection. Two additional types of residual risk exist. Some agents, which probably are present among our blood donors, may or may not cause infection via blood transfusion (e.g.: chronic infection with *Coxiella burnetii*, vCJD and HTLV). In addition, some agents which are known to cause infection through blood transfusion, may or may not be silently imported by traveling blood donors (e.g.: West Nile Virus). Far-reaching safety measures have been implemented to decrease the three types of residual risk, although the cost-benefit ratios involved may be very high or unknown. The Dept of Blood-borne Infections has started a study on the definition and management of the residual risk of transfusion-transmitted infections.

Publications

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Cees L van der Poel MD PhD
c.l.vanderpoel@umcutrecht.nl

Transfusion Technology Assessment

Academic staff
CL van der Poel MD PhD (PI)
GA de Wit PhD (Head MTA dept.)

Post doc
MP Janssen PhD

PhD students
BA Borkent-Raven PhD
W Oei MD

Other contributors
Prof GJ Bonsel MD PhD
M Kretzschmar PhD
WP Schaasberg
Prof BA van Hout PhD

Secretariat
MC Goettsch
T +31 88 75 68165
E m.c.goettsch@umcutrecht.nl

Address
Transfusion Technology Assessment Unit
Sanquin Research & Julius Center for Health Sciences and
Primary Care of Utrecht University
Stratenum 6.1.31 room 7.117
University Medical Center Utrecht
P.O. Box 85500
NL-3508 GA Utrecht
The Netherlands
T +31 88 755 3246
E c.l.vanderpoel@umcutrecht.nl
W sanquinresearch.nl
W umcutrecht.nl

Transfusion Technology Assessment

Program description

The Transfusion Technology Assessment (TTA) group was established in 2004 as an ongoing collaboration of Sanquin with Medical Technology Assessment Department of the Julius Center for Health Sciences and Primary Care at Utrecht University with the mission to perform risk assessments and cost-utility analyses on blood safety, to establish models for clinical blood use and blood recipient profiles and to collate and analyze European data on blood use and supply. Apart from scientific publications, the TTA group provides proprietary information to Sanquin Executive Board and Sanquin Plasma Products.

Optimal Blood Safety

Blood products are very safe, but remain material from human origin, the donors of which are exposed to changing environments. Emerging infectious diseases (EID) may require new interventions. Also newly identified risks for recipients, such as the shelf life of blood products may require new approaches. The risk of negative health outcomes for recipients of blood needs to be assessed on a regular basis. Dutch government policy is to aim at 'optimal blood safety'. Cost-utility analyses can provide rational decisions for new safety interventions. Such questions are answered by mathematical modeling of data on the spread and properties of EID´s, donor epidemiology and donation behavior, infectious load of donated blood, test characteristics, processing and pathogen inactivation steps and distribution characteristics of end products to different categories of recipients in hospitals.

Optimal Blood Use

Modeling recipient outcomes requires national data on clinical blood use and blood recipient profiles, including recipient survival after transfusion. In collaboration with hospitals and the National Statistics Bureau (CBS) datasets are maintained on the use of blood products to different categories of recipients and on recipient morbidity and mortality. In addition blood use becomes clear, which hospitals can use for benchmarking. European data elaborated by TTA provide additional comparisons.

Optimal Blood supply

Aging of donors may diminish supply and aging of recipients may increase demand. Donor population characteristics and collection processes are modeled based on Sanquin datasets. From hospital data, the blood use and outdating are modeled in order to signal trends in blood use over different recipient groups. Ongoing monitoring of such trends and statistical predictions allow blood supply management to be timely informed.

Optimal Methodology

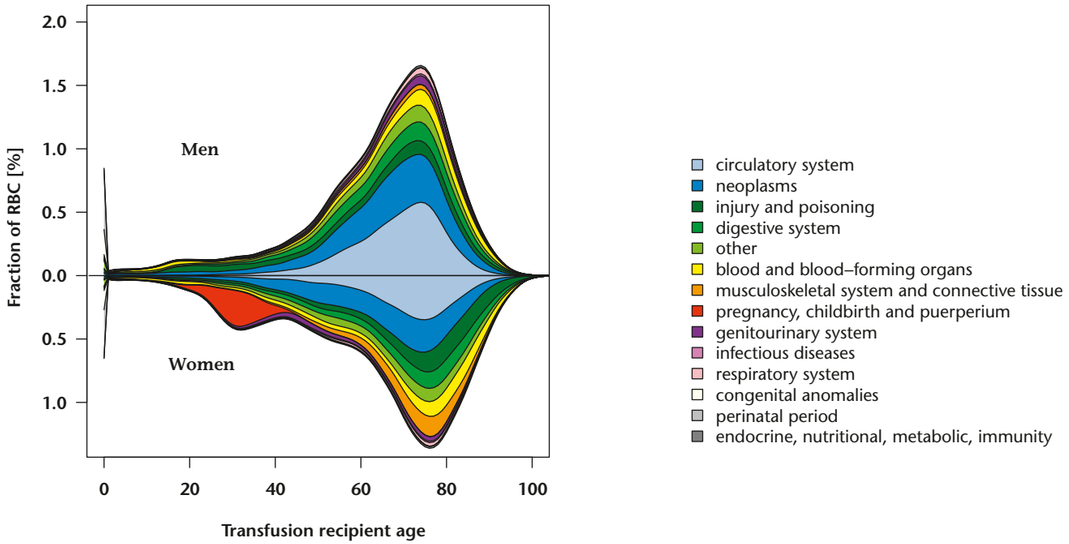
Application of existing modeling methods for transfusion chain data reveals that methodology could be further improved. Such studies are primarily initiated for problem solving within the primary TTA objectives, and if appropriate submitted for publication in statistical journals.

Profiles of Transfusion Recipients (PROTON-study)

Information on blood recipients from 1995-2006 was collected in 20 Dutch hospitals to establish the PROTON dataset, containing 2.4 million transfusions and recipients thereof. Data were linked to mortality databases (GBA) and hospital discharge diagnoses databases (LMR) at Statistics Netherlands (CBS). PROTON encompasses 28% of total Dutch blood use. Detailed analysis of clinical subgroups is envisioned for 2011 by a new post-doc position. It is envisioned that after PROTON, updated analyses are needed. In collaboration with Clinical Consultancy Services of Sanquin Blood Bank and the AIM tool developed by America´s Blood Centers, TTA offered for a program of Data ANalysis for ongoing OPtimization of the TRAnsfusion chain DANOPTRA), with the vision to provide an infrastructure for ongoing modeling studies.

Prediction of national blood demand and supply (PREDICT study)

The second evaluation of the Dutch Blood Supply Act *(ZONMW)* concluded that demographic changes could affect



The relationship between age and diagnosis of red blood cell recipients.

demand and supply. The Executive Board of Sanquin asked TTA to identify relevant parameters in the PROTON dataset. The PREDICT study reported on PROTON and Sanquin data and demographics of CBS. Based on demographic changes alone, an increase of RBC demand over 2008 to 2015 was expected. However modeling both demographics and trends in clinical RBC use predicted a decrease of RBC demand by 8%. Such predictions are envisioned to be repeated regularly with DANOPTRA datasets.

Modeling the shelf-life of blood components in relation to adverse outcomes

TTA collaborated with LUMC on observational research based on PROTON data for possible associations of storage time of blood products and the risk of TRALI. Generalized linear models suggest that extended storage of platelets in plasma may be associated with increased TRALI risk. Storage of FFP for 2 years and red blood cells for 35 days was not associated with increased TRALI risk. Further studies of recipient outcomes (mortality) in relation to shelf-life of products are envisioned.

Modeling Infections in the Transfusion Chain (MITCH-study)

The Utrecht Center for Infection Dynamics (UCID) performs mathematical modeling on infectious disease epidemiology, and is a collaboration of the Center for Infectious Disease Control (RIVM/CIb) and the Julius Center. Collaboration of TTA with UCID strengthens the knowledge base for blood safety modeling. Generic Modeling of Infections in the Transfusion Chain (MITCH) is in development, encompassing all relevant parameters for biological and epidemiological characteristics of infections in the transfusion chain. MITCH will allow ad hoc introduction of actual EID parameters, yielding timely assessment for risk management. In collaboration with UCID and Sanquin Dept of Blood-borne Infections modeling of Q-fever risk during outbreaks in the Netherlands was started. Given recent Q-fever, Chikungunya and Dengue outbreaks in Europe, the European Centers of Disease Control (ECDC) asked TTA to develop a European Up-Front blood Risk Assessment Tool (EUFRAT), a web based EID modeling tool to provide quick access by EU experts and decision makers.

Risk modeling for plasma-derived medicinal products

European legislation (EMEA guidelines) requires viral risk assessments of all plasma products, and TTA developed probabilistic risk assessment methods for this. Manufacturers of plasma products must also report to EMA on frequencies of HIV, HBV and HCV in donor populations. A monitoring tool and statistical tests were developed to check changes in the donor populations at national and regional levels. The developed tests are generic and can be applied by any blood establishment or plasma fractionation institute. TTA provided advice for further implementation at Sanquin Plasma Products, IPFA and EMEA.

Cost-effectiveness of blood safety measures in the Netherlands

Additional blood screening tests such as Triplex NAT and HTLV-I/II antibody testing were not uniformly added in Europe to the classical serological testing for HBV, HIV and HCV. These additional tests were evaluated by cost-effectiveness analyses (CEAs) in the framework of a grant from the Dutch Medical Research Council. Cost-effectiveness was analyzed of: Triplex NAT for HBV, HCV and HIV; anti-HTLV-I/II tests and HAV NAT. Disease progression of the infections was described in new Markov models. In the Netherlands, the incremental cost-effectiveness ratio (ICER) of Triplex NAT in mini pools of 6 donations is € 5,199,220 per QALY and € 4,647,062 per QALY for individual donation NAT. ICER of testing all donations for anti-HTLV-I/II is € 45,182,666 per QALY and € 2,234,041 per QALY for testing only new donors. The ICER of HAV NAT is € 18,562,483 per QALY.

Methodological studies

Survival after transfusion (SAT) is an important variable to the QALY's gained by blood safety interventions. TTA found marked differences in PROTON datasets between patient survival after the first transfusion *versus* the SAT for each individual component transfusion. Evaluating methods for estimating SAT yielded that the Kaplan-Meier method may provide correct estimates but confidence intervals are incorrect. Probabilistic risk models have outcomes with margins of uncertainty. Value of information (VOI) modeling allows systematic quantification of the expected gains of additional information by hypothetical research aimed at reduction of margins of uncertainty.

Council of Europe Reporting on the collection, testing and use of blood and blood components in Europe

Annual reporting on the collection, testing and use of blood components in Europe was assigned by the Council of Europe to the TTA group since 2001. The 2006, 2007 and 2008 reports and a Trend Analysis for 2001-2005 data were finalized after review by Member State experts. Reports are published by the Council of Europe, EDQM Department of Biological Standardization, OMCL Network and Healthcare, Strassbourg.

Key publications

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Janny de Wildt-Eggen PhD
j.dewildt@sanquin.nl

Transfusion Monitoring

Academic Staff

J de Wildt-Eggen PhD (PI)
C Bosgoed MSc
JThM de Wolf MD PhD
MJ Dijkstra-Tiekstra PhD
S Hazelaar PhD
WM Smid MD PhD MBA

Technical staff

M Kraan BSc
AC Setroikromo
J Slegers-Kühne

Guests

Prof E Vellenga MD PhD

Address

Sanquin Research
Department of Transfusion Monitoring
Hanzeplein 1
NL-9713 GZ Groningen
P.O. Box 1191
NL-9701 BD Groningen
The Netherlands
T +31 50 361 0655
F +31 50 361 9039
E j.dewildt@sanquin.nl
W tram.sanquin.nl

Transfusion Monitoring

Improvement of storage of blood products

Improving materials and methods

Margriet Dijkstra-Tiekstra PhD, m.dijkstra@sanquin.nl

The department Transfusion Monitoring (TraM) of Sanquin Research in Groningen has organized a study for the BEST (Biomedical Excellence for Safer Transfusion) Collaborative to evaluate the effect of overnight storage of whole blood or buffy coats on *in vitro* quality of platelets concentrates. Included in this study were six centers located in Europe and Northern America.

Besides this, TraM did also research projects for the stem cell department. A study is performed to optimize freezing conditions of stem cells. The influence of sample materials on the *in vitro* quality of stem cell simulated products using the current freezing program was evaluated, as well as the addition of DMSO. Additionally, pilots were done to find a way to validate HPC apheresis.

For the production department TraM studied the influence of prolonged separation of whole blood on amount of hemolysis. Hemolysis is significantly higher for red cells after prolonged separation and all hemolytic concentrates derived from units with prolonged separation.

Quality Improvement

Margriet Dijkstra-Tiekstra PhD, m.dijkstra@sanquin.nl

Every year department TraM organizes send arounds to compare the results of different instruments used within the different departments of Sanquin Blood Bank. Samples were prepared centrally at TraM and distributed to the participants. Afterwards the results were collected and analyzed. Reports with conclusions were written and sent to the different departments. In 2010 again five national send arounds were executed. (I) comparison of cell counters for counting platelets in platelet concentrates and storage solution, (II) comparison of flowcytometric instruments for counting leukocytes in plasma, red cell concentrates, platelet concentrates and platelet storage solution, (III) comparison of blood gas analyzers for measuring pH in platelet concentrates, (IV) comparison of flow cytometric instruments for measuring platelet activation in platelet concentrates using CD62p expression and Annexin V binding and (V) comparison of flow cytomteric instruments for counting platelets using CD41 expression.

Key publications

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Development of new products

Cryoseal®

Sandra Hazelaar PhD, s.hazelaar@sanquin.nl

From 2008 cryoseal, a fibrin glue of single donor plasma which Sanquin has started to produce as an alternative for autologous or pooled plasma fibrin glues, is produced in a routine setting. The storage life of cryoseal is one year and is based on stability research performed by ThermoGenesis. In 2009 TraM has started a formal stability study to officially confirm the one year storage life of cryoseal. The second goal of this study is to collect data to extend the storage life to two years. Data of storage of cryoseal for up to 12 months is already available. Another part of this study is testing of the integrity of the overwrap of the syringes containing cryoseal which will be performed at the end of the stability study (2011).

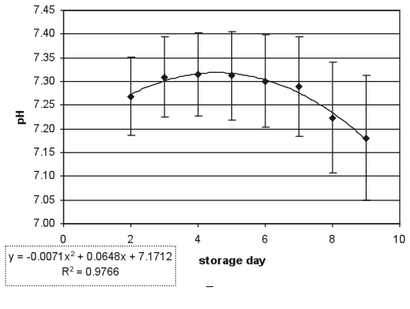
Other studies in correlation to cryoseal are:
The packaging of cryoseal: this is very important to minimize damage to the overwrap and syringes during storage and transport. A cardboard box has been developed for the cryoseal syringes in which they are firmly packed and the barcodes well scannable. The validation of these boxes has been completed and the boxes are now used as standard packaging material.
Transport of cryoseal: To guarantee the quality of the product it is absolutely mandatory that the temperature remains below -18°C during all the different steps of packaging and transportation. A start was made with the mapping of the freezing and thawing curve of the different cryoseal volumes. Also, a simulation was made of the cryoseal route after production to monitor the temperature of the product during transportation.
Homogeneity: The homogeneity of the 4 syringes of one batch was determined. No difference was found between the 4 syringes indicating that the cryoprecipitate and thrombin is mixed well before it is divided over the syringes.

A new proposal was initiated for a randomized multi-center study for the use of cryoseal in knee replacement and approved. This proposal is a collaboration between the Sanquin Blood Bank in Leiden and Groningen, with JA van Hilten as Principal Investigator (SW). Preparations were made for the start of the study in 2011.

Non-invasive pH measurement in PCs

Christa Bosgoed MSc, c.bosgoed@sanquin.nl
Margriet Dijkstra-Tiekstra PhD, m.dijkstra@sanquin.nl

TraM has performed a large field evaluation involving five blood bank departments and four participating hospitals. Blood Cell Storage, Inc. (BCSI) has developed a detection system to measure, in a non-invasive way, the pH in platelet concentrates (PCs).
During 15 months (June 2009-September 2010) all PCs were prepared in this especially designed storage bag. This storage bag enables pH measurement at any time during storage without sampling. In the study the BCSI pH1000 meter is evaluated for routine use. Also the pH of the PCs were studied to determine whether or not there is a correlation with the *in vitro* quality and/or bacterial contamination, storage time and the platelet increment after transfusion. TraM coordinated production, logistics, execution of the experiments, data collection, and overall analysis. It appeared that the BCSI pH1000 is easy to use in routine settings. A correlation has been found between storage day of the PCs and pH, but not between pH and bacterial contamination. Also no correlation was found between pH at day of transfusion and the platelet increment after transfusion. For this is need to be remarked that almost all transfused PCs appeared to have a pH between 6.9 and 7.4, while an effect can be expected for the lower pH ranges (6.4-6.8).



Mean pH profile of PCs (n=13963) during storage as measured using the BCSI pH1000.

BCSI pH 1000

Transfusion monitoring

Blood group discrepancies

Margriet Dijkstra-Tiekstra PhD, m.dijkstra@sanquin.nl

Blood groups can be determined using several techniques. Most going techniques are the so-called tube technique and column technique. Besides this blood group type sessions take place on large scale using the micro plate method and typing on the basis of DNA is also possible. From the hospitals a few cases of red cells with a mixed field blood group are reported to our blood bank. It is known that in some cases a certain blood group (mostly A) is found using the tube technique, whereas with column technique a 'mixed field' response is observed. The aim of this project is to study the frequency of these discrepancies and give a recommendation how to concern.

Clinical project: Platelet transfusions: prophylactic versus on demand

Joost de Wolf MD PhD, j.dewolf@sanquin.nl

RMH Kivit (Clinical Consultancy Department of Sanquin Blood Bank in Groningen) will finish a study with the University Medical Center Groningen and Medical Center Leeuwarden related to platelet transfusions. In leukemia for patients treated with the same HOVON protocols 2 different platelet transfusion policies will be studied: in UMCG patients are transfused prophylactic and in MCL on demand. End points of study are the number of platelet transfusions used during chemotherapy cycles and bleeding diathesis.

Clinical project: Enhancing quality of autologous stem cell products in cooperation with UMCG's department of haematology

Martin Smid MD PhD, m.smid@sanquin.nl

This project aims for enhancing the quality and quantity of stem cells in autologous transplants and was executed with financial support of Tekke Huizinga Fonds. Over the years Sanquin performs stem cell collection and processes the autologous products, executed in close cooperation with the UMCG that treats and transplants the patients.

Key publications

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Prof Anneke Brand MD PhD
a.brand@sanquin.nl

Prof Dick J van Rhenen MD PhD
d.vanrhenen@sanquin.nl

Transfusion Medicine

Academic staff

Prof A Brand MD PhD (PI)
Prof DJ van Rhenen MD PhD (PI)
Prof E Briët MD PhD
J Scharenberg PhD
H Schonewille PhD
Y van Hensbergen PhD
JA van Hilten PhD
LMG van de Watering MD PhD
JG van der Bom MD PhD
PF van der Meer PhD

PhD students

M Lenselink
RA Middelburg
BW Prick MD
LS Sitniakowsky
L Trannoy PhD
D van Stein MD
M van der Garde
E Verduin MD
S Zalpuri

Technical staff

M. Bogaerts
F El Barjiji
A Honohan
S Hoek
A Javadi
J Lorinser
B Meijer
M Slot
T van Boxsel
T van der Heide
R Wordragen-Vlaswinkel

Other contributors

YM Bilgin MD
JL Kerkhoffs MD (Clinical Consultancy Dept)
CM Khodabux MD
MM Lange MD, dept Surgery LUMC
C So-Osman MD (Clinical Consultancy Dept)
BAS Tomson MD (Clinical Consultancy Dept)
JM van Beckhoven (Cord Blood Bank)
JC Wiersum-Osselton (TRIP)

Specialist training transfusion medicine

JAE Somers
A Willemze

Secretariat

A Cabenda
J Vogel-Bakker

Contact information

Sanquin Research
Department of Transfusion Medicine
Plesmanlaan 1a
NL-2333 BZ Leiden
The Netherlands
T +31 71 568 5053
F +31 71 568 5191
E a.brand@sanquin.nl
W tm.sanquin.nl

Transfusion Medicine

The research line of transfusion medicine encompasses randomized controlled clinical trials, observational studies and translational studies.
The main mission of the Dept of Transfusion Medicine is to support clinicians with evidence-based answers to questions regarding transfusion management. For this purpose randomized controlled trials (research line 1) and observational studies (research line 2) are used. For the understanding of intended and unintended transfusion effects, translational research (research line 3) supports the clinical research.

Randomized Clinical Trials

The results of a RCT evaluating the efficacy of amatosalen pathogen-reduced platelet (PR) transfusions (HOVON 82; project leaders: DJ van Rhenen, A Brand) showed a decreased efficacy of PR-treated platelets (Kerkhoffs et al., 2010). This led to the conclusion that implementation of this method for routine platelet preparation cannot be advised. This initiated a new multicenter study evaluating PR using riboflavin (vitamin B2), the PREPAReS study (see below).

In 2010, the results of another RCT, the TRIGGER study (project leaders: A Brand, RHHG Nelissen) comparing a strict transfusion trigger with an operational hospital based trigger in orthopedic surgery showed that the Quality of Life in the immediate postoperative period was not related to the patients' hemoglobin level (So-Osman et al., 2010).

In 2010 the final follow-up data of the TOMAAT study (Optimal blood management in orthopedic surgery) were completed. The final analysis of the study is in progress.
Another study that ended in 2010 evaluated the effect of intravenous immunoglobulin (IVIG) to prevent exchange transfusions in hemolytic disease of the newborn due to Rh-antibodies. The study found no benefit of IVIG treatment.
Four RCTs were still ongoing in 2010 and two are in preparation, a summary of the RCTs and their status at the end of 2010 is as follows:
In the WOMB study (Well being of Obstetric patient on Minimal Blood transfusions, project leader: DJ van Rhenen) women with post-partum anemia are randomized to a restrictive and liberal transfusion trigger to compare post-partum fatigue and functioning.
Rationale: Approximately 3% of women after clinical delivery receive one or more blood transfusions. It is widely assumed that post-partum anemia leads to severe fatigue impairing breast-feeding and function (Prick et al. Bio Med Central Pregnancy and Childbirth 2010, 10:83). In the Womb study patients, stratified for mode of delivery, are randomized to a liberal and restrictive transfusion trigger and up to 6 weeks scored at regular intervals on validated QoL scales for fatigue and functioning. Additional support from the Landsteiner Foundation for Transfusion Research was obtained to complete the study. Inclusion of the 500 patients could be completed in early 2011.

In the multicenter, semi-blinded, randomized FIBER (Fibrin Induced Blood Exposure Reduction study; project leaders: JA van Hilten, G Tavilla) study in CABG (coronary artery bypass graft) surgery the effect of Sanquin's single donor plasma product fibrin sealant (Cryolijm) on reduction of transfusion needs and ICU stay is investigated. 1285 evaluable patients are required. By the end of 2010, 955 patients had been included in 7 centers.

Cost-effectiveness of allo-immunization by pre-emptive extended (Rh, K, Fy-a, Jk-a, M & S) antigen-matching of red blood cell (RBC) transfusions is the primary outcome question of the MATCH study (project leaders: H Schonewille, A Brand). Alloimmunization against RBC antigens occurs in 1-10% of patients after a first transfusion event but, once immunized, 20-25% develop multiple RBC antibodies after subsequent transfusions. Identifying these patients before the first transfusion event is pivotal to define the target group for extended preventive matching. In the MATCH study, naive patients and patients who previously formed an antibody are stratified and randomized to either standard or extended matched RBC units. Of 1120 required patients, by the end of 2010, 415 patients have been included.

The PREPAReS (Pathogen Reduction Evaluation & Predictive Analytical Rating Score study, project leaders: JA van Hilten, A Brand) is a non-inferiority, single blinded study to compare the clinical efficacy of the Mirasol (riboflavin/vitamin B2)-pathogen reduced platelet concentrates with standard platelet concentrates. The study started accrual end of November 2010 in the HAGA hospital (the Hague). Two other Dutch centers and centers in Norway and Canada intend contribution to the required intake of 618 patients.

The FIRST (Fibrin Sealant in Total Knee Replacement surgery trial (project leaders: JA van Hilten, RGHH Nelissen) is a multi-center, semi-blinded, study to investigate whether application of (single donor) Fibrin Sealant after total knee replacement surgery reduces post-surgical bleeding, swelling and pain. By the The Harris' knee score as primary outcome postoperative knee function, estimated in flexion and extension at 2 and 6 weeks after surgery is evaluated. The study requires an intake of 500 evaluable patients. In the first week of 2011 inclusion started.

The RBC-storage time study on clinical outcome in high risk intensive care patients (ABLE-NL, project leaders: LMG van de Watering, P Henny) is embedded in an international study (Canadian ABLE study). The presumed deleterious effect of stored RBC on clinical outcome, was mainly evaluated in observational studies and a few pilot-RCTs. The correct interpretation of observational studies is crucial and we identified some most important pitfalls in this studies and identified conflicting outcomes. The national ABLE arm extends to medium risk ICU-patients.

Key publications

Kerkhoffs JL, van Putten WL, Novotny VM, te Boekhorst PA, Schipperus MR, Zwaginga JJ, van Pampus LC, de Greef GE, Luten M, Huijgens PC, Brand A, van Rhenen DJ. Clinical effectiveness of leucoreduced, pooled donor platelet concentrates, stored in plasma or additive solution with and without pathogen reduction. Br J Haematol 2010; 150(2):209-17.

So-Osman C, Nelissen R, Brand R, Brand A, Stiggelbout AM. Postoperative anemia after joint replacement surgery is not related to quality of life during the first two weeks postoperatively. Transfusion 2011; 51(1):71-81.

Middelburg RA, van de Watering LM, van der Bom JG. Blood transfusions: good or bad? Confounding by indication, an underestimated problem in clinical transfusion research. Transfusion 2010; 50(6):1181-3.

Observational studies

TRALI

Transfusion Related Acute Lung Injury (TRALI) is the leading cause of transfusion related mortality. A study aiming to quantify the contribution of female and allo-exposed blood donors, both as markers for leukocyte antibodies, to the occurrence of TRALI (project leaders: JG van der Bom, E Briët) started in 2006 in cooperation with the Dept of Clinical Epidemiology at the Leiden University Medical Center.
The key finding in this project is the observation that allo-exposed donors of plasma rich products (FFP or the plasma unit of donor platelets) confer an increased risk of TRALI, while allo-exposed donors of plasma poor products (in RBC or platelets) do not. The overall percentage of TRALI cases that could have been prevented by the deferral of all allo-exposed donors (irrespective of product type) was 51% (95% CI: 14% - 88%) (Middelburg et al., 2011). Further results from the project include determination of leukocyte antibody prevalences in subgroups of allo-exposed and non-allo-exposed donors.
Overall the conclusion from the project is that the male-only plasma measure prevents a substantial part of all TRALI, but plasma poor products still cause TRALI in leukocyte antibody independent ways.
Donor leukocyte reactive antibodies were shown to activate cognate recipient neutrophils, which cause vascular injury by the release of oxygen radicals. Donor leukocyte reactive antibodies (against HNA, HLA class I+II) are mainly found in (multiparous) female donors. As a precautionary measure only plasma from non-transfused male donors is used for single donor Fresh Frozen Plasma in the Netherlands since July 2007 in order to eliminate antibodies able to activate cognate recipient neutrophils, which cause vascular injury by the release of oxygen radicals. Such donor leukocyte reactive antibodies are mainly found in (multiparous) female donors.
In the TRALI syndrome study (project leaders: DJ van Rhenen, EAM Beckers) from January 2005 till January 2009 all TRALI cases reported to Sanquin Blood Bank were investigated for patient, donor and product characteristics.
Despite the precautionary measure of male-only plasma, the frequency of TRALI reports did not change; possibly because of the higher awareness of TRALI. In addition, non-immune causes might be more important, which are obviously not influenced by male-only plasma polices. In our series, despite extensive and deliberate testing for incompatible (HNA, HLA class I and II) donor antibodies, the majority (40/75) consisted of non-alloimmune TRALI cases (van Stein et al. 2010).

Red blood cell antigen immunization

Two studies unravel the susceptibility for red blood cell (RBC) antigen immunization.

LOTUS study

The first is the LOTUS study (project leaders: H Schonewille, A Brand in collaboration with the LOTUS consortium) evaluating the incidence and long-term persistence of fetal versus (intra-uterine) transfusion induced antigen exposure and its relationship with HLA-antigens, HLA-antibodies and fetal chimerism in a large cohort of women treated for hemolytic disease of the newborn between 1987-2009. Within the Lotus consortium the effect of severe fetal anemia on long-term neurodevelopment impairment (NDI) is evaluated (project leader: E Lopriore). By the end of 2010, 281 mother-child pairs were included and being evaluated.

R-fact study

The second study is a multi-center case cohort study, the R-fact (Risk factors for alloimmunization after erythrocyte transfusion; project leaders: JG van der Bom, JJ Zwaginga) which aims to examine the association between clinical, environmental and genetic characteristics of recipients of erythrocyte transfusions and the risk of immunization against RBC allo-antigens. In 2010 a pilot study with 42 participants showed the feasibility of the medical and logistical aspects, including the use of a participant questionnaire and a voluntary blood sample collection. Besides the Leiden Medical University Center, seven additional large red blood cell product using hospitals are preparing for cooperation.

Umbilical cords

The last clinical cohort study investigates whether transplantation of two umbilical cords (UCB) improves hematopoietic engraftment and outcome in adults with high risk leukemia or bone-marrow disease (project leaders: JJ Cornelissen, A Brand). In 2009 this became a HOVON (no 106) study (principle investigator: JJ Cornelissen). By the end of 2010, 24 of the 40 anticipated patients had been included.

Key publications

Middelburg RA, van Stein D, Atsma F, Wiersum-Osselton JC, Porcelijn L, Beckers EAM, Briët E, van der Bom JG. Allo-exposed blood donors and transfusion-related acute lung injury; A case-referent study. Transfusion 2011; Accepted for publication.

Van Stein D, Beckers EAM, Sintnicolaas K, Porcelijn L, Danovic F, Wollersheim JA, Brand A, van Rhenen DJ. Transfusion-Related Acute Lung Injury (TRALI) reports in the Netherlands: an observational study. Transfusion 2010; 50:213-20.

Translational Research

Unraveling increased mortality by leukocyte-containing transfusions during cardiac surgery (project leaders: LMG van de Watering, A Brand) by cytokine induction was studied in 346 cardiac valve surgery patients, who had participated in an RCT comparing buffy coat versus filtered erythrocyte transfusions. Pre- and post-surgery cytokine levels were determined. Multivariate analysis showed higher IL-6 concentrations associated with multiple organ dysfunction syndrome and both higher IL-6 and IL-10 concentrations associated with hospital mortality in the group that had received multiple non-leukocyte-depleted RBC compared to the group that received LD-RBC. These findings suggest that leukocyte-containing RBC interfere with the postoperative cytokine pro- and anti-inflammatory cytokine cascade, which may enhance the development of complications after cardiac surgery. Further analysis on the role of leukocytes in blood transfusions was evaluated in transplant patients (project leaders: FHJ Claas, A Brand) and showed by functional and microarray decreased immune effector mechanisms and regulatory effects by HLA-DR shared transfusions, whereas in presensitized patients prolonged memory T cell reactivity was observed (Eikmans et al., 2010).

Transfusion related immunomodulation

A study further evaluating transfusion related immunomodulation by gene array (GE) expression evaluation (project leaders JA van Hilten) showes in a wide range of transfusion recipients (cardiac surgery, orthopedic surgery, anemia of prematurity) only minor effect of leukocyte-depleted RBC transfusion on cytokine gene expression profiles. However, increased GE of TGF-α, TNFSF10 and -13B transcripts were observed after cardiopulmonary bypass surgery. A relationship with post-operative complications is further explored.

Platelet transfusions

The possibility to predict the need for platelet transfusions in acute myeloid leukemia patients (*Ex vivo* Determination of Bleeding risk; project leaders: R Middelburg, JJ Zwaginga) is of great importance to establish appropriate indications for platelet transfusions for these patients. Within this project a flowcytometry based assay for the assessment of platelet function *ex vivo* in whole blood, has been validated in 2010. Correlation with clinical bleeding (within the PREPAReS study) starts in 2011.

Predictive value of platelet product characteristics and clinical efficacy regarding bleeding and transfusion increment of platelet transfusions (project leader: PF van de Meer) will correlate a composite score of stored and/or pathogen-treated platelet products within the randomized PREPAReS study. In 2010 the technique of platelet product scoring was validated.

Preventing surface sialic acid loss of the platelet GPIbareceptor increases platelet lifespan *in vivo* and after transfusion (pilot study) (project leader: G Jansen) aims to investigate the relationship between sialic acid loss and loss of the vWFR complex in human platelets and platelet life span. Preliminary results showed a relationship between loss of GPIbaafter activation with neuraminidase in human platelets. This, however, has to be investigated further.
The current study is divided into three sections. First part is focused on validation of the tests. Secondly we would like to investigate the relationship between platelet storage and loss of sialic acid and loss of platelet receptors. The third part is to investigate platelet characteristics of patients with thrombocytopenia with special interest in sialic acid.

RBC alloantibodies

The mechanism of HLA restriction in RBC allo-antibody formation (project leaders: H Schonewille, IIL Doxiadis) is further explored. An (IgG) antibody response generally requires initial activation of antigen-specific T helper (CD4) cells via the indirect (by self-HLA class II) pathway through effective presentation by antigen-presenting cells. By the end of 2010, more than 1000 patients with various (multiple) antibody specificities were HLA class-II typed. This study will reveal whether the role of HLA antigens in RBC immunization is of such extent that they have to be taken into account for preventive matching strategies.

Double cord transplantation

Understanding single cord blood dominance after double cord transplantation (project leader: Y van Hensbergen, A Brand). After double cord transplantation in most instances one of the two cords dominates. We validated a method using monoclonal HLA-single allele specific antibodies to follow the development of cells in the early post-transplantation period with the aim of early detection of (the mechanism of) a winning UCB.

Key publications

Bilgin YM, van de Watering LM, Versteegh MI, van Oers MH, Brand A. Effects of allogeneic leukocytes in blood transfusions during cardiac surgery on inflammatory mediators and postoperative complications. Crit Care Med 2010; 38(2):546-52.

Eikmans M, Waanders MM, Roelen DL, van Miert P, Anholts JDH, de Fijter H, Brand A, Claas FHJ. Differential effect of pretransplant blood transfusions on immune effector and regulatory compartments in HLA-sensitized and non-sensitized recipients. Transplantation 2010; 90:1192-99.

Wim LAM de Kort MD PhD
w.dekort@sanquin.nl

Donor Studies

Academic staff
WLAM de Kort MD PhD (PI)
F Atsma PhD
P van Noord MD PhD
IJT Veldhuizen PhD

PhD students
AM Baart
K Peffer
A van Dongen-Turman
A Wevers

Research assistents
K Habets
K van den Toren
E Wagenmans

Students
MC Janssen

Other Contributors
E Brummelkamp (Radboud University, Nijmegen)
P Pasker-de Jong PhD (Radboud University, Nijmegen)
E Rombout-Sestrienkova
J Wollersheim MD

Secretariat
J Hendriks-van Heijst

Address
Sanquin Research
Department of Donor Studies
Geert Grooteplein Zuid 34
NL-6525 GA Nijmegen
P.O. Box 1013
NL-6501 BA Nijmegen
The Netherlands
T +31 24 327 9034
F +31 24 327 9471
E w.dekort@sanquin.nl
W ds.sanquin.nl

Donor Studies

The research of the Dept of Donor Studies runs along two related research lines: 1) Donor Recruitment and Retention, and 2) Donor characteristics and health issues regarding blood donations, including donor deferrals.

Donor Recruitment and Retention

Behavioral studies

Ingrid Veldhuizen PhD, i.veldhuizen@sanquin.nl

Psychosocial and behavioral aspects of blood donation are the key issues within this research line.
The determinants of donation behavior are important for the donor career. Gaining insight into voluntary withdrawal among new blood donors is important for developing a long-term donor career. Behavioral determinants were measured amongst 5000 new donors at three different points in their donor career. This longitudinal study provides a thorough understanding of which factors at what point in time influence a decrease in motivation leading to early voluntary withdrawal. These insights allow for tailored interventions to improve donor retention. The first results indicate that with regard to the first blood donation, experiencing a physical reaction in itself does not decrease the donation motivation. Instead, the subjective severity of the reaction can negatively influence the motivation to donate.
In 2009 a PhD project started on show/no-show behavior of donors after receiving an invitation to donate. In 2010 information was gathered about no-show behavior of donors in different ways, namely: exploring existing literature for a review article, analyses of cohort data among blood donors (Donor Insight research), and analyses of blood bank data to calculate show-rates in different ways. Furthermore, in cooperation with the donor administration, a study was designed to clarify how many donors cancel their invitation for a donation and for what reason. Additionally, 90 donors were interviewed about their satisfaction with the invitation system, reasons of previous no-show behavior and negative donation experiences. In preparation of the second year a brainstorm session was held about possible theories for the questionnaire that will be send to a random sample of donors. This questionnaire has been sent out and we are awaiting responses.

DOnor MAnagement IN Europe, DOMAINE

DOMAINE (Donor Management IN Europe) is a European Union co-funded project, in which blood establishments from 18 European member states and one patient-driven organization join their forces on donor management. DOMAINE aims to compare and recommend good donor management practice. It focuses on various aspects of donor management (including cultural differences): donor recruitment strategies, donor retention strategies, deferral procedures and blood bank policy regarding patients requiring long-term transfusion. In the first phase – performed in 2008/2009 – of the project, a survey was conducted to analyze donor management practice in Europe. In total, 48 questionnaires have been sent to 37 European countries, with a response rate of 88%. The (confidential) survey report has been finalized in May 2009. The report has served as a template for the manual on good donor management, which has been presented in a specially organized session at the XXXIst ISBT Berlin meeting in June 2010. The third and final phase will concentrate on setting up a training program for blood donor management professionals on the manual.

Key publications

De Kort W, Wagenmans E, van Dongen A, Slotboom Y, Hofstede G, Veldhuizen I. Blood product collection and supply: A matter of money? Vox Sanguinis 2010; 98:e201-8.

Donor characteristics and donor deferrals

Femke Atsma PhD, f.atsma@sanquin.nl
Paul van Noord MD PhD, p.vannoord@sanquin.nl

Donor characteristics and health effects related to blood donation are the main issues within this research line. Furthermore, there is a strong focus on donor deferral.

Donor characteristics and health effects of blood donation
Donor InSight is a large study, which started in 2007. The primary aim of Donor InSight is to gain insight into donor characteristics. In total, 50,000 whole blood and plasma donors were invited to fill in an extensive questionnaire on many topics, like demography, lifestyle, health and disease, donor motivation and donor satisfaction. Mid 2009 data collection was completed; 50,000 donors received the questionnaire of which 63% returned the questionnaire. Furthermore, the research in this field focuses on health and disease within donors. The donor population is said to reflect a relatively healthy subset of individuals. In order to gain insight into the health status of donors, a description of the donor pool in terms of demographic characteristics and cardiovascular risk factors has been made, using Donor InSight data and data from the general population as reference (Atsma et al.). Additionally, a PhD project on donation frequency and cardiovascular disease has started at the end of 2009. First, the occurrence of cardiovascular disease will be explored by linking donation data with morbidity and mortality data from Statistics Netherlands. Second, the effect of donation frequency on cardiovascular disease will be investigated by comparing number of donations between medically deferred donors and non-medically deferred donors. Furthermore, the effect of number of donations on metabolic factors, such as

blood pressure, lipid levels, and insulin sensitivity, will be investigated within active and newly registered donors.

Donor deferral

Low hemoglobin level (Hb) is an important reason for donor deferral. In the Netherlands, in total, about 10% of the donors visiting a collection center, is deferred. Within the deferred group, from 2-3 % of male donors up to 5-7 % of female donors are being deferred for low Hb. Since deferral is a proven reason of donor lapse, reducing this percentage is paramount. Hb is known to be related to several factors, including: gender, physical condition, iron status, Body Mass Index, nutrition, but also environmental conditions, such as environmental temperature and donation history. To disentangle these complex relations we recently started an extensive statistical modeling study on prognostic factors of Hb. In 2010 the first modeling results have been published (Baart et al.). In a pilot study among donors, substantial iron depletion – measured as Zinc Protoporphyrin (ZPP) levels – was observed. ZPP is an anticipated predictor of iron depleted Hb production.

Key publications

Baart M, de Kort WL, Moons KGM, Vergouwe Y. Prediction of low hemoglobin levels in whole blood donors. Vox Sanquinis 2011; 100:204-11.

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Product and Services Departments

For more information: www.sanquin.nl/productandservicesdepartments

Product Development

Sanquin Plasma Products

Anky Koenderman PhD, a.koenderman@sanquin.nl

The product development strategy of Sanquin Plasma Products aims primarily at maintaining the state-of-the-art level of its plasma derivatives portfolio and production processes. To that end, the product and process development program is regularly evaluated and updated if needed. Besides, opportunities for development of new (plasma) products are being explored in feasibility studies which may evolve into full-blown development projects when considered to be economically feasible.

Product Development Division

CAF-DCF, Brussels, Belgium

Ruth Laub PhD, info@caf-dcf-redcross.be

The CAF-DCF Product Development Division (former R&D) is located in Brussels. For its staff, research and development means ensuring both the efficacy of plasma-derived medicinal products and their biological safety as regards pathogens, pollutants, and accompanying proteins. Focusing on therapeutic proteins (IVIG, albumin, AGP, FVIII) and their excipients in plasma and concentrates, the division develops both immunological methods and biochemical-biophysical techniques and exploits them in industrial applications. The paradigm of plasma derivative safety is approached through projects in different areas: NAT screening, collection and statistical evaluation of critical virus epidemiological data, neutralization by specific antibodies, virus infectivity testing in a cell model, virus inactivation/virus elimination validation studies, and pathogen reduction techniques (including UVC irradiation developed in our R&D Division).

Medical Department

Sanquin Plasma Products

Paul FW Strengers MD, p.strengers@sanquin.nl

The Medical Department is, in its applied research activities, responsible for the design and conduct of clinical trials with (recently developed) plasma products. The Medical Department closely cooperates with clinical investigators in the Netherlands e.g. the Netherlands Inter-University Working Party on the Study of Immune Deficiencies and the Dutch Haemophilia Treatment Centers, and with investigators abroad. In 2010, three clinical studies with intravenous immunoglobulin, Nanogam®, were ongoing in order to study the efficacy and safety of Nanogam® in different clinical conditions. Moreover, a clinical study with apotransferrin was initiated, and preparations were made for a clinical study with a newly developed FVIII product.

Sanquin Reagents

Roel Melsert MSc, r.melsert@sanquin.nl

Sanquin Reagents developed a broad range of blood grouping and immunology reagents for laboratories, including several innovative products for diagnostic use and for clinical research. These reagents are available worldwide through a network of distributors, and bulk reagents for manufacturing are supplied directly from Amsterdam. The Reagents Unit is ISO 9001 and ISO 13485 certified and is committed to introduce new products on a continuous basis. New products are the outcome of R&D projects, some of which are executed in close collaboration with departments within Sanquin and/or with other companies and institutions.

Sanquin Pharmaceutical Services

Peter C van Mourik, p.vanmourik@sanquin.nl

Sanquin Pharmaceutical Services (SPS) is a business unit specialized in a broad array of pharmaceutical services aiming at the development and quality testing of biologicals intended for therapeutical application in humans. These services include the development of adequate production processes, contract production of mammalian cell products (monoclonal antibodies and/or r-DNA) as well as safety testing and designing validation studies for assays and processes, including viral reduction studies.

Sanquin Diagnostic Services

Ruben Baumgarten MD PhD, r.baumgarten@sanquin.nl

Sanquin Diagnostic Services excels in routine and top-reference specialized testing in the field of blood-related diseases and immune-mediated disorders. The blood sample testing is carried out in Amsterdam and is available to all Health Care Institutions and commercial companies in the Netherlands and abroad. The division aims to work according to the highest quality standards in order to function as a diagnostic reference center in the fields mentioned above, in national as well as in international settings. With its fully certified laboratories, Sanquin Diagnostic Services can provide a vast array of both routine and tailor-made diagnostic tests. Sanquin Diagnostic Services is committed to continuous innovation reflected by introduction of new diagnostic tests. New tests are often developed and validated in house, in R&D projects, most of which are carried out in close cooperation with Sanquin Research.

Sanquin Consulting Services

W Martin Smid MD PhD MBA, m.smid@sanquin.nl

The mission of Sanquin Consultancy Services (SCS) is to provide guidance and advice services to restricted economy countries.

Objectives are

1. support restricted economy countries in developing safe, efficacious and sustainable blood supply systems based on current quality principles,
2. provide modular training programmes on transfusion medicine for restricted economy countries focused on the managerial and quality aspects of the transfusion chain, and
3. extend and strengthen the training and consultative potential within the Sanquin organization.

Patent portfolio and valorization

Theo Ariëns, t.ariens@sanquin.nl

In addition to our efforts to publish our research work in scientific publications, Sanquin also disseminates knowledge in the form of patents and other forms of know-how. In 2010 Sanquin focused its IP efforts on the enhancement of clinical efficacy of biologicals both in the area of blood coagulation factors, IVIG and C1-esterase inhibitor; monoclonal antibodies and cellular vaccines.

Most often in-licensing parties both seek the opportunity of patent protection for future product pipelines and the expertise of our inventors and their research groups. Therefore a license contract in general incorporates a joint research agreement enabling Sanquin to generate funding for its research and enabling third parties to evaluate the patent proposition.

An overview of the valorization status of Sanquin Research patents and hybridoma's is shown. Most patents/hybridoma's listed have a primary therapeutic application.

Patents/Patent Applications	Status 2010
Enhancement efficacy blood coagulation factors a.o.	
'FVIII mutants'	3rd party out licensed
'FVII-LRP antagonists'	Licensing discussions ongoing
'Anti-FVIII antibodies' (improving half life)	Open for licensing
'C1-est inhibitor in AMI'	3rd party out licensed
'IVIG' (improving therapeutic efficacy)	Open for licensing
Enhancement efficacy monoclonal biologicals	
'Anti Antibody Stability' (improving ADCC efficacy)	Open for licensing
'SIRPalfa interferentie' (improving CD20 efficacy)	
'Anti-antibodies' (improving efficacy anti-TNFs)	Discussions ongoing
'Monitoring of Immunoglobulin receptor (-like) genes'	Open for licensing
Enhancement Cellular Therapies & Vaccine Development	
'HOBIT-transcription factor for killer cell activation'	Open for licensing
'MHC Multimers'	Open for licensing

Sanquin hybridoma's	Status 2010
RAG_35_201	Open for licensing
CD 97	Open for licensing
CD3 human IgM, IgG1, IgG2, IgG3, IgG4, IgA, IgE	Open for licensing
Anti c-1q / Anti c3-2 / 2C8	Open for licensing
4-7B	3rd party licensed semi-exclusively
CD70	3rd party licensed
IL 6,_8,_12,_14,_16	partly out-licensed semi-exclusively

Funding and sponsors

Various organizations, charities and industries have contributed towards research of Sanquin by funding investigators, travel expenses, equipment or offering free materials:

Landsteiner Laboratory

Sanquin Research and the Academic Medical Center of the University of Amsterdam collaborate in the joint Sanquin – AMC Landsteiner Laboratory for Blood Transfusion Research, housed in Sanquin's premises in Amsterdam.

Jon J van Rood Center for Clinical Transfusion Research

Sanquin Research and the Leiden University Medical Center collaborate in the joint Sanquin – LUMC Jon J van Rood Center for Clinical Transfusion Research

Dutch & European Research Council

Dutch Medical Research Council (ZON/MW)
Earth and Life Sciences NWO
European Commission
Netherlands Genomics Initiative (NROG)
Netherlands Organization for Scientific Research (NWO)
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Charities, private funding organizations

Dutch Cancer Foundation (KWF)
Netherlands Heart Foundation
Dutch Blood Transfusion Society
Dutch Society of Thrombosis and Hemostasis
Pediatric Cancer Research Foundation
Friends of Research on MS
Gratama Foundation
Joghem van Loghem Foundation
Landsteiner Foundation for Blood Research (LSBR)
Leiden University Fund
Ministry of Public Health, Welfare and Sport
Tekke Huizinga Foundation

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Future Diagnostics
Genentech
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Philips
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Région de Bruxelles-Capitale
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Roche Pharmaceuticals
Radboud University Nijmegen
Schering Corporation
Staten Serum Institute
Siemens A.G.
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VU University Medical Center, Amsterdam
Wageningen University and Research Center
Wyeth-Pfizer

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Other sources of funding

Ministry of Economic Affairs (WBSO)

Publications

Papers in peer reviewed journals

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Academic Staff Index

Colophon

Further information may be obtained from:

Corporate Staff Office

Sanquin Blood Supply
P.O. Box 9892
NL-1006 Amsterdam
The Netherlands
T +31 20 512 3000
F +31 20 512 3303
E research@sanquin.nl
W sanquinresearch.nl

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Editors

René van Lier
Anneke de Regt
Jan Willem Smeenk
Florine van Milligen

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