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Sanquin Scientific Report 2007

Sanquin

07

Scientific Report

Blood and Beyond

Scientific Report 2007

Contents

Words of welcome

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In our 2007 scientific report you will find information on our organization and our research policies. We describe the major research lines and give you some key publications for further reference.

In 2007 we introduced the system of Principal Investigator (PI), where senior scientists are responsible for their own program as well as for personnel and funding. To get PI-status, the experienced scientist must have his own program with a series of high profile publications, he has demonstrated to be successful as a mentor for PhD students and he should have acquired external funding for his group during a number of years. In this report all Sanquin PI's are listed with their staff, students and contact information.

In 2007 the research programs at our Blood Bank research departments were further strengthened. The department of Research and Development of the South East Region is concentrating on donor related studies, with expertise from epidemiology and the social sciences, in order to have our donor policies scientifically based. The South West Region is concentrating on clinical studies in close collaboration with academic hospitals, especially Leiden University Medical Center, as well as the larger general hospitals in the Netherlands. The North West Region is dedicated towards transfusion technology in close collaboration with the Sanquin Research division. In the North East Region we are now focusing on validation of new technologies.

We are pleased to be able to continue the long standing collaboration with the Academic Medical Center of the University of Amsterdam in the joint Sanquin – AMC Landsteiner Laboratory for Transfusion Medicine. A new long-term agreement was signed in December, expanding the joint research program with epidemiology of blood transmitted infections, and hemostasis and thrombosis, while continuing research in the fields of immunology and hematology as well as strengthening translational research.

Our collaboration with Utrecht University on coagulation and transfusion technology assessment was continued, as was the collaboration with Leiden University Medical Center with respect to clinical research

In 2007 two eminent Sanquin researchers retired: dr Ruby Pietersz and dr Jan van Mourik. On both occasions a farewell symposium was organized. Both were attended very well. The lead of dr Pietersz' research group is taken over by dr Dirk de Korte, while dr Van Mourik's research group is continuing under the leadership of dr Bas de Laat. On the occasion of her retirement dr Pietersz received a knighthood: "officier in de orde van Oranje Nassau" for her contribution to transfusion medicine in – especially – developing countries.

Dr Ellen van der Schoot was appointed professor of Experimental immunohematology at the University of Amsterdam and held her inaugural lecture in November 2007.

Prof Hanneke Schuitemaker left Sanquin at the end of 2007 for the Academic Medical Center, where her research group will continue to contribute to the research program of the joint Landsteiner Laboratory. Prof Schuitemaker remains part time associated with Sanquin as an advisor on issues of viral safety of the blood supply.

In April 2007 the second Sanquin Spring Seminar was held under the title 'Antibodies in disease, diagnosis, and treatment'. The conference was very well attended and preparations for the third Sanquin Spring Seminars in 2009 are in full swing. The theme of the 2009 conference is Cellular therapies: insights and new horizons.

Kind regards,
Sanquin Executive Board,
Prof Ernest Briët, MD, PhD
Director of Sanquin Research.

Hoogtepunten: onderzoek Sanquin in vogelvlucht

Binnen Sanquin werken bijna 250 wetenschappers en ontwikkelaars. Gezamenlijk bestrijken zij een breed scala van onderzoek op het gebied van bloed en bloedproducten. Ter introductie van dit wetenschappelijk jaarverslag zetten wij enkele hoogtepunten uit 2007 op een rij.

De veiligheid van bloedproducten is één van de belangrijke aspecten van het onderzoek bij Sanquin. Vandaar dat verbetering van de diagnostiek van bloedoverdraagbare infecties hoog op de agenda staat. Een mooi voorbeeld is het snel opsporen van bacteriële besmettingen van bloedproducten. “Dankzij de vele maatregelen die vanaf de jaren negentig zijn genomen is de virusveiligheid van onze producten onder controle”, aldus Dirk de Korte, manager Onderzoek & Ontwikkeling bij Sanquin Bloedbank Regio Noordwest. “Sindsdien focussen we ook sterk op de bacteriële veiligheid. Vooral bloedplaatjes vormen wat dit betreft een bottleneck, omdat plaatjes bij kamertemperatuur (22°C) bewaard moeten worden. Bij lagere temperaturen gaan ze als het ware stuk en verliezen ze hun biologische activiteit.”
Sinds 2001 gebruikt Sanquin kweektests voor de detectie van bacteriën in alle bloedplaatjesconcentraten. Nadeel daarvan is dat het enkele dagen duurt voordat de uitslag bekend is. Aangezien bloedplaatjes slechts kort houdbaar zijn, worden ze wel al direct uitgegeven aan ziekenhuizen. De Korte: “Plaatjesproducten worden als veilig beschouwd totdat het tegendeel bewezen is. Heel soms moet je dan producten terugroepen, en dat willen we gaan voorkomen met snellere screening. Daartoe hebben we in het afgelopen jaar een real-time PCR-methode geoptimaliseerd. Het gaat om een zogeheten 16S rDNA-test, waarmee we een ribosomaal DNA-fragment kunnen aantonen dat heel specifiek is voor bacteriën. Daardoor kunnen we nu binnen vier uur de meest voorkomende bacteriën in plasma en plaatjes opsporen, zoals Bacillus-, Staphylococcus- en Proprioni-stammen. Aan de PCR-test hebben we zelf een extra controle toegevoegd: een stukje DNA van een zogeheten lambda-faag. Daardoor is de assay nu in principe bruikbaar voor diagnostiek, al zullen nog de nodige hordes genomen moeten worden voordat we met deze test onze plaatjesvoorraad routinematig kunnen screenen.”

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Donorstudies

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Vanzelfsprekend vond afgelopen jaar ook onderzoek plaats rond de kurk waar Sanquin op drijft: de donors die vrijwillig bloed geven. Zo deed onderzoeker Pieter van der Meer contractresearch naar een naald die tijdens een donatie de afgifte van bloed versnelt. “In deze nieuwe high-troughput naald is een kleine vernauwing weggehaald tussen de naald en de afnameslang naar de bloedzak. Daardoor stroomt het bloed bijna 20 procent sneller door de nauwe doorgang”, legt Van der Meer uit. “Dat klinkt misschien heel eenvoudig, en dat is het ook wel. Maar het effect is opvallend. Wij hebben de naalden grondig getest in de praktijk en ze bleken bijzonder goed te werken: de gemiddelde afnameduur ging van acht naar zeven minuten per donatie. Heel prettig voor de donor natuurlijk. Bovendien halveerde het aantal donaties dat langer dan twaalf minuten duurde. Belangrijk, want de bloedplaatjes uit het bloed van zo’n donatie kunnen we niet gebruiken – er bestaat een risico dat ze geactiveerd zijn. En dan hebben we het over een paar duizend donaties per jaar, waarvan we de plaatjes voortaan tóch kunnen verwerken tot bloedproducten.”

Vanuit Nijmegen coördineert Bloedbank Regio Zuidoost de landelijke en Europese Sanquin-studies over donors. “Nederland telt zo’n 430.000 actieve donors, en dat bestand willen we op peil houden om te voorkomen dat er op termijn een tekort ontstaat. In sommige Europese landen is dat al het geval”, aldus sociaal wetenschapper Ingrid Veldhuizen, die samen met de Nijmeegse divisiedirecteur Wim de Kort verschillende donorstudies begeleidt. “Hoe raar het ook lijkt, Sanquin kent zijn donors eigenlijk nog niet goed genoeg”, zeggen Veldhuizen en De Kort eensgezind. “Wat zijn hun karakteristieken? Waarom geven ze bloed, en waarom stoppen sommige mensen daar op een gegeven moment toch weer mee? Via de zogeheten Donor InSight-studie hopen we daarover meer duidelijkheid te krijgen. Daartoe hebben we tot nu toe bijna 28.000 donors vragenlijsten toegestuurd. In de loop van dit jaar verwachten we de eerste resultaten van die enquête te presenteren. Een belangrijk onderwerp blijft donorretentie: hoe houd je mensen gemotiveerd om donor te blijven? Jaarlijks valt vijf à tien procent van de donors uit ons bestand. Deels door natuurlijk verloop, want je mag maar tot je 70ste bloed geven. Maar deels doordat mensen gewoon niet meer komen. Uit onderzoek blijkt dat maar liefst de

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helft van die groep in principe nog wel donor wil zijn. Daarom willen we via speciale interventieprogramma's onze donors nóg beter gaan informeren en hopen we ze langer aan Sanquin te kunnen binden. Verder hebben we afgelopen jaren onze brochures onder de loep genomen en aangepast, zodat ze motiverender werken. Een heel andere strategie is het werven van nieuwe donors via bestaande donors. Ook dat lijkt inmiddels de eerste vruchten af te werpen."

Verbeterd transfusiebeleid

Meer in de kinderschoenen staat een onderzoeksproject dat moet leiden tot verbeterde toepassing van bloedtransfusies. Samen met vier ziekenhuizen participeert Sanquin sinds 2004 in de langlopende TOMaat-studie (acroniem voor 'Transfusie Op Maat'), een gezamenlijk initiatief van het LUMC in Leiden en Sanquin Bloedbank Regio Zuidwest. Eind 2007 waren in totaal bijna 1850 patiënten in deze studie geïncludeerd en maakte een tussentijdse analyse duidelijk dat het onderzoek op dezelfde voet kan worden voortgezet. "In het najaar van 2008 hopen we de beoogde 2500 patiënten geïncludeerd te hebben", vertelt onderzoekster Anneke Brand van Sanquin Bloedbank Regio Zuidwest. "Die grote aantallen zijn nodig om straks goede evidence based keuzes te kunnen maken over het gebruik van bloedbesparende transfusies. We kijken bij deze trial naar alternatieven voor donorbloed, in dit geval bij patiënten die een orthopedische operatie ondergaan waarbij een knie- of heupgewricht wordt vervangen."

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Bij de TOMaat-studie wordt een vergelijking gemaakt tussen het middel erythropoïetine – beter bekend als EPO – en zogeheten re-infusie van zogeheten autoloog bloed (of bloedcomponenten) van de patiënt zélf. Beide methoden zouden het gebruik van 'klassieke' allogene bloedtransfusies na de operatie kunnen verminderen en daardoor minder complicaties opleveren bij de patiënt. De toekomst zal leren of dit inderdaad het geval is. De gerandomiseerde studie moet verder onder meer duidelijk maken of de aanpak kosteneffectief is en of de kwaliteit van leven van patiënten er door verbetert. Naar verwachting zullen de resultaten van de TOMaat-studie eind 2009 bekend zijn.

Stamcellen

Een hot topic in de transfusiewereld is stamceltherapie, waarbij stamcellen getransplanteerd worden om defecte cellen te vervangen. Bijvoorbeeld in het beenmerg, de plek waar bloedcellen worden aangemaakt. Men spreekt dan van hematopoietische stamceltransplantaties. Binnen Sanquin werken hier verschillende groepen aan. Onder andere bij de afdeling Moleculaire Celbiologie, waar celbiologe Paula van Hennik fundamenteel onderzoek doet naar de manier waarop stamcellen zich bewegen door het lichaam van een patiënt.

"Cruciaal bij een beenmergtransplantatie is dat zoveel mogelijk stamcellen terechtkomen in het beenmerg", vertelt Van Hennik. "Het lichaam regelt dit via zogeheten chemoattractie, waarbij receptoren heel specifiek bepaalde boodschappermoleculen binden zodat cellen naar de juiste plaats bewegen. Ons onderzoek richt zich op dit proces, wat in bloed-stamcellen voornamelijk gereguleerd wordt door het chemoattractant SDF-1 en zijn receptor CXCR4.

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Daarnaast was al bekend dat ook chemorepulsie onder invloed van het eiwit Slit en zijn receptor Robo een rol speelt bij de beweging van lymfocyten. Dit is een bepaald type witte bloedcellen, waarvan de migratie geremd wordt door Slit. Met behulp van in vitro studies hebben wij onderzocht of Robo/Slit een vergelijkbare rol speelt bij stamcelmigratie. Dat lijkt inderdaad zo te zijn.

"Hopelijk geeft dit in de toekomst mogelijkheden om deze signalering bij te sturen, zodat we meer controle krijgen op de migratie van stamcellen. Dit onderzoek is ook belangrijk voor andere aandoeningen waarbij vergelijkbare processen van belang zijn, zoals ontstekingsprocessen en het uitzaaien van kankercellen. Vandaar dat wij eveneens onderzoek doen naar de 'intracellulaire' eiwitten die door Slit en Robo worden beïnvloed."

Van Henniks collega, medisch biologe Carlijn Voermans, doet bij de afdeling Experimentele Immunohematologie eveneens stamcelonderzoek. "Na een stamceltransplantatie moet het beenmerg weer herstellen en duurt het ongeveer twee weken voordat de productie van witte bloedcellen weer op gang komt. Wij proberen de signaalmoleculen te achterhalen die ervoor zorgen dat het beenmerg na een transplantatie weer de oude wordt", aldus Voermans. "Door de moleculaire routes in de hematopoetische niche beter te doorgronden hopen we er meer grip

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op te krijgen en zo het herstel te kunnen verbeteren en te versnellen."

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Een meer toegepaste onderzoekslijn van Voermans richt zich op het invriezen en bewaren van stamcellen. Deze cryopreservatie vindt al jaren plaats met behulp van een vriesbeschermend middel DMSO (dimethylsulfoxide), maar daarbij gaat desondanks een deel van de ingevroren (stam)cellen dood. Bovendien leidt het na transplantatie vaak tot vervelende bijwerkingen bij patiënten. Zij kunnen bijvoorbeeld last krijgen van misselijkheid, ademhalingsproblemen en allergische reacties. Voermans: "Wij hebben afgelopen jaar verbeterde invriesprotocollen ontwikkeld, die gebaseerd zijn op een nieuw theoretisch model van onderzoekers van Wageningen UR. Hun model beschrijft de osmotische processen die plaatsvinden wanneer tijdens het invriezen van stamcellen het water in de cellen wordt vervangen door het invriesmengsel met DMSO. Op basis van het Wageningse model hebben we een optimale 'invriescurve' ontwikkeld en hebben we het effect vergeleken van verschillende DMSO-concentraties op de kwaliteit van het stamceltransplantaat. Onze studies laten zien die kwaliteit verbetert dankzij de nieuwe aanpak, waardoor patiënten bij een transplantatie méér – en betere – stamcellen ontvangen."

Auto-immuunziekten

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Eén van de wetenschappelijke hoogtepunten van 2007 was een publicatie in Science van Rob Aalberse en zijn collega's van de afdeling Immunopathologie. Samen met onderzoekers van de Universiteit Maastricht en het Utrechtse bedrijf Genmab ontdekten de Sanquin-immunologen als eersten een verklaring voor de instabiliteit van bepaalde antistoffen. Deze Y-vormige moleculen (van het type IgG4, om precies te zijn) blijken in onze bloedbaan fragmenten van hun 'armen' met elkaar te kunnen uitwisselen. De onderzoekers ontdekten het moleculaire mechanisme van deze wisseldans. Hun vondst heeft mogelijk impact op nieuwe therapieën tegen bijvoorbeeld auto-immuunziekten. De eerste klinische trials met recombinante antistoffen zijn inmiddels onderweg.

Dit mooie fundamentele onderzoek van Aalberse liet afgelopen jaar opvallend weinig stof opwaaien in de media. Dat zou weleens heel anders kunnen lopen bij een andere immunologische studie van Sanquin, die wordt uitgevoerd door

immunopatholoog Lucien Aarden. Zijn onderzoekgroep doet onderzoek naar infliximab (Remicade®), een medicijn dat te boek staat als een wondermiddel voor mensen met reumatoïde artritis en de ziekte van Crohn. Normaal gesproken krijgen patiënten die lijden aan deze ontstekingsziekten methotrexaat, prednison of een andere ontstekingsremmer, maar als ze daar niet op reageren wordt zwaarder geschut ingezet. Infliximab bijvoorbeeld. Deze peperdure antistof bestaat uit een stukje van een muizeneiwit en een menselijk eiwit. Kosten: 15.000 à 45.000 euro per patiënt per jaar. “Samen met het Jan van Breemen Instituut, het Slotervaart Ziekenhuis, de VU en het AMC hebben wij afgelopen jaar ontdekt dat infliximab minder goed werkt dan wordt geclaimd”, aldus Aarden. “Meer dan de helft van de patiënten blijkt antistoffen aan te maken tegen de antistof, waardoor het effect van de therapie vermindert. Wij hebben inmiddels een test ontwikkeld waarmee we de concentratie van het geneesmiddel én de antistoffen ertegen kunnen monitoren. Ons uiteindelijke doel blijft een test waarmee patiënten dit zelf kunnen doen.”

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Veilige moedermelk

Een wat vreemde eend in de bijt lijkt het onderzoek van viroloog Fokke Terpstra, één van de Sanquin-promovendi van het afgelopen jaar. Als onderdeel van studies naar virusveiligheid deed hij contractresearch naar veilige moedermelk. “In de VS zijn de zogeheten melkbanken ongerust over het risico dat infectieziekten worden overgedragen via moedermelk”, licht Terpstra toe. “Vandaar dat die melk wordt gepasteuriseerd om bacteriën te doden en virussen te inactiveren. Wij hebben daartoe twee technieken met elkaar vergeleken: de standaard Holder-pasteurisatie waarbij moedermelk een half uur lang wordt verhit bij 63°C en een nieuwere high-temperature short-time (HTST) pasteurisatie waarbij de melk slechts zestien seconden wordt verhit bij 72°C. Uit ons onderzoek blijkt dat de snelle methode minstens zo veilig en effectief is. Niet alleen worden bacteriën geïnactiveerd, maar ook virussen zoals HIV en het pseudorabies-virus. Omdat de hittebehandeling korter duurt, mag je verwachten dat de kwaliteit van de moedermelk er beter door blijft. Al hebben wij dat aspect zelf niet onderzocht. De HTST-technologie heeft in de praktijk nog wel een nadeel: het is veel duurder dan een klassieke pasteurisatie.”

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Prenatale diagnostiek

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Van moedermelk is het een logische stap naar het werk van Ellen van der Schoot van de afdeling Experimentele Immunohematologie. Namens Sanquin maakt zij deel uit van een Europees netwerk (SAFE) dat onderzoek doet naar niet-invasieve diagnostiek bij ongeboren kinderen. “Een bekend voorbeeld zijn rhesus-baby’s, waarbij het eerste kind D-positief bloed heeft, terwijl hun moeder D-negatief is”, vertelt de arts-onderzoeker. “Tijdens de eerste zwangerschap maakt de moeder antistoffen aan tegen die rhesusfactor. Bij een volgende zwangerschap is het dan cruciaal om te achterhalen of de foetus eveneens D-positief is, want dan zou het immuunsysteem van de moeder immers in actie kunnen komen – met alle gevolgen van dien. Is het kind D-negatief, dan is dat een hele geruststelling voor de ouders. Het idee achter SAFE is dat je dit soort diagnostiek wil doen zonder dat de ongeboren baby er last van heeft. Gelukkig kan dit via het bloed. Een placenta schilfert als het ware langzaam af, waardoor cellen van de foetus terechtkomen in de bloedbaan van de moeder. De kunst is om het kleine beetje DNA van de foetus te traceren tussen de overmaat aan DNA van de moeder.” Van der Schoots onderzoeksgroep ontwikkelde een test die dit foetale DNA kan aantonen. Daardoor is al na negen weken zwangerschap duidelijk of het om een D-positieve baby gaat. “Belangrijk, want D-negatieve zwangere vrouwen die nog geen antistoffen hebben aangemaakt krijgen in de dertigste week ‘anti-D’. Dit voorkomt dat hun immuunsysteem het eventueel zelf gaat aanmaken. Veertig procent is echter zwanger van een D-negatief kind, dus ondergaat de behandeling voor niets. Met onze test kun je deze onnodige behandeling beperken.”

Meer informatie kunt u vinden in het Engelstalig Scientific Report 2007.
U kunt dit downloaden van de website (www.sanquin.nl; onder actueel) of aanvragen bij het directiesecretariaat Sanquin Research, e-mail: research@sanquin.nl; telefoon: +31 20 512 3224.

Highlights: a brief sketch of research at Sanquin

Nearly 250 scientists and developers work at Sanquin. Together they cover a broad range of research in the field of blood and blood products. A number of highlights of 2007 are presented below as an introduction to this Scientific Report.

Blood safety is one of the key aspects of Sanquin's research. This is why improving the diagnosis of blood-transmitted infections is high on the agenda. A good example is the early identification of bacterial contamination of blood products. "Thanks to the many measures taken since the nineties, the virus safety of our products is under control", says Dirk de Korte, Research and Development Manager at Sanquin Blood Bank North West Region. "Since then, our strong focus has been on bacterial safety. Platelets are a major bottleneck here, because they need to be stored at room temperature (22°C). At lower temperatures, they break down and lose their biological activity."
Since 2001, Sanquin has been using culture methods to detect bacteria in all platelet concentrates. The disadvantage of these techniques is that it takes several days to obtain the results. Since platelets have a limited shelf life, they are directly shipped to hospitals. De Korte: "Platelet products are considered safe until proven otherwise. Sometimes you need to recall products, and this is what we want to prevent through faster screening. Over the past year, we optimized a real-time PCR method to achieve this: a so-called 16S rDNA test that allows us to detect a ribosomal DNA fragment that is highly specific for bacteria. This means we are able to identify within four hours the most common bacteria present in plasma and platelets, such as Bacillus, Staphylococcus and Propioni strains. We have added our own double-check to the PCR test – a DNA segment from a so-called lambda phage. This makes the assay potentially useful as a diagnostic tool, although we are still facing a number of hurdles before it can be used to routinely screen our platelet stock."

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Donor studies

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In 2007 research at Sanquin also focused its attention on the mainstay of its activities: the donors who voluntarily donate blood. Researcher Pieter van der Meer conducted contract research regarding a needle that speeds up the transfer of

blood during donation. “In this new high-throughput needle, a small narrowing between the needle and the transfer tube to the blood bag is removed. This allows the blood to flow through the narrow passage almost 20 percent faster”, explains Van der Meer. “That may sound simple, and it is. But the effects are remarkable. We performed extensive field tests with the needles, and they proved very effective; average transfusion time dropped from eight to seven minutes per donation, which is more comfortable for the donor. Additionally, the number of donations that lasted more than twelve minutes was cut by half. This is important because platelets from those donations cannot be used, as there is a risk that they are already activated. We're talking about a few thousand donations per year for which the platelets can now be used as blood products after all.”

Blood Bank South East Region in Nijmegen coordinates national and European donor studies. “There are 430,000 active donors in the Netherlands, and we want to maintain this pool so that we are not faced with shortages in the long term. This is already an issue in some European countries”, according to psychologist Ingrid Veldhuizen, who together with division director Wim de Kort, provides support for various donor studies. “Strange as it may seem, Sanquin does not yet know its donors well enough”, say Veldhuizen and De Kort unanimously. “What characteristics do they share? Why do they give blood, and why do some people stop after a while? We hope the Donor InSight study will shed some light on these questions. To date, we have sent questionnaires to more than 28,000 donors.

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We expect to present the initial results of this survey during the course of this year. Donor retention remains a key topic: how do you keep people motivated to continue donating blood? Five to ten percent of donors drop out of our pool every year. Some of this is normal turnover, as you can only give blood until the age of 70. But part of it is people who simply stop coming. Research indicates that at least half of these individuals are in principle willing to remain donors. For this reason we want to provide our donors with even better information through special intervention programs, with the aim of binding them to Sanquin for a longer period. Over the past few years, we have also taken a close look at our brochures and adapted them to make them more motivating. An entirely new strategy is recruiting new donors via existing donors, an approach that now seems to be bearing fruit.”

Improving transfusion policy

A new research project that aims to improve the application of blood transfusions is just getting under way. Since 2004, Sanquin has been cooperating with four hospitals in the long-term TOMaat study (acronym for Transfusie Op Maat, tailor-made transfusion), a joint initiative of the LUMC in Leiden and Sanquin Blood Bank South West Region. By late 2007, almost 1,850 patients had been included in the study, and an interim analysis showed that the study can be continued as planned. "We hope the target of 2,500 included patients will be reached by late 2008", says researcher Anneke Brand of Sanquin Blood Bank South West Region. "The large numbers are necessary in order to be able to make good, evidence-based choices about the use of blood-saving transfusions in the future. We are looking for alternatives for donor blood in this trial, in this case in patients undergoing orthopedic surgery for knee or hip joint replacement."

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The TOMaat study compares the use of the drug erythropoietin – better known as EPO – and so-called re-infusion of so-called autologous blood (or blood components) that belongs to the patient himself. Both methods have the potential to decrease the use of 'classic' allogenic postoperative blood transfusions, thereby reducing complications for patients. The future will show whether this is the case. The randomized study must also clarify whether the approach is cost-effective and improves patient quality of life. The results of the TOMaat study are expected by late 2009.

Stem cells

Stem cell therapy is a hot topic in the transfusion world. In this form of treatment, stem cells are transplanted to replace defective cells (for example in bone marrow, where blood cells are created). These transplants are called hematopoietic stem cell transplants. Various groups within Sanquin are conducting research in this field, including the Molecular Cell Biology Department. Cell biologist Paula van Hennik is conducting fundamental research into the way stem cells move through a patient's body.

"In bone marrow transplants, it is crucial that as many stem cells as possible end up in the bone marrow", explains Van Hennik. "The body mediates this via so-called

chemo-attraction, in which receptors bind highly specific messenger molecules that make sure cells migrate to the right place. Our research focuses on this process, which in blood cells is mostly regulated by the chemo-attractant SDF-1 and its receptor, CXCR4. Additionally, chemorepulsion was already known to play a role in lymphocyte migration via the Slit protein and its Robo receptor. Lymphocytes are a specific type of white blood cell whose migration is inhibited by Slit. We used in vitro studies to examine whether Robo/Slit plays a similar role in stem cell migration. This seems to be the case."

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"Hopefully, this will open up new avenues for influencing these signal pathways in the future, allowing us to gain better control of stem cell migration. This research is important for other conditions involving comparable processes, such as inflammatory processes and cancer cell metastasis. That is why we are also studying the 'intracellular' proteins influenced by Slit and Robo."

Van Hennik's colleague, medical biologist Carlijn Voermans, conducts other stem cell research at the Experimental Immunohematology Department. "The bone marrow needs to recover after a stem cell transplant. It takes about two weeks for production of white blood cells to start up again. We are trying to identify the signal molecules that restore bone marrow function after a transplant", says Voermans. "By gaining a better understanding of the molecular pathways in the hematopoietic niche, we hope to speed up and improve recovery."

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A more applied line of research conducted by Voermans focuses on freezing and storing stem cells. This cryopreservation has been taking place for years using a substance, DMSO (dimethyl sulphoxide), to protect the cells from freezing. However, some (stem)cells do not survive freezing despite this protection. Additionally, it frequently causes unpleasant side-effects in patients following transplantation, such as nausea, breathing problems and allergic reactions. Voermans says, "Over the past year we have been using a new theoretical model by researchers from Wageningen UR to develop improved freezing protocols. Their model describes the osmotic processes that occur when water in the stem cells is replaced with DMSO from the freezing solution during the freezing process. Based on the Wageningen model, we developed an optimal 'freezing curve', and compared the effect of DMSO concentrations on the quality of the stem cell

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transplant. Our studies show that quality improves thanks to the new approach, so patients receive more – and better – stem cells during a transplant."

Autoimmune diseases

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One of the scientific highlights of 2007 was a publication in Science by Rob Aalberse and his colleagues from the Immunopathology Department. Together with researchers from Maastricht University and the Utrecht company Genmab, the Sanquin immunologists were the first to explain the instability of certain antibodies. These Y-shaped molecules (specifically of type IgG4) were found to have the capacity to swap fragments of their 'arms' with each other. The researchers identified the molecular mechanism underlying this switch. Their discovery has the potential to affect new therapies for conditions such as autoimmune diseases. The first clinical trials using recombinant antibodies have since been initiated.

Aalberse's fantastic fundamental research project received surprisingly little media attention. This may not be the case for another one of Sanquin's immunology research projects, currently being conducted by immunopathologist Lucien Aarden.

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His research group is investigating infliximab (Remicade®), a medicine considered a wonder drug for people with rheumatoid arthritis and Crohn's disease. Normally, patients suffering from these inflammatory conditions receive treatment with methotrexate, prednisone or other anti-inflammatory drugs, and heavier drugs, such as infliximab, are used if they fail to respond. This extremely expensive antibody consists of fragments of a mouse protein and a human protein. The cost: 15,000 to 45,000 euros per patient per year. "Over the past year, together with the Jan van Breemen Institute, the Slotervaart Hospital, the VU University and the AMC we discovered that infliximab does not work as well as claimed", according to Aarden. "Over half of patients make antibodies against the antibody, which decreases the effect of the therapy. We have since developed a test that can be used to monitor both the concentration of the drug as well as the antibodies against it. Our final goal is to develop a test that patients can use themselves."

Safe breast milk

One of the seemingly oddball projects of the past year was research by virologist Fokke Terpstra, one of Sanquin's researchers obtaining their PhD in 2007. He conducted contract research into safe breast milk as a part of investigations into virus safety. “In the US, the so-called milk banks are worried about the risks transmission of infectious agents via breast milk”, explains Terpstra. “That's why the milk is pasteurized to kill bacteria and inactivate viruses. We compared two techniques – the standard Holder pasteurization method, in which breast milk is heated at 63°C for half an hour, and a newer high-temperature short-time (HTST) pasteurization method, in which the milk is heated at 72°C for sixteen seconds. Our research shows that the fast method is at least as safe and effective. Not only are bacteria inactivated, so are viruses such as HIV and the pseudorabies virus. Because the heat treatment is shorter, it is to be expected that the quality of the breast milk remains better, although we did not examine this aspect. The HTST technique does have one practical disadvantage – it is much more expensive than classic pasteurization.”

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Prenatal diagnostics

The logical step from breast milk leads us to Ellen van der Schoot's work at the Experimental Immunohematology Department. Representing Sanquin, she is a member of a European network (SAFE) conducting research into non-invasive diagnostics in unborn children. “A well-known example is rhesus babies, where the first child has D positive blood while the mother is D negative”, explains the doctor-researcher. “During the first pregnancy, the mother creates antibodies against the rhesus factor. It is essential during a subsequent pregnancy to determine whether the fetus is also D positive, as the mother's immune system could activate if this is the case – with all the consequences that this brings. If the child is D negative, the parents can be reassured. The idea behind SAFE is to be able to perform this kind of assay without affecting the unborn child. Fortunately, this can be done via the mother's blood. A placenta basically 'flakes' away over time, so fetal cells end up in the mother's bloodstream. The trick is to trace that tiny amount of fetal DNA in the sea of maternal DNA.” Van der Schoot's research group developed a test to identify

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this fetal DNA. This allows doctors to determine whether a baby is D positive from the ninth week of pregnancy onwards. “That's important, because D-negative women who have not yet made antibodies receive 'anti-D' in their thirtieth week. This prevents their own immune system from creating it. However, forty percent are pregnant with a D negative child, and receive unnecessary treatment. Our test means that unnecessary treatment can be avoided.”

More information is available in the English language Scientific Report 2007. It may be downloaded from the website (www.sanquin.nl; under 'news') or requested from the Sanquin Research Management Secretariat, e-mail: research@sanquin.nl; telephone: +31 20 512 3224.

Introduction

Introduction

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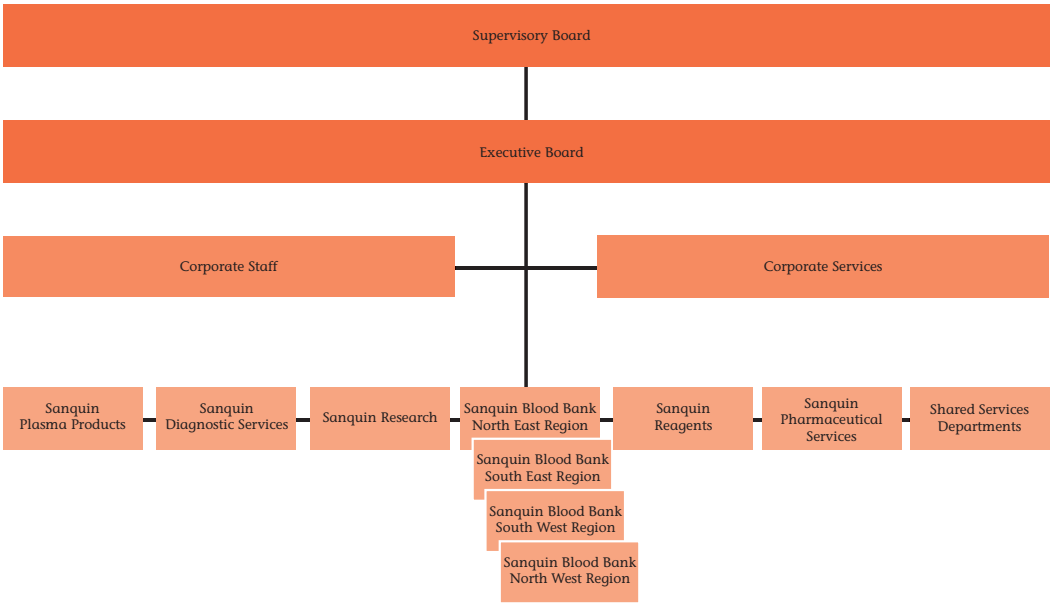
Sanquin Blood Supply Foundation

Sanquin Blood Supply Foundation consists of seven divisions and two business units. 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. Four Blood Bank divisions each have their own department of Research and Education. Finally, at the Amsterdam premises we find Sanquin Diagnostic Services, Sanquin Plasma Products, Sanquin Research, and the business units Sanquin Pharmaceutical Services and Sanquin Reagents.



Principal investigators

As explained in the words of welcome, Sanquin introduced the system of Principal investigator (PI). You will find more information on the following PI's research groups:

Principal investigator	Department
Prof Rob Aalberse PhD	*Immunopathology, Sanquin Research*
Prof Lucien Aarden PhD	*Immunopathology, Sanquin Research*
Prof Anneke Brand MD PhD	*Research & Development, Sanquin Blood Bank South West Region*
Wim de Kort MD PhD	*Research & Development, Sanquin Blood Bank South East Region*
Dirk de Korte PhD	*Research & Development, Sanquin Blood Bank North West Region*
Peter Hordijk PhD	*Molecular Cell Biology, Sanquin Research*
Prof Koen Mertens PhD	*Plasma Proteins, Sanquin Research*
Timo van den Berg PhD	*Blood Cell Research, Sanquin Research*
Cees van der Poel MD PhD	*Transfusion Technology Assessment, Sanquin Research and Julius Center, Utrecht University*
Prof C Ellen van der Schoot MD PhD	*Immunohematology, Sanquin Research*
S Marieke van Ham PhD	*Immunopathology, Sanquin Research*
Prof Dick van Rhenen MD PhD	*Sanquin Blood Bank South West Region*
Arthur Verhoeven PhD	*Blood Cell Research, Sanquin Research*
Jan Voorberg PhD	*Plasma Proteins, Sanquin Research*

Research Programming Committee

The Research Programming Committee is advising the Executive Board on strategic issues and on selection of projects funded from Sanquin's own resources. A yearly call for proposals on product and process development is issued, and projects are reviewed by international referees before selection to guarantee the quality of the research proposals. In 2007 further actions were continued to improve the quality of research proposals to be submitted to external funding agencies and charities by internal review meetings and procedures.

The Research Programming Committee consisted of five members representing four product/market combinations: Blood Banks (Prof DJ van Rhenen MD PhD, HJC de Wit PharmD), Plasma Products (PFW Strengers MD), Diagnostic Services (C Aaij PhD) and Research (Prof E Briët MD PhD), supported by an executive secretary, JW Smeenk MSc).

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 2007 the Scientic Advisory Board consisted of:

Prof E Briët MD PhD (Chairman, Sanquin Executive Board & Universities of Amsterdam and Leiden)

Prof A Brand MD PhD (Sanquin Blood Bank South West Region & Leiden University)

C de Visser PhD (Netherlands Organization for Scientific Research)

Prof RRP de Vries MD PhD (Leiden University)

Prof DE Grobbee MD PhD (Utrecht University)

Prof MM Levi MD PhD (University of Amsterdam)

Prof DKF Meijer PhD (University of Groningen)

Prof DJ van Rhenen MD PhD (Sanquin Blood Bank South West Region & Erasmus University Rotterdam)

JW Smeenk MSc, Executive secretary (Sanquin Corporate Staff)

Research Assessment

All research groups of Sanquin are visited by a peer review committee once in every five years. In earlier years these site visits were organized by departments. Following the advice of the Scientific Advisory Board, in 2006 site visits were organized thematically, basically in line with the research lines presented in this report. With the introduction of the Principal Investigator, it was decided to reorganize the site visit system again. In 2008 the first site visits based on research groups of PI's will take place. The task of the Scientific Advisory Board was strengthened with a more hands-on annual evaluation of Sanquin's Research management.

As was already the case for blood bank divisions, a number of critical performance indicators were developed for the research groups. These CPI's are available on an annual basis for both the Scientific Advisory Board and the international peer review committees.

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.
At many Dutch universities, staff from various Sanquin divisions is 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 specialising in transfusion medicine and/or 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 North East Region runs a postgraduate masters program, under the heading of Academic Institute for International Development of Transfusion Medicine (IDTM). Sanquin is WHO Collaborating Organization for Transfusion Medicine.

Professorships Sanquin Staff

Prof Rob Aalberse PhD (Biological immunology, Subfaculty of Biology, University of Amsterdam)

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 Ernest Briët MD PhD (Epidemiology of blood transfusion, Leiden University Medical Center and Medicine, Academic Medical Center, University of Amsterdam)

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 Dick van Rhenen MD PhD (Blood transfusion medicine, Erasmus University Medical Center, University of Rotterdam)

Prof Hanneke Schuitemaker PhD (Virology, especially viro-pathogenesis of AIDS, Academic Medical Center, University of Amsterdam)

Prof C Ellen van der Schoot MD PhD (Experimental Immunohematology, 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

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).

A new long-term agreement was signed in December 2007, expanding the joint research program with epidemiology of blood transmitted infections, and hemostasis and thrombosis, while continuing research in the fields of immunology and hematology as well as strengthening translational research.

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. In 2007 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

During 2007 most of the laboratories of Sanquin Research have been subject tot an internal audit by the QOE (Quality Occupational Health and Environmental safety) department. The scope of these audits was ISO 17025 as explained in T31 by the Dutch Accreditation Council, ISO 14001 and OHSAS 18001.

At Sanquin Reagents, two ISO certificates were renewed in 2007 (ISO 9001 & ISO 13485).

The departments of Virus Safety Services, Clinical Monitoring and Blood Transfusion Technology were visited by the Dutch Accreditation Council (RvA) and the CCKL in april 2007 and prolonged their accreditation according to ISO 17025 and certification according to the CCKL Code of practice version four with four years.

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 auditteam 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.

In 2007 the department of Research & Development, Sanquin Blood Bank North East Region was added to the ISO 9001-2000 system. The Stem cells department of this division has put much effort towards the preparation for JACIE accreditation expected in 2008.

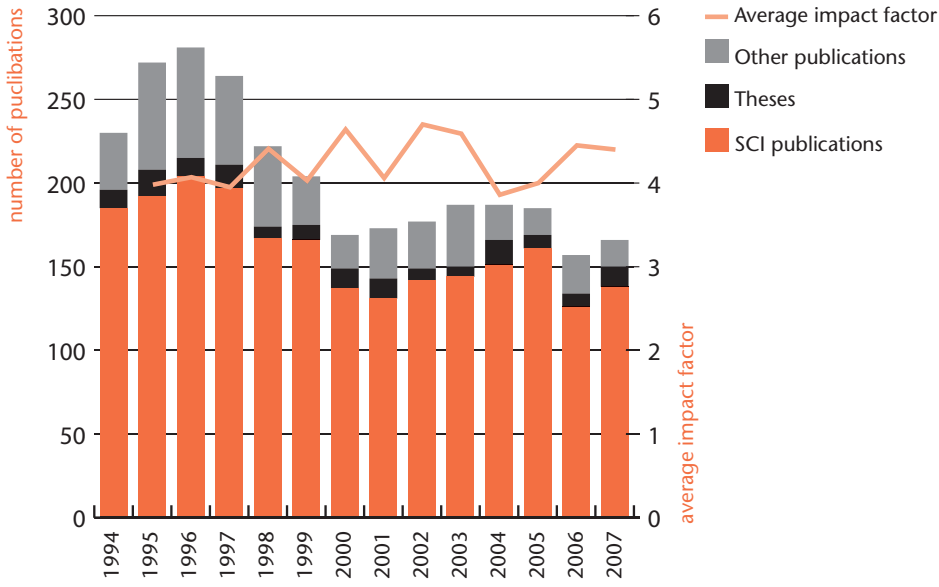
Publications

The number of papers in peer reviewed journals was higher than in previous years. The number of citations in the five years after publishing (2002) was much higher, 3042 for 142 papers, an average of 21,4 citations per paper.

Scientific publications

Year	*Total number*	*SCI publications*	*Theses*	*Average impact factor*
1994	230	185	11	na
1995	272	192	16	4,0
1996	281	204	11	4,1
1997	264	197	14	4,0
1998	222	167	7	4,4
1999	204	166	9	4,0
2000	169	137	12	4,7
2001	173	131	12	4,1
2002	177	142	7	4,7
2003	187	144	6	4,6
2004	187	151	15	3,9
2005	185	161	8	4,0
2006	157	126	8	4,5
2007	166	138	12	4,4

Scientific publications and average impact factor



Articles* published in 1993 through 2001 annual reports cited** in five full years after publication

Publications from year	*Total citations*	*Number of SCI publications*	*Average number of citations per publication*
1994	3599	185	19,5
1995	3215	192	16,7
1996	3057	204	15,0
1997	2962	197	15,0
1998	3448	167	20,6
1999	2910	166	17,5
2000	2699	137	19,7
2001	2220	131	16,9
2002	3042	142	21,4

* Only SCI publications are included

** Excluding self citations

Articles* published in 1993 through 2001 annual reports cited** in five full years after publication

	Citations in year												
Publications from year	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
1994	596	894	871	686	552								
1995		484	736	732	641	622							
1996			491	736	685	615	530						
1997				369	661	657	656	619					
1998					646	811	768	646	577				
1999						468	726	677	543	496			
2000							442	614	580	552	511		
2001								349	510	513	434	414	
2002									498	667	735	594	548

* Only SCI publications are included ** Excluding self citations

Funding

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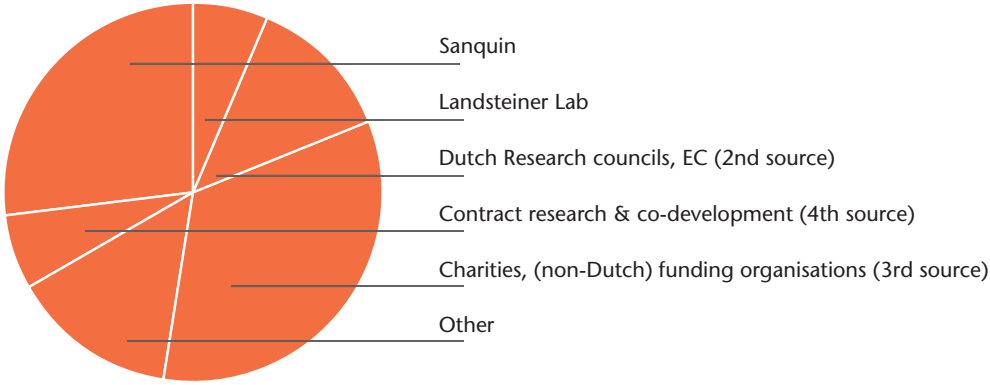
In 2007 Sanquin researchers were again successful in obtaining external funding (See page 186 for an overview of our sponsors). As European Research funds become more and more important, Sanquin invests in external consulting services to assist our staff in forming consortia and writing proposals for the seventh Framework Program that started in 2007, and to assist in administrative and organizational matters. Several projects were applied for. The group of Dr De Kort was awarded a Public Health Program on Donor Management (DOMAINE). Contract Research income was comparable to that in the years before. Income from charities increased due to a grant from the Landsteiner Foundation for Blood Transfusion Research, LSBR.
Seven research projects 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. Unfortunately more then fifteen good proposals could not be funded, due to lack of resources. The available funds for product- and process development within the organization are expected to grow slightly in the years to come.

Sources of funding of research projects (direct costs only)



Valorization

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In 2007 Sanquin continued its policy to stimulate researchers to come forward with suggestions for co-development, contract research with third parties and with innovative ideas for patent application. Income generated from cooporation with for instance Pharma, Biotech end Diagnostic companies and from out-licensing of patents/hybridoma's (both for therapeutic and diagnostic use) form additional funding for research. On page 186 you will find an overview of commercial parties with whom Sanquin Research collaborated through the years. On page 184 you will find an overview of out-licensed and available cell lines & published patents.

Sanquin joined the virtual Life Sciences Center Amsterdam, that comprises research institutes, universities, (startup) companies and funding opportunities in the Amsterdam region. LSCA created a shared Technology Transfer Office, combining expertise from the various participants with respect to intellectual property and knowledge transfer.

Research lines

Hematology

Alloimmunization against blood group antigens

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Alloantibody research

The humoral immune response against RhD was studied at single cell level. Intriguingly we found that in the peripheral blood of hyperimmunized anti-D donors a large memory B-cell pool against the RhD antigen exists in an IgM+ CD27- negative B-cell subset. However, this subset was found to be negative for the ABCB1 transporter, which discriminates naïve from memory cells. A similar observation was done in anti-tetanus toxoid donors. Using TT as a model, we are now developing a method to directly sort the antigen specific B cells during the hyperimmunization response, to be able to follow the kinetics of the different B cell subsets during hyperimmunization.

Maternal Human Platelet Antigen-1a alloantibodies (HPA-1a Abs) causing neonatal alloimmune thrombocytopenia (NAIT) can bind also to endothelium, via the β3-integrin (CD61), which may contribute to the bleeding disorder as observed in NAIT. We investigated the influence of HPA-1a-Abs on endothelial cell function, with emphasis on monolayer integrity. A CD61 monoclonal antibody (MoAb) was used as a model for the HPA-1a Abs and all results were confirmed with purified IgG fractions from HPA-1a alloimmunized women. The effect of these antibodies was examined by monitoring the adhesion, spreading, and monolayer integrity of primary human umbilical vein endothelial cells (HUVECs) using classical adhesion assays as well as ECIS (Electrical Cell-Substrate Impedance Sensing). We found that both the MoAb CD61 and the HPA-1a Abs caused a significant reduction in HUVEC spreading. Addition of the MoAb CD61 and the HPA-1a Abs antibodies prior to or following formation of a stable endothelial monolayer also negatively affected endothelial monolayer integrity, which could be mimicked by inhibiting Rho kinase and was accompanied by a redistribution of junctional proteins. Our data suggest that HPA-1a alloantibodies have a direct effect on endothelial cell spreading and monolayer integrity, which may contribute to the increased bleeding tendency in children with NAIT.

Key publications

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Dohmen SE, Muit J, Ligthart PC, Verhagen OJ, Van der Schoot CE. Anti-e found in a case of hemolytic disease of the fetus and newborn make use of the IGHV3 superspecies genes. Transfusion 2008; 48(1):194-5.

Berry J, Allen D, Porcelijn L, De Haas M, Kekomaki R, Kaplan C, Ouwehand WH, Metcalfe P. Collaborative studies to establish the first World Health Organization International Standard for detection of human antibody against human platelet antigen-3a. Vox Sang 2007; 93(4):309-15.

Van Wamelen DJ, Klumper FJ, De Haas M, Meerman RH, Van Kamp IL, Oepkes D. Obstetric history and antibody titer in estimating severity of Kell alloimmunization in pregnancy. Obstet Gynecol 2007; 109(5):1093-8.

Placental FcRn

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The transport of antibodies across the placenta is mediated by FcRn. The same receptor is also crucial for maintaining a high IgG serum concentration (~10 mg/ml) by prolonging IgG half life, FcRn is. Human IgG3 has a short half life, suggesting FcRn-mediated IgG salvage to be defective for IgG3. In the present study we used human placenta-derived JAR cells that express endogenous FcRn, human A375 cells devoid of FcRn-expression, and A375 cells transfected with human FcRn to study human IgG transport in an entirely human system. We in particular investigated the differences between FcRn mediated transport of IgG1 and IgG3, and the effect of the amino acid differences at position 435. Various sources of IgG were used: IVIg, myeloma IgG, and V-gene matched recombinant wild type IgG1 and IgG3 and mutated variants thereof differing only at position 435. Despite the differences in half life of IgG1 and IgG3 in plasma due to different binding characteristics of these IgG's of FcRn, a similar transport rate was observed *in vitro*. However, in the presence of both IgG1 and IgG3 the IgG3 transport rate is inhibited. By making

mutants of IgG1 and IgG3 we could demonstrate that this effect was dependent on the presence of His and Arg at position 435 in IgG1 and IgG3, respectively. To study if these observations also apply *in vivo*, we analyzed sera from agammaglobulinemic patients devoid of endogenous IgG production, who receive regular IVIg replacement therapy. We measured the relative IgG subclass levels found in IVIg and sera four weeks after receiving the last IVIg dose. Although not commonly found in Europe, we found detectable levels of the H435 containing IgG3 G3m(s,t) allotype in IVIg 37-39. In agreement with our *in vitro* findings, we found that this allotype was enriched in these patients compared to the total IgG3 levels that mainly consisted of the R435-containing G3m(b) and G3m(g) allotypes, strongly indicating that the R435 is also responsible for the low half life of IgG3 *in vivo*. IgG3 engineered with the isoallotypic H435 variation may therefore be a potent candidate for *in vivo* monoclonal antibody therapies.

IgG1 and IgG2 have a similar half life, yet IgG2 is the only subclass with a lower fetal than maternal concentration at term. All residues known to be important for FcRn binding are preserved between IgG1 and IgG2. Using the same model as described above we investigated FcRn mediated transcytosis of VH-matched WT IgG1 and IgG2 and mutated variants thereof lacking FcγR binding (B2G antibodies). We observed that FcγR binding properties were not required for transport and that FcRn transported IgG2 to a lesser extent than IgG1. Transport of IgG1 with a shortened lower hinge (ΔGly236) like WT IgG2, was reduced to levels that equaled that of IgG2. Likewise, IgG2 with an extended lower hinge like IgG1 (+Gly236) was increased to levels observed for WT IgG1. Gly236 is not a contact residue between IgG and FcRn, suggesting the lack of Gly236 to lead to an altered configuration of the IgG, possibly due to less flexible Fab that is closer to the Fc portion. This may sterically interfere with FcRn binding. We conclude that the lack of Gly 236 is sufficient to explain the lowered transcytosis rates for IgG2 that may also account for the low maternal / fetal IgG2 ratios at term.

Molecular Blood group polymorphisms

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Gestur Vidarsson PhD, g.vidarsson@sanquin.nl

Genetic research

In a collaborative European study (BloodGen) the first prototype of a so-called 'blood-chip' was launched during the ISBT-meeting in Capetown, September 2006. In 2007 more than 1000 blood samples were tested and the genotype was correlated to the serological results. All observed differences except two could be resolved in favor of the Bloodchip, showing the robustness of the genetic approach. In 2007 the bloodgroup antigens still missing on the array were identified, and steps to implement these assays in a second generation assay were taken.

The mechanism of anti-D prophylaxis in Hemolytic Disease of the Fetus and Newborn is not clear yet, neither is the mechanism of anti-D (hyper)immunization, although it is known to be a multifactorial process not just depending on the antigen. We analyzed different genetic factors that might be involved. Frequency of HLA-DRB1 alleles, an IGHV insert/deletion polymorphism at the IGH locus containing two IGHV3 superspecies genes, and FCGR II(A,B, C) and FCGRIII (A,B) polymorphisms were determined. We examined two different study groups; 50 hyperimmunized anti-D donors and 165 D-negative pregnant women producing anti-D despite anti-D prophylaxis, which were compared to Dutch bloodbank donors and 193 D-negative pregnant women without anti-D, respectively. Genotyping of the HLA-DRB1 locus was performed by standard typing assays. IGHV insert polymorphism was determined by an insert-specific PCR. FCGR polymorphisms were determined by multiplex ligation-dependent probe amplification. A significant association between the HLA-DRB1*015 positive phenotype and hyperimmunization against anti-D (42% versus 26% in controls, ($p = 0.01$)) was observed. The gene frequency was also higher in D-negative pregnant women who made anti-D (allele frequency = 0.18) than in controls (0.12, $p = 0.03$. The HLA-DRB1*04 phenotype was found to have a protective effect for RhD immunization during pregnancy (21% in immunized women versus 34% in controls, $p = 0.01$). No significant differences were found in any group for the IGHV insert and FCGR polymorphisms.

Better understanding of more genetic factors influencing anti-D immunization might lead to a better management of alloimmunization against RhD.

In 2007 transduced murine ES cells were obtained, which have to be selected for the propogation of RhD transgenic mice. In parallel these transduced ES lines will be used to produce murine RhD expressing erythrocytes *in vitro*.

In the European Network of Excellence on Special non invasive Advances in Fetal and neonatal Evaluations (SAFE) the application of new technological approaches for non-invasive prenatal genotyping on fetal DNA present in maternal blood is further investigated. The application of hypermethylated markers as fetal identifiers was investigated. In 2007 non-invasive fetal DNA typing assays for K, Rhc, RhE and HPA-1a were developed and/or evaluated for its diagnostic accuracy.

Key publications

Avent ND, Martinez A, Flegel WA, Olsson ML, Scott ML, Nogués N, Písăcka M, Daniels G, Van der Schoot E, Muñiz-Diaz E, Madgett TE, Storry JR, Beiboer SH, Maaskant-van Wijk PA, Von Zabern I, Jiménez E, Tejedor D, López M, Camacho E, Cheroutre G, Hacker A, Jinoch P, Svobodova I, De Haas M. The BloodGen project: toward mass-scale comprehensive genotyping of blood donors in the European Union and beyond. Transfusion 2007; 47(1 Suppl):40S-6S.

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Fetomaternal and Transfusion-induced alloimmunization

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Red cell alloimmunization

Within the regional hospitals this is an ongoing research issue. We identified that patients who developed red cell antibodies upon a first transfusion event are high responders towards a second event. This observation may be helpful to identify patients eligible for future extended matched RBC transfusions. This will be investigated in a randomized multicenter study, assigning patients to ABO-D or more extended matched red cell transfusions, started in 2006. Another group for whom extended matching is urgently needed are females receiving intrauterine transfusions for alloimmune hemolytic disease. Despite Rh-K-matching they develop broad RBC alloimmunization. In 2007 a survey on all females that received intrauterine transfusions from 1988-2006 started, on the mechanim(s) involved in the high incidence and persisting maternal antibody response evaluating T cell epitopes and persistent chimerism. To reduce high maternal RBC alloimmunization in the future we currently investigate donor availability for extended (FY, JK and S, besides RH and K) compatible IUTs for females transfused for hemolytic disease of the fetus.

The issue of extended matching is related to the Bloodchip project in which a large cohort of individuals drawn from across the EU were genotyped to demonstrate the accuracy and improvement of molecular genetic techniques over standard serological testing.
The CE marking of the chip is now being finished by typing 3000 samples divided among the participants of the consortium. When the testing for the CE marking is done, Bloodchip will become available for commercial use. A special problem is posed by the presence of RHD positive alleles in serologically typed D-negative donors. The number of D-negative and C-and/or E-positive samples that were collected and analysed till December 2007 is 831, i.e. 619 C positive, 453 E positive and 8 CE positive. In addition 4992 D-negative and CE-negative (rr) samples were collected and analysed (see below). Of every sample in which an RHD allele was found, results were confirmed with a second sample, which was also used to extent

the molecular typing by RHD-exon-specific sequencing. It will be investigated whether an informative look-back study can be performed, tracing D-neg patients who received blood from weak D donors typing negative by serology

D neg	Total number	RHD-gene present	RHD-allele causing weak D expression	Cause of weak D expression
C pos	619	18	7	1x Del; 1x weak D type 30 1x weak D type11 1x weak D type 48 3 x chimera
E pos	453	10	1	1x Del RHD (IVS3+2T>A) 2x chimera (needs to be confirmed)
CE pos	8	2	2	1x weak D type 2 1x weak D type 15
ccee	4992	2	1	1x chimera

Platelet alloimmunization

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Platelet alloimmunization can affect fetuses and newborns due to maternal antibodies against a paternal platelet antigen on fetal platelets (FNAIT). Initially, affected children were treated with intrauterine platelet transfusions, but gradually it became clear that clinical outcome and severe thrombocytopenia with bleeding occurred less using non-invasive maternal IVIG treatment. On the other hand we showed that for newborns with thrombocytopenia due to NAIT improved faster and better with compatible platelet transfusions with HPA 1a and/or 5b negative platelets as compared with IVIG.

In case of alloimmune platelet refractoriness anti-HPA antibodies play a minor role, wheras anti-HLA antibodies are still the most common cause of transfusion failure. The non-radioactive tracing of transfused platelets by the use of monospecific human monoclonal HLA antibodies was further validated in 2007 and nine

monoclonals that can discriminate more than 80% of the random population are now available in sufficient amounts.

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Phagocytes

Phagocyte activation

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Macrophages and neutrophils recognize pathogens by means of a variety of cell surface receptors. These include non-opsonic pattern recognition receptors (PRRs) for microbial structures, such as scavenger receptors, Toll-like receptors (TLR) or NOD-like receptors (NLR), and opsonic receptors, such as complement or Fc receptors that recognize complement fragments and antibodies covering the microbes.

CD163

We studied a potential role of the scavenger receptor group B cysteine-rich scavenger receptor family member CD163 as a receptor for microbes. CD163 is highly and preferentially expressed by mature tissue macrophages and has previously been identified as a receptor for hemoglobin-haptoglobin complexes. Our findings have demonstrated that CD163 mediates recognition of intact gram-positive of and gram-negative bacteria. Furthermore, the binding of bacteria to CD163 on macrophages resulted in a potent cytokine response, suggesting that CD163 may function as a sensor for bacterial infection in tissues *in vivo*. In addition, CD163 was also identified as a cell-cell adhesion receptor. In particular, CD163 was shown to act as an erythroblast adhesion receptor that mediates interactions between macrophages and erythroblastic progenitor cells in erythroblastic islands in the bone marrow where erythroblasts develop. The interaction between CD163 and erythroblast was found to support erythroblast expansion and/or survival. While most populations of macrophages express CD163 *in vivo*, the receptor functions as a typical marker for M-CSF-driven M2 macrophages. In chronic inflammatory conditions, such as multiple sclerosis, proteolytic cleavage of CD163 from monocytes occurs and surface regulation of CD163 was identified as a prognostic marker for glucocorticoid responsiveness.

TLR and NLR in neutrophil function

Members of the TLR and NLR families act as receptors for conserved microbial components, such as lipopolysaccharide (LPS) and others, and their role in

macrophage activation is relatively well documented. We are focussing on the role of TLR and NLR in neutrophil function. Studies with neutrophils from an individual suffering from deficiency of IRAK-4, a protein that interacts with MyD88 and is pivotal for the MyD88-dependent pathway of TLR signaling, indicate that IRAK-4 forms an absolute and common requirement for TLR signaling in neutrophils. Also activation of the NADPH oxidase and resultant intracellular killing of *Salmonella typhimirium* was shown to depend on complement receptor-3-mediated uptake and subsequent TLR4 triggering by LPS in the neutrophil phagosome. We are also studying the role of NLR protein family members in phagocytes. NLR proteins act as putative sensors for microbial and host components that, together with caspase-1 and adaptor proteins, form complexes termed inflammasomes. Upon activation, these inflammasomes mediate the cleavage of pro-IL1β into active IL1β. Sustained activation of inflammasomes, either by excess of ligand (e.g. uric acid crystals in gout) or by activating mutations, leads to autoinflammatory syndromes. Work in collaboration with the group of Prof Jurg Tschopp (Lausanne, Switzerland) has shown that phagocytosis, even of inert particles such as asbestos or silica, constitutes a stimulus for inflammasome activation in macrophages and this process required the formation of reactive oxygen species by a NADPH oxidase other than the classical phagocyte NADPH oxidase (i.e. NOX2). Our results also provide evidence for the existence of functional inflammasomes in neutrophils and research to investigate the regulation and function of inflammasome components in these cells is ongoing.

Complement and FcReceptors

Opsonization by complement of antibodies is often required for an effective uptake and removal of microbes by phagocytes. This involves receptors for complement, such as CR3 or Fc-gamma receptors (FcγRs) that recognize the Fc region of antibodies. The wide variation in copy number and functional isoforms is presently studied in patients suffering from various autoinflammatory and autoimmune diseases, such as idiopathic immune thrombocytopenia, pediatric vasculitis (Kawasaki disease), and rheumatoid arthritis, in which intravenous immunoglobulin infusions (IVIG) or chimeric Ig-based 'biologicals' are being used to neutralize factors (anti-TNFα) or deplete B cells (CD20 monoclonal antibodies).

Regulation of phagocyte activation

While much attention is focussed on the receptors and pathways that trigger phagocyte activation, clearly less is known about the mechanisms that control this process. We are investigating the role of SIRPα in this context. SIRPα is a typical inhibitory receptor that is selectively expressed on myeloid and neuronal cells. It acts as a receptor for the broadly expressed surface molecule CD47, and the ligation of SIRPα 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 SIRPα. We propose that, in analogy to MHC class I molecules that restrict NK cell function via killer inhibitory receptors, CD47 acts as a 'self' molecule to control phagocyte functions. Indeed, there is solid evidence now that CD47-SIRPα interactions negatively regulate the clearance of erythrocytes and platelets by macrophages *in vivo*. We are investigating whether also other phagocyte activities are controlled via SIRPα. In line with this idea, our experiments performed in collaboration with the group of Prof V Everts (Dept Oral Cell Biology, ACTA, Amsterdam) provide evidence that osteoclast generated *in vitro* from SIRPα-mutant mice that carry a defect in SIRPα signaling have an increased bone resorption. This is not due to an enhanced formation of osteoclasts, but rather to a diminished inhibition of the bone resorption capacity of mature osteoclasts. As a result SIRPα-mutant mice are osteopenic. In addition, we have demonstrated that SIRPα is a negative regulator of the phagocyte oxidative burst. Over-expression of full-length SIRPα in PLB985 cells strongly suppresses the oxidative burst, and this appears due to a down-regulation of gp91phox. Furthermore, macrophages and granulocytes from SIRPα-mutant mice display an enhanced oxidative burst and an enhanced expression of gp91phox. This suggests that SIRPα limits activity of the phagocyte NADPH oxidase to prevent excessive 'collateral' oxidatived damage to the host during infection. Work is ongoing to investigate the contribution of SIRPα signaling in microbial infection models.

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Immunodeficiencies

A variety of genetic defects in phagocyte function give rise to an increased susceptibility to bacterial and fungal infection. We investigate in (pediatric) patients the genetic basis of such immune defects and their consequences for phagocyte development and function.

Chronic granulomatous disease

Chronic granulomatous disease (CGD) is caused by mutations in genes encoding the subunits of the phagocyte NADPH oxidase (NOX2) that plays a pivotal role in the oxidative killing of microorganisms in the phagosome. Apart from a number of previously undescribed mutations in NADPH oxidase genes, we have identified a number of patients with a novel subtype of CGD, termed partial CGD, characterized by strongly diminished oxidative burst in response to bacterial peptide f-Met-Leu-Phe (fMLP), but relatively normal responses to phorbol ester and serum-treated zymosan (STZ). We are currently characterizing the defect(s) in partial CGD in more detail. Activation of the NADPH oxidase is controlled by assembly of the complex at the phagosomal membrane, but the dynamics and requirements of this process have not been properly documented. We have generated a panel of cell

lines expressing fluorescently tagged NADPH oxidase components and are using fluorescence resonance energy transfer (FRET) and other advanced fluorescent imaging techniques to study this at the single-cell level. This is done in collaboration with the group of dr C Otto (University of Twente).

Shwachman-Diamond Syndrome

Apart from the research on CGD and the patient registration in a European Genetic Database on CGD, current efforts are also directed to characterize other neutrophil defects in greater detail. For instance, Shwachman-Diamond Syndrome (SDS) is an autosomal, recessively inherited disorder characterized by bone marrow failure with significant risk of developing pediatric acute myeloid leukemia (AML) and myelodysplasia (MDS), which is often refractory to treatment. Neutropenia and defective neutrophil chemotaxis are the most frequently observed hematological abnormalities in SDS patients. In this respect, the role of SBDS (the protein defective in SDS) is studied in normal myeloid differentiation and function by transfection and knock-down strategies. We have found that SBDS co-localizes with the microtubule organizing center as well as with the mitotic spindle, suggesting a potential role for SBDS in regulating chromosome segregation during mitosis. The interactions of SBDS with nuclear and cytoplasmic SBDS-associating 'partners' or cellular structures is the focus and we anticipate that defective SBDS function results in cell migration and division defects, potentially contributing to the increased risk of developing leukemia.

In terms of migration defects of neutrophils, our studies on LAD-1/variant syndrome (different form LAD-III) and defective CR3 activation are ongoing in order to clarify the precize genetic background of the disease.

Key publications

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Apoptosis

Under normal conditions mature neutrophils have a short life-span and die by apoptosis. Neutrophil apoptosis is an important mechanism for limiting the inflammatory response. Our previous findings have provided evidence for an important role of mitochondria during neutrophil apoptosis, as was recently consolidated by the very detailed and excellent studies on the specific differences in the respiratory chain of and altered complex formation in neutrophil mitochondria. G-CSF, which enhances the development of immature granulocytes and the mobilization neutrophils from the bone marrow, also prolongs the survival of mature neutrophils. However, the mechanism underlying this effect has remained elusive. Our findings show now that G-CSF delays the apoptotic process, and also that G-CSF acts at a level downstream of caspase-8 and mitochondria, and upstream of the caspases-9 and -3. In particular, G-CSF controls the increased calcium (Ca^{2+}) influx during apoptosis, thereby preventing activation of Ca^{2+}-dependent calpain. Calpain inhibition resulted in the stabilization of the X-linked inhibitor of apoptosis (XIAP) and hence inhibited caspase-9 and -3 in human neutrophils. We are

characterizing other calpain-dependent and -independent pathways that contribute to the delay in apoptosis caused by G-CSF.
Also in the development of myeloid cells, apoptosis is believed to play an important role in neutropenia syndromes, such as in Shwachman-Diamond syndrome in which neutropenia, myelodysplasia and myeloid leukemia may develop. We have thus far focused on the myeloid inhibitory receptor SIRPα that seems to provide pro-apoptotic signals in myeloid cells. This may be relevant during acute myeloid leukemia (AML). SIRPα is expressed on hematopoietic stem cells and throughout myeloid development, but expression on AML is relatively low as observed for both SIRPα mRNA and protein. This appeared due, at least in part, by (indirect) epigenetic silencing of SIRPα gene expression as shown by studies with inhibitors of DNA methylation and histone deacetylation and by methylation analysis of the SIRPα promoter region. Reconstitution of expression and triggering of SIRPα by agonistic antibodies induces apoptosis in AML cell lines. This suggests that the growth of myeloid leukemic cells may be controlled by SIRPα, and that a low SIRPα expression on AML may contribute to their uncontrolled proliferation and survival.

Key publication

Van Raam BJ, Sluiter W, De Wit E, Roos D, Verhoeven AJ, Kuijpers TW. Mitochondrial membrane potential in human neutrophils is maintained by complex III activity in the absence of supercomplex organisation. PLoS ONE 2008; 3(4):e2013.

Granulocyte transfusion

Granulocyte transfusion provides support for neutropenic patients suffering from life-threatening infections. The large numbers of granulocytes required can be mobilized from the bone marrow of donors by treatment with G-CSF and dexamethasone. We have shown that this treatment does not significantly affect the major effector functions of neutrophils. Moreover, G-CSF and dexamethasone prolong the potential storage time *in vitro*. In order to identify components or signaling pathways involved in extended survival, we have performed microarray analysis of neutrophils before and after G-CSF plus dexamethasone treatment and this has identified several candidate proteins that are currently being tested for functional relevance.

These data have already led to the start of further studies on the pro-inflammatory and anti-inflammatory properties with which G-CSF pre-activation *in vivo* seems to endow the neutrophils present in the granulocyte concentrates. Phagocytosis, TLR and NLR signaling as well as the production and release of interleukins of these neutrophils have our attention which may be of importance for the patients receiving granulocyte transfusions for reason of prolonged febrile neutropenia and life-threatening infections.

Key publications

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Signaling in transendothelial migration

Molecular mechanisms of hematopoietic stem cell migration

Hematopoietic stem cell (HSC) transplantation is applied to treat various (non-) hematological diseases. The speed of hematological recovery after HSC transplantation depends on the number and the quality of HSCs in the graft, but also on the capacity of the transplanted HSC to migrate efficiently to the bone marrow (BM) cavity (i.e. homing). It is well established that the chemokine Stromal Derived Factor-1 (SDF-1/ CXCL12) and its receptor CXCR4 are critical in the homing of HSC to the BM and also control the migration of other cell types (e.g. lymphocytes, neurons, breast cancer cells). Interestingly, the chemorepellent Slit proteins and the Roundabout (Robo) receptors, initially identified in the developing brain Drosophila, inhibit the CXCL12-induced migration of leukocytes. These data make it conceivable that a balance between positive and negative migratory cues regulate the migratory behavior of hematopoietic (stem) cells to and from tissues.

Our goal is to 1) unravel the signaling mechanisms involved in CXCL12-induced migration of hematopoietic (stem) cells and 2) establish the mechanism mediating the apparent inhibitory migratory signal provided by Slit and Robo.

This research will provide new insights in the control of HSC migration, and expand our knowledge on the role of negative migratory signals in (stem) cell migration. This work may thus lead to novel therapeutic targets to improve HSC transplantation protocols, and may offer alternative ways to block migration in inflammatory diseases or cancer cell metastasis.

Signaling mechanisms involved in CXCL12-induced migration of hematopoietic (stem) cells

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CXCR4 signaling is abrogated as soon as the receptor is endocytosed in response to the signaling cascade initiated after ligand binding. Earlier results obtained by our group showed that specific inhibition of Rac1 GTPase signaling, induced a rapid and specific, ligand-independent internalization of the CXCR4 receptor. Initial experiments will focus on the characterization of the kinetics of the downregulation and the fate of the endocytosed receptor (degradation or re-cycling to the membrane). In addition, we were able to show that the full-length CXCR4

C-terminus interacts with endogenous Rac1 in various (non-) adherent cell-types using a peptide-based proteomics approach. The functional interaction between these proteins needs to be confirmed and characterized.

Signaling by Slit and Robo

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Four Robo homolog and 3 homologs of the Slit protein were identified in the vertebrate system. However, nothing was known about the expression pattern of the Robo and Slit homologs in the human hematopoietic system. Results of quantitative mRNA expression analysis revealed that cells that define the micro-environment of the HSCs, i.e. endothelial and mesenchymal stromal cells, express Slit2 and -3, as well as Robo1, -2, -3. The simultaneous expression of the ligand and the receptor by these cells suggest that there might be an autocrine mechanism. In hematopoietic cells, CD34+ hematopoietic stem and progenitor cells (HSPCs), showed the broadest pattern of expression of the Robo homologs. Furthermore, cell surface Robo1 expression was shown to be dynamic and downregulated during myeloid differentiation of human CD34+ HSPCs *in vitro*. In addition, we observed that Robo2 expression is lost upon monocyte differentiation, corresponding to the absence of Robo2 expression on mature, primary monocytes.
In CXCL12-induced Transwell migration and adhesion assays, recombinant Slit3 had only a small and variable effect on migration of cord-blood or mobilized peripheral blood-derived CD34+ cells, while the directional migration of the leukemic cell line HL60 was inhibited. Interestingly, the migration of primary monocytes was enhanced by pre-incubation with Slit3. In addition, stimulation of Robo by Slit3 induced contraction of these cells, resulting in reduced spreading when adhered to fibronectin overnight. This observed morphological change induced by Slit3 in monocytes suggests (relative) activation of the small GTPase RhoA.
Another part of the project focuses on the intracellular proteins that mediate the Slit/Robo response. Preliminary results of biochemical peptide-based experiments show an interaction between the SH3-domain containing proteins Grb2, Csk, Ras-Gap and p130Cas, and one or more of the four conserved domains in the intracellular tail of the Robo1 protein. The conformation of these previously unknown interactions and the functional relevance is currently under investigation.

Cell polarity and migration

Cell adhesion, polarization and directional migration (i.e. chemotaxis) are key aspects of (patho)physiological events such as morphogenesis, vasculogenesis, inflammation, wound healing and tumor cell metastasis. Directional cell migration is critically dependent on proper orchestration of cytoskeletal dynamics within different parts of the cell. The cytoskeleton, in turn, is regulated by small GTPases of the Rho family. These GTPases act as molecular switches, cycling between an inactive and active conformation, as a result of signaling initiated by adhesion or chemotactic stimuli. It is well established that proper targeting of GTPase activity within the cell is paramount to the induction of polarity and of sustained, directional migration. However, the mechanisms that control the targeting and localized activation of GTPase signaling are poorly understood.

PKA and Epac1 regulate endothelial integrity and migration through parallel and independent pathways

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The vascular endothelium provides a semi-permeable barrier, which restricts the passage of fluid, macromolecules and cells to the surrounding tissues. Cyclic AMP promotes endothelial barrier function and protects endothelium against pro-inflammatory mediators. This study analyzed the relative contribution of two cAMP targets, PKA and Epac1, to the control of endothelial barrier function and endothelial cell migration. Real-time recording of transendothelial electrical resistance showed that activation of either PKA or Epac1 with specific cAMP analogs increases endothelial barrier function and promotes endothelial cell migration. In addition, reduction of Epac1 expression showed that Epac1 and PKA control endothelial integrity and cell motility by two independent and complementary signaling pathways. We demonstrate that integrin-mediated adhesion is required for PKA, but not Epac1-Rap1-driven stimulation of endothelial barrier function. In contrast, both PKA- and Epac1-stimulated endothelial cell migration requires integrin function. These data show that activation of Epac1 and PKA by cAMP results in the stimulation of two parallel, independent signaling pathways that positively regulate endothelial integrity and cell migration, which is important for recovery after endothelial damage and for restoration of compromized endothelial barrier function.

Key publication

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Microtubule dynamics and Rac1 signaling independently regulate barrier function in lung epithelial cells

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Cadherin-mediated cell-cell adhesion controls the morphology and function of epithelial cells and is a critical component of the pathology of chronic inflammatory disorders. Dynamic interactions between cadherins and the actin cytoskeleton are required for stable cell-cell contact. Besides actin, also microtubules target intercellular, cadherin-based junctions and contribute to their formation and stability. Here, we studied the role of microtubules in conjunction with Rho-like GTPases in the regulation of lung epithelial barrier function using real-time monitoring of transepithelial electrical resistance. Unexpectedly, we found that disruption of microtubules promotes epithelial cell-cell adhesion. This increase in epithelial barrier function is accompanied by the accumulation of β-catenin at cell-cell junctions, as detected by immunofluorescence. Moreover, we found that the increase in cell-cell contact, induced by microtubule depolymerization, requires signaling through a RhoA/Rho kinase pathway. The Rac1 GTPase counteracts this pathway, because inhibition of Rac1 signaling rapidly promotes epithelial barrier function, in a microtubule- and RhoA-independent fashion. Together, our data suggest that microtubule-RhoA-mediated signaling and Rac1 control lung epithelial integrity through counteracting, independent pathways.

Key publication

Lorenowicz MJ, Fernandez-Borja M, Van Stalborch AM, Van Sterkenburg MA, Hiemstra PS, Hordijk PL. Microtubule dynamics and Rac-1 signaling independently regulate barrier function in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 2007; 293(5):L1321-31.

Rac1-induced cell migration requires membrane recruitment of the nuclear oncogene SET

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The Rho GTPase Rac1 controls cell adhesion and motility. The effector loop of Rac1 mediates interactions with downstream effectors, whereas its C-terminus binds the exchange factor β-Pix, which mediates Rac1 targeting and activation. Here, we report that Rac1, through its C-terminus, also binds the nuclear oncogene SET/I2PP2A, an inhibitor of the serine/threonine phosphatase PP2A. We found that SET translocates to the plasma membrane in cells that express active Rac1 as well as in migrating cells. Membrane-targeting of SET stimulates cell migration in a Rac1-dependent manner. Conversely, reduction of SET expression inhibits Rac1-induced migration, indicating that efficient Rac1 signaling requires membrane recruitment of SET. The recruitment of the SET oncogene to the plasma membrane represents a new feature of Rac1 signaling. Our results suggest a model in which Rac1-stimulated cell motility requires both effector loop-based downstream signaling and recruitment of a signaling amplifier, i.e. SET, through the hypervariable C-terminus.

Key publications

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Regulation of the inflammatory response of endothelial cells by RhoB

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Pro-inflammatory mediators such as the cytokines TNFα, IL1α and the bacterial product lipopolysaccharide (LPS) are released during pathogen infection and chronic inflammation. These mediators activate the inflammatory response of the endothelium by inducing the expression of leukocyte-adhesion molecules which are required for the binding and extravasation of circulating leukocytes. We found that stimulation of human primary endothelial cells with pro-inflammatory mediators induces the upregulation and activation of the small GTPase RhoB, which localizes to endosomes. This specific localization of RhoB was previously reported in other cell types where RhoB was shown to regulate the intracellular traffic of signaling

molecules. Similarly, we found that RhoB regulates the intracellular traffic of the TNF-receptor in experiments in which we silenced RhoB expression in endothelial cells with specific small interfering RNA. Furthermore, RhoB silencing prevented TNF-induced activation of the mitogen-activated kinases ERK, p38 and JNK. This suggests that RhoB regulates TNF-dependent signaling through the control of the intracellular traffic dynamics of the TNF-receptor. Currently, we are analyzing the effects of RhoB signaling on the regulation of the integrity of the endothelial monolayer in inflammatory conditions by studying the effect of RhoB silencing in endothelial junction stability upon stimulation with TNFα.

Role of the cellular prion protein in the adhesion and migration of human endothelial cells and leukocytes

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The cellular prion protein (PrPC) is a highly conserved glycoprotein expressed on multiple cell types. An abnormally folded isoform of PrP, PrP-scrapie, is thought to be the causative agent of fatal neurodegenerative disorders known as prion diseases, which include bovine BSE, sheep scrapie and human CJD. The disease pathology is thought to be caused by a toxic gain-of function of endogenous PrP when converted to PrP-scrapie. However, the physiological role of the normal host-encoded PrP has not been fully clarified. Various studies have shown that PrP is able to induce intracellular signaling. Since PrP is a GPI-anchored protein and has no intracellular domain, PrP-dependent signaling probably requires PrP association with transmembrane signaling molecules. The observation that PrP can bind several molecules involved in cell adhesion supports current models proposing that PrP signals as part of a plasma membrane-located multicomponent protein complex that generates outside-in signals from the extracellular matrix to the intracellular milieu. Adhesion-induced signals have a crucial role in cell migration and survival. In 2007, we started a new project to study the role of PrP in the human hematovascular system, specifically on the role of PrP in cell adhesion and migration. First, we detected PrP on human endothelial cells, on CD34-positive hematopoietic stem cells and on human leukemic cells. We found that stimulation of endothelial cells with TNFα or phorbol ester induces the downregulation of PrP protein expression. Furthermore, we showed that endothelial cells internalize surface PrP inefficiently,

which may explain the resistance of these cells to prion infection. We are currently investigating the role of PrP in human endothelial cell adhesion and migration by small interference RNA-mediated silencing of PrP expression. Furthermore, we have studied the role of PrP in the chemotaxis of leukocytes towards CXCL12 in an in vitro assay. We found that addition of anti-PrP antibodies results in a decreased leukocyte chemotaxis. This suggests that PrP regulates leukocyte adhesion and/or migration. Further studies will include the use of pharmacological inhibitors to explore which intracellular pathways may be affected by the anti-PrP antibodies.

Dynamic interactions between endothelial cells and leukocytes

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The migration of leukocytes across the endothelial lining of the vascular wall requires a complicated series of adhesion and signaling events. Endothelial Ig-like cell adhesion molecules (IgCAMs) such as ICAM-1, play an important role, not just as ligands for leukocyte integrins, but also as signaling initiators. Clustering these IgCAMs triggers a wide range of events in the endothelial cells' interior, of which activation of Rho-like GTPases, induction of cytoskeletal changes and the transient modulation of cell-cell contact are key events. Specific clustering of ICAM-1 on the endothelium induces the formation of apical cup-structures, also known as docking structures, dynamic membrane protrusions that partially surround adherent leukocytes. We have shown that RhoG is activated downstream from ICAM-1 engagement and that this requires the intracellular domain of ICAM-1. ICAM-1 co-localizes with RhoG and binds to the RhoG-specific guanine-nucleotide exchange factor SGEF. This interaction is mediated by the SH3 domain of SGEF and the proline-rich sequence motif in the intracellular tail of ICAM-1. Depletion of endothelial RhoG by siRNA or deletion of the intracellular tail of ICAM-1 did not affect leukocyte adhesion but did decrease the formation of apical cups and inhibited leukocyte transendothelial migration. The data indicate that the intracellular tail of ICAM-1 and RhoG are actively involved in facilitating the passage of leukocytes. Silencing SGEF also resulted in a significant reduction in cup formation and TEM. Together these results identify a new signaling pathway involving RhoG and its exchange factor SGEF downstream from ICAM-1 that is critical for leukocyte TEM.
Additionally, our results show that RhoA is activated downstream from ICAM-1

clustering. Using RNAi, we could show that RhoA is required for ICAM-1-induced RhoG activation, indicating that RhoA acts upstream from RhoG.
Currently, our research is focused on the details of the mechanism that regulates the formation of the apical cup-structures and the recruitment of ICAM-1 to sites of leukocyte adhesion. 4-D (3-dimensional in time) confocal laser scanning microscopy is used in an attempt to visualize the formation of the cup-structures around leukocytes in real-time. In addition, transendothelial migration and adhesion assays under physiological flow were set-up and will be used to further quantify the contribution of the small GTPases RhoG and RhoA to leukocyte adhesion to and migration across the endothelium.

Key publications

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Hemostasis and thrombosis

Biosynthesis of factor VIII-von Willebrand factor complex

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Vascular endothelial cells line the vessel wall thereby providing a tightly regulated barrier that separates blood from the underlying tissues. The dynamic properties of the endothelial barrier allows for diverse processes such as extravasation of leukocytes to the underlying tissues, neovascularization in response to vascular injury, regulation of vascular tone and control of hemostasis. Both hemodynamic changes and inflammatory mediators have been shown to dramatically influence gene expression profiles within endothelial cells of different vascular beds. This positions the endothelium as a highly flexible barrier that is capable of modifying its phenotypic characteristics in response to local or systemic 'danger signals'. Apart from its ability to reprogramming transcriptional pathways endothelial cells have developed intracellular storage pools for pro-thrombotic and pro-inflammatory mediators that allow them to rapidly respond to changes in their micro-environment. Weibel-Palade bodies (WPB), rod-shaped organelles that have originally been identified in aortic endothelial cells, comprize one of the best characterized storage compartments within endothelial cells. The major constituent of WPB is the multimeric protein von Willebrand factor (VWF) that is involved in the adhesion of blood platelets to sites of vascular injury. Besides VWF, these Weibel-Palade bodies contain a number of other proteins, including P-selectin, angiopoietin-2, osteoprotegerin and a number of other components. Interestingly, the composition of Weibel-Palade bodies can be modified in response to inflammatory stimuli. Upon stimulation of endothelial cells with the cytokine interleukin-1ß, synthesis of the chemotactic cytokine interleukin-8 is upregulated and part of the synthesized IL-8 is co-stored with VWF in Weibel-Palade bodies. We have performed a quantitative analysis of the entry of IL-8 into Weibel-Palade bodies using pulse-chase analysis and subcellular fractionation studies. We observed that only a small percentage of the synthesized IL-8 is stored in Weibel-Palade bodies. Equimolar amounts of IL-8 and VWF were present in these organelles suggesting that monomeric VWF contains a single binding site for IL-8. We also showed that IL-8 can interact with VWF in a pH-dependent manner. Binding was optimal at pH 6.2; a value corresponding to the pH in the Golgi-apparatus and

trans Golgi network. Our findings suggest that direct interaction of VWF with IL-8 in the trans Golgi network provides a basis for the co-sorting of IL-8 to Weibel-Palade bodies. Similarly, other Weibel-Palade body constituents may be recruited by virtue of their capacity to bind to VWF in the trans Golgi network.

Factor VIII and von Willebrand Factor (VWF) circulate in plasma in a non-covalent complex. It is generally assumed that VWF acts as a molecular chaperone that protects factor VIII from proteolytic degradation in the circulation. This is illustrated by the decreased factor VIII levels that are found in plasma of patients with von Willebrand disease variants that fail to interact with factor VIII. In these patients, factor VIII levels are reduced to approximately 20% of that observed in normal plasma, resulting in a mild bleeding tendency. Conversely, elevated levels of VWF have also been linked to increased levels of blood coagulation factor VIII. Elevated levels of factor VIII have been shown to increase the risk for venous thrombosis. This observation underscores the important role for VWF in the regulation of circulating levels of factor VIII. VWF is synthesized by vascular endothelial cells and megakaryocytes whereas factor VIII is synthesized in multiple tissues in liver and kidney.
Presently, it cannot be excluded that factor VIII and VWF have at least in part a common cellular origin. We studied the requirements for storage of factor VIII in Weibel-Palade bodies employing primary endothelial cells and human embryonic kidney (HEK293) cells expressing VWF. Co-expression studies in HEK293 cells revealed that a fluorescently labeled FVIII-YFP variant was stored in VWF containing storage organelles that resemble authentic Weibel-Palade bodies. Similarly, expression of FVIII-YFP resulted in the appearance of FVIII-YFP in Weibel-Palade bodies in endothelial cells. We subsequently addressed whether a Tyr1680Phe variant which does not display high-affinity binding to VWF is also directed towards VWF containing storage organelles in HEK293 and primary endothelial cells. Surprisingly, also Tyr1680Phe FVIII-YFP was co-stored in VWF containing storage organelles.
Our results suggest that co-trafficking of factor VIII to Weibel-Palade bodies is not dependent on high affinity binding to VWF. Future studies will focus on the precize mechanism by which factor VIII is recruited to VWF containing storage organelles.

Key publications

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Structure and functions of enzyme-cofactor complexes

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The coagulation cascade comprises several serine proteases that act in combination with a non-enzymatic cofactor. During the past 10 years we have been studying the mechanism by which activated factor IX (factor IXa) assembles with its cofactor, factor VIII. The function of factor IXa in the blood coagulation cascade is to activate factor X in a process that requires the presence of phospholipid surface, calcium ions and activated factor VIII (factor VIIIa). Factor IXa alone is a very poor protease that is 'switched on' to a fully active serine protease upon binding to factor VIIIa. The activated cofactor is a heterotrimer composed of A1, A2 and A3-C1-C2 subunits, and multiple domains contribute to the assembly of the factor X activating complex on phospholipid membranes. It has been firmly established that activated platelets provide a catalytic surface for factor X activation by the factor VIIIa/factor IXa complex. We have previously shown that factor Xa generation can also proceed on the surface of resting endothelial cells. The inherent instability of factor VIII due to dissociation of the A2 subunit from the active hetero-trimer causes rapid dampening of factor Xa generation on phospholipids vesicles and activated platelets. Remarkably, persistent factor Xa generation is observed on endothelial cell surfaces, which is most likely caused by the continuous replacement of surface-bound factor VIIIa by non-activated factor VIII present in solution. Potentially, factor Xa generation

on the surface of endothelial cells can be affected by the presence of VWF. Addition of an excess VWF did not affect factor Xa generation by the factor VIIIa/factor IXa complex in which factor VIII was activated by thrombin. In contrast, an increase in the lag phase of factor Xa generation was observed for factor Xa generation that was initiated by factor Xa-activated factor VIII. These results show that VWF can delay the initiation of factor Xa formation when factor Xa is used to activate factor VIII. To substantiate these findings we used a factor VIII variant harboring a Tyr1680Phe substitution which abolishes high affinity binding to VWF. No delay in factor Xa generation by VWF was observed when factor Xa activated FVIII-Tyr1680Phe was used as a cofactor for factor IXa on the surface of endothelial cells. These results show that VWF can delay the generation of factor Xa formation by the factor VIIIa/factor IXa complex on the surface of endothelial cells. Under physiological conditions this protective effect of VWF most likely contributes to an increase of the threshold for activation of pro-coagulant enzymes in the circulation.

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Circulating antibodies to blood coagulation

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Pathogenic antibodies to coagulation factors may develop as a consequence of factor replacement therapy in patients with clotting factor deficiencies. This response is triggered by the administration of a compound which is recognized by the immune system as a foreign agent. Alternatively, pathogenic antibodies against self proteins may arise in previously healthy individuals as a result of the loss of tolerance to self proteins by as yet poorly understood mechanisms. Within this line of research pathogenic antibodies against blood coagulation factor VIII and factor IX that arise in patients with the X-linked bleeding disorder hemophilia A are subject of study. This side-effect occurs in approximately 25% of the patients with severe hemophilia A, and in about 5% of the patients with hemophilia B. Inhibitor development renders patients unresponsive to coagulation factor replacement therapy. Several studies have shown that inhibitory antibodies target the A2, A3-C1 and C2 domain of factor VIII. Binding to the A2 and A3-C1 domains prohibit the formation of a functional factor VIIIa-factor IXa complex whereas anti-C2 domain antibodies interfere with the binding of factor VIII to phospholipids. Tolerance to factor VIII in hemophilia A patients with inhibitors can be restored by the frequent administration of high dosages of factor VIII. Initially during treatment a rise in inhibitor titer is observed which is most likely explained by the activation of factor VIII-specific memory B cells. The mechanisms underlying the successful induction of tolerance have not yet been defined. Studies in a murine model for heophilia A have shown that high dosages of factor VIII interfere with the restimulation of factor VIII specific memory B cells. This prompted us to explore the presence and frequency of FVIII-specific memory B cells in peripheral blood derived from patients with hemophilia A. CD19+ B cells were isolated and stimulated by a mixture of cytokines derived from mitogen-stimulated T cells in the presence of cells expressing CD40 ligand. Factor VIII specific IgG coud be detected after 9-10 days in culture supernatants derived from cultures of 1000 B cells. The frequency of factor VIII specific memory B cells in peripheral blood samples of hemophilia A patients was found to range between <0.01-0.35% of the total number of peripheral memory B cells. These percentages are similar to that observed following vaccination and

viral infections. No or only very low numbers of factor VIII specific memory B cells were observed in hemophilia A patients without inhibitors and in patients who were successfully tolerized. Our findings therefore suggest that restoration of tolerance in hemophilia A patients with inhibitors employing high dosages of factor VIII results in the elimination of peripheral factor VIII-specific memory B cells.

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. Previous findings have shown that the spacer domain harbors a major binding site for anti-ADAMTS13 antibodies. As yet the diagnosis of patients with acquired TTP relies on the measurement of ADAMTS13 activity using a small fluorogenic substrate derived from the amino acid sequence within the A2 domain of VWF that is cleaved by ADAMTS13. This so-called FRETS-VWF73 substrate allows for rapid, real-time monitoring of ADAMTS13 activity. A potential drawback is provided by the quenching of the fluorescent probe by bilirubin thereby interfering with adequate measurement of ADAMTS13 activity. We explored whether the addition of bilirubin oxidase to plasma samples provides an improvement for measuring ADAMTS13 activity in 'hyperbilirubinemic' plasma samples. The results from our study suggest that addition of bilirubin oxidase to 'hyperbilirubinemic' plasma samples results in a strongly decreased coefficient of variation for ADAMTS13 activity as measured by FRETS-VWF73. Based on these findings we propose to apply bilirubin oxidase for more accurate determination of ADAMTS13 activity levels in 'hyperbilirubinemic' plasma samples. Patients suffering from the anti-phospholipid syndrome often develop antibodies directed against β2-glycoprotein I. Patients suffering from this syndrome have a history of vascular thrombosis and/or pregnancy associated morbidity. Many groups have investigated the epitope specificity of antibodies

directed β2-glycoprotein I. It appears that antibodies directed towards domain I of β2-glycoprotein I are clinically most relevant. In future studies we will address the mechanism of action of this important class of pathogenic anti-phospholipid antibodies.

Key publications

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Cellular receptors involved in clearance of factor VIII

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Dysfunction of the mechanism by which factor VIII is removed from the circulation may cause elevated or reduced factor VIII levels, and thus disturb the hemostatic balance. It is therefore of critical importance to gain insight into this mechanism. Moreover, knowledge on the clearance mechanism could provide the basis for prolonging factor VIII half-life, which could be beneficial for factor VIII replacement therapy of patients with hemophilia A. In 1999, we and others established that factor VIII binds to the low-density lipoprotein receptor-related protein (LRP). This

receptor is a member of the LDL-receptor family, which is involved in the binding and cellular uptake of a variety of ligands. Since then we established that factor VIII also interacts with other LDL-receptor family members, including very-low density lipoprotein receptor (VLDLR), low density lipoprotein receptor (LDLR) and megalin (LRP2). We were able to assess the *in vivo* relevance of these interactions using mice with multiple receptor deficiencies. By this approach, we have demonstrated that LRP and the much smaller LDLR are involved in the catabolism of FVIII. In the multi-domain protein factor VIII, which consists of a heavy chain (A1-A2 domains) and a light chain (A3-C1-C2 domains), two LRP binding regions were identified. We have demonstrated that the light chain of factor VIII contains the main LRP interactive region. Others reported that the A2 domain of factor VIII comprises a LRP binding region. We have recently shown that this region is only exposed for interaction with LRP after proteolytic cleavage of the factor VIII heavy chain. We believe therefore that the role of the A2 domain may be limited to the clearance of activated factor VIII. In non-activated factor VIII, LRP binding seems exclusively driven by the factor VIII light chain. In view of the notion that von Willebrand factor (VWF) protects factor VIII from premature clearance, it seems likely that part of the putative LRP-interactive sites in factor VIII are buried in the factor VIII–VWF complex. As we described previously, one LRP-binding region resides in the A3-domain. Using an antibody that inhibits the interaction of factor VIII with both VWF and LRP *in vitro*, we now have identified an additional LRP-binding element, which is located in the C1-domain. We propose that these sites drive the clearance of factor VIII once dissociated from its complex with VWF.

Another question that remained was how these LDL receptor-related proteins bind factor VIII. Each family member contains compact small ligand-binding domains that are clustered in distinct regions within the molecule. LRP contains four clusters of these so-called complement-type repeats for binding their ligands. Direct binding studies revealed that cluster II of 8 repeats (CR3-10) and cluster IV of 11 repeats (CR21-31) were most effective in binding FVIII light chain. To further pinpoint which complement-type repeat mediates the binding, we constructed sub-fragments of the clusters comprising three consecutive repeats. However, surface plasmon resonance analysis revealed that multiple three-repeat containing fragments bound

FVIII. The binding could therefore not be attributed to a specific set of CR domains. We further found that the binding affinity for FVIII light chain of a full length cluster was markedly higher than that of a three-repeat containing fragment. These findings suggested that there is a cooperative binding mechanism between a cluster of repeats and factor VIII light chain. The next issue that will be addressed is the identification of the residues of factor VIII involved in binding the CR domains of LRP and LDLR.

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Immunology

Immunoglobulins

Dimers in intravenous immunoglobulin (IVIg)

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Intravenous immunoglobulin (IVIg) is being used not only for replacement therapy in patients with antibody deficiency, but also in other conditions such as idiopathic thrombocytopenia, Kawasaki syndrome and Guillain-Barre. In applications other than replacement therapy, the mechanisms of action are largely uncertain. Possibilities 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).

IVIg contains a certain amount of dimers in the preparation. The function of these dimers is as yet unclear. Therefore the characteristics of dimers in IVIg are investigated.

The stability of the IgG dimers present in IVIg under different physical conditions was investigated by size-exclusion chromatography and sodium dodecyl sulphate (SDS) electrophoresis. Most dimers dissociate rapidly at conditions mimicking those in patients after administering IVIg. Of the remaining dimers, one type will dissociate upon SDS denaturation and comprise dimers in dynamic equilibrium with monomers as well as dimers that are stable upon dilution. Another type is SDS-resistant. The subclass distribution of the dimeric fraction was found to be skewed towards IgG3, rather than IgG2, which was unexpected in view of a report on covalent dimers in IgG2.

Dimer formation can be the result of Fc-Fc interactions for immunoglobulins of the IgG4 isotype. Several assays including Biacore analysis indicate that this type of interaction requires conformational changes prior to binding. While this potential source of dimers is virtually absent in Nanogam (IVIg manufactured by Sanquin), this property of IgG4 might be relevant *in vivo* to compensate for its functional monovalence as monomer.

Structural and functional properties of human IgG4

Human IgG4 was found to exchange half-molecules with other IgG4 in the blood, which usually results in asymmetric antibodies (i.e. with two different antigen-combining sites). IgG4 is an exception among the IgG molecules, since it does not

activate complement, it binds poorly to Fcγreceptors and cannot form large immune complexes. Altogether this results in an immunoglobulin without inflammatory properties. The inability to form large immune complexes can be explained by the asymmetry of the antibody as a result of exchange. Such an exchange reaction is not observed upon mixing IgG4 antibodies in buffer. This suggested that the process, which involves breaking not only disulphide bonds, but also strong hydrophobic interactions, is catalyzed *in vivo*. In close collaboration with Genmab, two IgG1/IgG4 sets of chimeric mouse/human monoclonal antibodies to two soluble, non-cross-reactive antigens were prepared, as well as a chimeric molecule consisting of IgG1 with the core hinge of IgG4 and/or the CH3 domain of IgG4. Mixtures of these antibodies were found to exchange of half molecules, both *in vivo* (in a mouse model) and *in vitro* (in the presence of glutathione as catalyst).

Key publication

Van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK; Martínez-Martínez P, Vermeulen E, Den Bleker TH, Wiegman L, Vink T, Aarden LA, De Baets MH, Van de Winkel JG, Aalberse RC, Parren PW. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 2007; 317(5844):1554-7.

Inflammation

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The Inflammation Research group focuses on complement activation and on preclinical, *in vitro* testing of new drugs. It was found that covalent fixation of activated C4 and C3 to C1q occurred during classical pathway activation and not during activation of other complement pathways. A differential antibody sandwich ELISA was optimized, and levels of these novel activation products were measured in various diseases. Patients with rheumatoid arthritis (RA) were demonstrated to exhibit higher expression levels of these activation parameters and plasma levels appeared to correlate with disease activity. A quantitative assay to measure C4 fixation by the MBL route was established as well as new methods to measure protein levels and biological activities of C4A and C4B. Furthermore we analyse the properties of anti-C1q antibodies in SLE sera.

Next to complement we are working on in vitro system for preclinical testing of drugs. We analyse the effect of these drugs in whole blood assays, in TLR-transfected cells and in mononuclear cell cultures. Testing a variety of therapeutic plasma proteins we have seen nice correlation with pyrogenicity as measured in the rabbit pyrogen test and in the LAL test. Moreover we observed strong synergy between TLR ligands and (1-->3)-beta-D-glucans.

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Immune regulation

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In this research line we focus on the regulation of immunological cascades that involve proteases by protease inhibitors.

C1-Inhibitor

A major inhibitor of classical complement pathway is C1-inhibitor (C1-Inh), a serine protease inhibitor (serpin). In this research line we focus on i) the generation of recombinant C1-Inh to gain more insight in the structure-function relationships of C1-Inh and ii) to investigate possible new fields of clinical application for C1-Inh. This research is performed in close collaboration with Sanquin Plasma Products.
i) The work on the structure and function of the C1-Inh was continued by comparing the function of plasma-derived C1-Inh to that of recombinant yeast-derived wild

type human C1-Inh and mutants thereof that lack the typically high number of carbohydrate groups. Both wild type and mutant C1-Inh were shown to be functionally active in *in vitro* assays. This year we studied the clearance rate and pharmacokinetics of recombinant C1-Inh infused in rabbits in comparison to plasma purified C1-Inh. We demonstrated that the removal of wild type recombinant C1-Inh from the circulation of rabbits is highly increased compared to plasma-purified C1-Inh, in that the recombinant C1-Inh disappeared from the circulation in minutes, whereas plasma-purified C1-Inh remained in the circulation for more than 18 hrs. Removal of the glycosylation sites in the recombinant C1-Inh doubled half life of C1-Inh in blood, but still was it removed from the circulation within 10 minutes. These data demonstrate that plasma-purified C1-Inh is highly superior in terms of persistence in the circulation compared to recombinant yeast-derived human C1-Inh. This makes plasma-derived C1-Inh the product of choice for treatment for Hereditary Angioedema (HAE) and for other potential clinical indications for C1-Inh, especially in a prophylactic setting where high maintenance levels of C1-Inh are needed. In addition the data show that removal of N-linked glycosylation in the recombinant product aids in increasing the circulation time of recombinant C1-Inh. This probably occurs via reduction of the non-eukaryotic, non-sialylated glycosylation sites that have been described to increase clearance. Currently, we are investigating the glycosylation patterns of plasma-derived and recombinant C1-Inh to confirm this. In addition, we have started a collaboration with dr Roel Bennink (Dept Nuclear Medicine, AMC) to investigate the actual biodistribution patterns of radiolabeled plasma-purified and recombinant (wild type and mutant) C1-Inh in rabbits. This to determine if the observed circulation rates are the result of actual clearance of these products from the circulation or whether it reflects binding of C1-Inh to the endothelium.

ii) We have extended our study whether and by which mechanisms C1-Inh has any cell-protective effects in a model that simulates the mechanical damage that venous cells experience when used in cardiac bypass constructions. We have observed a protective effect of C1-Inh against endothelial damage in this model. In addition, beneficial effects of C1-Inh against complement activation in venous bypass model in mice. This part of the project is performed in collaboration with prof Hans Niessen (Dept Pathology, VUMC) and prof Paul Quax (TNO, Leiden). In addition, in collaboration with prof Frank

Baas (Dept of Neurology, AMC), C1-In was observed to be limit complement activation and axonal damage in a rat animal model of peripheral nerve injury.

Granzymes

Next to the extracellular serpin C1-Inh we continued research on the function of intracellular serpins by studying the role of granzymes and granzyme-inhibiting serpins. Cytotoxic T cells and natural killer cells produce Granzyme A (GrA) and Granzyme B (GrB). In conjunction with perforin, GrA and GrB induce target cell apoptosis. The activity of granzyme B is regulated by the human intracellular serpin SerpinB9 and many cells are protected against the action of GrB via expression of SerpinB9. Expression of the granzymes, perforin or SerpinB9 may be indicative for the severity of inflammatory disease in which CTL and NK cells are the major effector cell types. In collaboration with Reinout Bem and dr Job van Woesel (Pediatric Intensive Care Unit, Emma Children's Hospital, AMC), we demonstrated that both Granzyme A and Granzyme B are activated in children with severe respiratory syncytial virus infection. CTL and NK play also an important role in rejection processes in organ transplantation. We investigated together with dr Ajda Rowshani and prof Ineke ten Berge (Dept Internal Medicine, AMC) whether the presence of Granzyme A and B, perforin and SerpinB in the urine of patients who have undergone renal transplantation correlates with transplant rejection. Indeed, next to GrB and perforin, especially GrA mRNA levels may be a predictive non-invasive biomarker or acute renal rejection. We are currently expanding our samples. For our functional studies on GrA function we generated active recombinant GrA in the yeast Pichia pastoris. We have demonstrated that GrA enters target cells via different plasma-membrane bound Glycosaminoglycans and enter the endosomal-lysosomal pathway. Without perforin, no target cell apoptosis is achieved as GrA needs to enter the cytosol from the endosomal/lysosomal pathway to exert its apoptotic function. Next, we are exploring if we can identify intracellular GrA substrates as well as GrA inhibitors. Finally, it was described that SerpinB9, the inhibitor of GrB, plays a role in protection of dendritic cells (DCs) against GrB action. We are investigating in which phase of CTL and DC interaction SerpinB9 exerts its major effects.

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Antigen presentation

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Antigen Presentation Research addresses the question how the humoral and cellular immune responses are regulated by MHC-mediated antigen presentation in B cells and dendritic cells.

B cells

1) In human B cells, effective class II-Ag presentation depends on MHC class II, but also on HLA-DM and HLA-DO, the chaperones that regulate the composition of the antigenic peptide repertoire. B cell chronic lymphocytic leukemia (B-CLL) is characterized by a chronic immune dysfunction of a.o. the T cell compartment. We previously demonstrated in collaboration with dr Arjan van de Loosdrecht and Martine Chamuleau (Dept Hematology, VUmc) aberrant class II antigen presentation in B cell chronic lymphocytic leukemia (B-CLL) and showed that this correlates with the known shift in the patients' T cell population towards the effector phenotype. This points an antigen driven process of immune activation. In our cohort T cell activation correlates better to parameters of aberrant MHC class II antigen presentation than to CMV-infection, a parameter previously described

to correlate to T cell activation in CLL. This year, we showed that transcriptional deregulation of HLA-DR, HLA-DM and HLA-DO results from hyperactivation of CIITA, the transcriptional master-regulator of the class II genes. Interestingly, especially mRNA levels of DOA, the alpha chain of the HLA-DO complex, seem to be predictive for survival of the patients. Last year, we showed that B cells behave as professional phagocytes of bacteria and particles when recognition is triggered via the specific B cell receptor (BCR). Now we demonstrated that phagocytosis of *Salmonella typhimurium*, 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. Thus, B cells may serve as a niche for survival of *S. typhimurium* from the innate immune system and a transport vehicle for systemic dissemination. In addition however, phagocytosis of Salmonella does lead to efficient antigen presentation of bacterial antigens to CD4+ T helper cells. This process aids in the formation of specific antibodies and therefore an efficient acquired immune response is mounted against *S. typhimurium.* We are currently investigating the effects of these observations in an animal model.

Dendritic cells

2) In our dendritic cell (DC) research program we aim to develop clinically approved, validated and cost-efficient monocyte-derived DC products. For the development of immuno-activatory DCs we extended our research on our newly developed DC maturation-cocktail. We demonstrated that the combination of MPLA, detoxified clinically-approved LPS, and IFN-γ, leads to mature DC that are both able to migrate and produce IL-12. Migration and Il-12 production are both prerequisites for anti-tumor immuno-activatory DC therapy. Migration is needed to allow the DCs to reach the lymph nodes upon administration. IL-12 production is needed to polarise the CD4+ Thelper cells in the lymph node towards the Th1 phenotype, necessary for optimal support of an effective and long-lasting anti-tumor CTL response. We now showed that these DCs indeed induce a strong Th1 response. In collaboration with dr Sheila Krishnadath and dr Francesca Milano (Dept Gastroenterology, AMC) and Prof Martien Kapsenberg (Dept Histology and Cell Biology, AMC) we are investigating the potential of anti-tumor DC therapy

in patients suffering from esophageal cancer. We showed that autologous DC can induce an immune response against esophageal cancer cells *in vitro*.
We have continued our collaboration with dr Carlijn Voermans and Prof Ellen van der Schoot (Dept of Experimental Immunohematology) and dr Hans Vrielink (Blood Bank North West Region) to isolate monocytes via a closed system through a specialised leukocytapheresis method (Haemonetics®) combined with the ElutraTM system. Currently we are optimising DC culture conditions in closed bags and validation freeze/thaw processes of the product.

T cells and antibody formation against biologicals

Finally, we started a new research line on the potential of tolerizing DC therapy in autoimmune disease and transplantation. For this we are investigating various methods to create stable DCs that are able to induce regulatory T cells.
3) Patients that suffer from RA are successfully treated with antibodies against TNFα In a large number of these patients however, therapy fails because patients mount an antibody response against the therapeutic antibody. We are investigating if we can identify the T cell epitopes in the therapeutic antibody Adalimumab that may play a role in antibody formation. For this research project we also aim to generate tetrameric MHC class II molecules as tools to monitor antigen specific T cells in relation to antibody formation against the therapeutic antibody. We are now able to express monomeric class II/peptide complexes in insect cells and are currently in the process of purification and tetramerization of the monomers.

Key publications

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Immunomodulation of blood transfusions in transplantation tolerance

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Aim of the study is by unraveling the blood transfusion effect to define requirements of blood transfusions intended for induction of allogenic tolerance.

In a retrospective study, in collaboration with the Leiden University Medical Centre, in a group of kidney-pancreas transplant patients (1996-2006), we observed that administration of pre-transplantation one-HLA-DR shared blood transfusions resulted in significant less severe acute rejection episodes, necessitating ATG treatment, compared to patients without a protocolled blood transfusion. This effect was irrespective of induction therapy with either ATG or daclizumab, which suggests that a pretransplant protocolled blood transfusion is still valuable combined with current post-transplant immunosuppressive drug therapy.
While our hypothesis is that Tregs maintaining tolerance are induced by indirect T cell stimulation after a protocolled transfusion, a model was sought to measure indirect antigen stimulation *in vitro* using (overlapping) peptides and allogeneic cell lysates. Since, we performed different experiments showing that indirect T cell stimulation tests as published in the literature are of questionable quality and lack appropriate controls for irrelevant peptides and direct stimulation. Extensive *in vitro* experiments applying published protocols show that validation of a model to measure indirect allorecognition by T cells is impossible and questions the results of other studies.
Since 2004 we collected from prospective combined kidney-pancreas transplantation patients who deliberately receive a 1 HLA-DR shared red blood cell concentrate with buffy-coat blood at serial intervals. Currently from 30 donor-patient combinations blood withdrawn prior, and 2 and 10 weeks after transfusion is available for analysis of Tregs and chimerism evaluation.

Key publication

Waanders MM, Heidt S, Koekkoek KM, Zoet YM, Doxiadis IIN, Amir A, Heemskerk MHM, Mulder A, Brand A, Roelen DL, Claas FHJ. Monitoring of indirect allorecognition: wishful thinking or solid data? Tissue Antigens 2008; 71(1):1-15.

Auto-immune diseases

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The Auto-immune Diseases research group aims to identify mechanisms that underlie the formation of auto-antibodies. Our hypothesis is that impaired clearance of dead cells leads to an increased risk for the formation of auto-antibodies against nuclear antigens which in their turn may lead to systemic lupus erythematosus (SLE). Clearance of dead cells is facilitated by a large number of plasma proteins. Indeed when apoptotic T cells are incubated with plasma or serum a number of plasma proteins such as IgM, C3, C4, SAP and clusterin bind to late apoptotic cells. As a side effect we noticed that incubation with plasma lead to rapid removal of nucleosomes from the dead cells. In the absence of serum this takes days but with as little as 5% serum the removal of nucleosomes is completed in 30 minutes.
In a large percentage of SLE sera this activity seems to be absent. We have now identified the responsible plasma protein to be the serine protease hyaluronic acid-binding protein-2 also called Factor VII-activating protein (FSAP). We have developed monoclonal antibodies to FSAP. Those antibodies are now used to affinity purify this protein from human plasma. Furthermore we have developed a sensitive quantitative elisa to measure FSAP levels in biological fluids such as plasma. Two of the monoclonal antibodies directed to the light chain of FSAP inhibit the nucleosomes releasing activity of serum confirming the role of FSAP in removal of nucleosomes from dead cells.
Necrotic cell death leads to release of the nuclear protein HMGB1. Released HMGB1 acts as an endogenous danger signal and seems to be important in a variety of inflammatory conditions. Because decent assays for HMGB1 protein levels are lacking we try to develop monoclonal antibodies to the protein. Differences between species are extremely small. Mouse and human differ in only two amino acids and human and horse HMGB1 are 100% identical. We have now purified horse HMGB1 and immunized mice. Moreover immunization of chickens was started. Because the difference between man and chicken HMGB1 is more extensive we expect a better humeral response in these animals.
As model for antibody formation in auto-immune conditions, antibody formation to the TNF inhibitor drugs infliximab, adalumimab and Enbrel in patients with

RA was investigated. Anti-TNF drugs are nowadays a standard treatment of RA, but clinical responses become limited over time in a large proportion of patients. In cooperation with the Dept of Rheumatology of the Vrije Universiteit Medical Center, the Academic Medical Center, the Slotervaart Ziekenhuis and the Jan van Breemen Institute levels of infliximab and anti-infliximab antibodies were measured in RA patients. Within a year about 50% of the patients developed antibodies to infliximab. Formation of antibodies indeed seems to be a major cause for diminished clinical efficacy. Infliximab is a chimeric monoclonal antibody whereas adalimumab is a fully human antibody. Therefore similar studies were performed in patients treated with adalimumab. Indeed we observed antibody formation in a lower proportion of patients. Nevertheless within a year almost 20% of patients developed antibodies to adalimumab leading to loss of efficacy. In about 200 patients treated with Enbrel, no indication of antibody formation was found. Presently we are expanding the assay repertoire to biologicals with other specificities such as rituximab, trastuzimab and omalizumab.

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Blood transmitted infections

Virological aspects of AIDS

Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection

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HLA B57 and the closely related HLA B5801 are over-represented among HIV-1 infected long-term nonprogressors (LTNPs). It has been suggested that this association between HLA B57/5801 and asymptomatic survival is a consequence of strong CTL responses against epitopes in the viral Gag protein. Moreover, CTL escape mutations in Gag would coincide with viral attenuation, resulting in low viral load despite evasion from immune control. In this study we compared HLA B57/5801 HIV-1 infected progressors and LTNPs for sequence variation in four dominant epitopes in Gag and their ability to generate CTL responses against these epitopes and the autologous escape variants. Prevalence and appearance of escape mutations in Gag epitopes and potential compensatory mutations were similar in HLA B57/5801 LTNPs and progressors. Both groups were also indistinguishable in the magnitude of CD8+ IFN-γ responses directed against the wild-type or autologous escape mutant Gag epitopes in IFN-γ ELISPOT analysis. Interestingly, HIV-1 variants from HLA B57/5801 LTNPs had much lower replication capacity than the viruses from HLA B57/5801 progressors, which did not correlate with specific mutations in Gag. In conclusion, the different clinical course of HLA B57/5801 LTNPs and progressors was not associated with differences in CTL escape mutations or CTL activity against epitopes in Gag but rather with differences in HIV-1 replication capacity.

Susceptibility of recently transmitted subtype B human immunodeficiency virus type 1 variants to broadly neutralizing antibodies

The ability of the broadly neutralizing human immunodeficiency virus type 1 (HIV-1) specific human monoclonal antibodies (MAbs) b12, 2G12, 2F5, and 4E10 to neutralize recently transmitted viruses has not yet been explored in detail. We investigated the neutralization sensitivity of subtype B HIV-1 variants obtained from four primary HIV infection cases and six transmission couples (four homosexual and two parenteral) to these MAbs. Sexually transmitted HIV-1 variants isolated within

the first 2 months after seroconversion were generally sensitive to 2F5, moderately resistant to 4E10 and b12, and initially resistant but later more sensitive to 2G12 neutralization. In the four homosexual transmission couples, MAb neutralization sensitivity of HIV in recipients did not correlate with the MAb neutralization sensitivity of HIV from their source partners, whereas the neutralization sensitivity of donor and recipient viruses involved in parenteral transmission was more similar. For a fraction (11%) of the HIV-1 variants analyzed here, neutralization by 2G12 could not be predicted by the presence of N-linked glycosylation sites previously described to be involved in 2G12 binding. Resistance to 2F5 and 4E10 neutralization did also not correlate with mutations in the respective core epitopes. Overall, we observed that the neutralization resistance of recently transmitted subtype B HIV-1 variants was relatively high. Although 8 of 10 patients had viruses that were sensitive to neutralization by at least one of the four broadly neutralizing antibodies studied, 4 of 10 patients harbored at least one virus variant that seemed resistant to all four antibodies. Our results suggest that vaccine antigens that only elicit antibodies equivalent to b12, 2G12, 2F5, and 4E10 may not be sufficient to protect against all contemporary HIV-1 variants and that additional cross-neutralizing specificities need to be sought.

Intravenous immunoglobulin (IVIG) treatment for modulation of immune activation in human immunodeficiency virus type 1 infected therapy-naive individuals.

We evaluated the ability of intravenous immunoglobulin (IVIG) to diminish immune hyperactivation, which is considered a major cause of CD4+ T cell loss during chronic HIV-1 infection and whether this affected CD4+ T cell counts and plasma HIV-1 RNA (pVL). Therefore, we treated six chronically HIV-1-infected, antiretroviral-therapy-naive patients with IVIG (0.4 g/kg) at weeks 0 and 4, with a follow-up of 12 weeks after the second dosage during which pVL, T cell numbers, and T cell activation were measured. At baseline median CD4+ T cell counts were 300 (range 200-460) x 10^6/liter and median pVL was 5.0 (range 3.2-5.2) log10 copies/ml. IgG plasma levels peaked during the first days after administration. We observed a decrease in the percentage of activated (CD38+ HLA-DR+) CD4+ and CD8+ T cells [3.5% (range 1-7%) and 5% (1-10%), respectively ($p = 0.027$)], but no effect on the fraction of proliferating CD4+ or CD8+ T cells as measured by Ki67 expression. CD4+ T

cell counts were significantly increased on day 4 (median +55 cells, range 0-150, p = 0.043). pVL was significantly increased on day 1 after IVIG infusion (median +0.13 log10, range 0.01-0.55, p = 0.028). All these parameters returned to baseline levels within 1 week after infusion. In conclusion, administration of IVIG caused a temporary decrease in T cell activation and an increase in CD4+ T cell counts, despite an increase in pVL. Our results support the hypothesis that T cell activation, rather than direct HIV-1 infection, mediates the loss of CD4+ T cells and suggest that immunomodulating therapy in HIV-1 infection could indeed be effective.

Key publications

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The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection

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The antiviral factor tripartite interaction motif 5α (Trim5α) restricts a broad range of retroviruses in a species specific manner. Although human Trim5α is unable to block HIV-1 infection in human cells, a modest inhibition of HIV-1 replication was reported. Recently two polymorphisms in the Trim5 gene (H43Y and R136Q) were

shown to affect the antiviral activity of Trim5α *in vitro*. In this study, participants of the Amsterdam Cohort studies were screened for polymorphisms at amino acid residue 43 and 136 of the Trim5 gene and the potential effects of these polymorphisms on the clinical course of HIV-1 infection were analyzed. In agreement with the reported decreased antiviral activity of Trim5α that contains a Y at amino acid residue 43 *in vitro*, an accelerated disease progression was observed for individuals who were homozygous for the 43Y genotype as compared to individuals who were heterozygous or homozygous for the 43H genotype. A protective effect of the 136Q genotype was observed but only after the emergence of CXCR4-using (X4) HIV-1 variants and when a viral load of 104.5 copies per ml plasma was used as an endpoint in survival analysis. Interestingly, naive CD4 T cells, which are selectively targeted by X4 HIV-1, revealed a significantly higher expression of Trim5α than memory CD4 T cells. In addition, we observed that the 136Q allele in combination with the -2GG genotype in the 5'UTR was associated with an accelerated disease progression. Thus, polymorphisms in the Trim5 gene may influence the clinical course of HIV-1 infection also underscoring the antiviral effect of Trim5α on HIV-1 *in vivo*.

Key publications

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Transfusion Technology Assessment

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Notwithstanding that blood products for transfusion are very safe, with new technologies there are always means to improve safety even further. As blood is an allogeneous human material of humans who are exposed to a changing environment with new threats, complete blood safety can never be obtained. In addition emerging infectious diseases may require new safety interventions. Dutch governmental policy is to balance such developments aiming at 'optimal blood safety' versus what is considered as 'maximum blood safety'. Optimal blood safety however is still not well defined, but cost-utility or cost-effectiveness evaluations of safety measures are included in the decision process. Risk assessments underlying these analyses draw attention not only to the decision models but also to communication of present risk – or safety – level to regulatory bodies and the public. With increasing pressure on cost containment in health care, cost-effectiveness analyses of blood safety interventions are internationally becoming more relevant. For these analyses nationally representative data on clinical blood use and blood recipient profiles, including recipient survival, are needed in addition to risk analyses of adverse outcomes of blood transfusion.
Given a new emerging infectious disease, the risk of disease or negative health outcome for recipients of different blood products needs to be assessed. Such assessments require modeling of the transfusion chain as well as costs and effects of given interventions: properties of the (new) infectious agent, donor epidemiology and donation behavior, test characteristics, processing and inactivation steps and distribution of the end products.

In collaboration with the Department of Medical Technology Assessment of the Julius Center for Health Sciences and Primary Care at the Utrecht University, a 'Transfusion Technology Assessment' group was formed, with the explicit mission to perform risk assessments and cost-effectiveness analyses on blood safety and to establish a nationally representative database of clinical blood use and blood recipient profiles. This means measurement and modeling of costs and effects associated with emerging threats given the national blood transfusion data and the evaluation of proposed blood safety interventions.

Ten years of blood transfusions: use, disease and survival (PROTON-study)

In the Netherlands about 954 500 blood component transfusions are given annually. As Sanquin and hospitals are separate organizations, and hospitals have diverse information systems, little quantitative information on transfusion recipient profiles is known. The distribution of various patient groups, underlying diseases, the amount and type(s) of components transfused, and the survival of the recipients are parameters required for evaluating the (cost-) effectiveness of blood safety interventions. Information on patients receiving blood components between 1st January 1995 and 31st December 2006 is collected from 18 Dutch hospitals. The dataset contains information on transfusions (component type, number of transfusions) and transfused patients (age, gender). In addition, some hospitals provided information on diagnosis and surgery. The data are linked to mortality and diagnoses databases (LMR) at Statistics Netherlands (CBS). Distributions of blood components, age and diagnoses are compared for various hospitals. The data were extrapolated to estimate the distributions of blood recipient characteristics in the Netherlands as a whole. The dataset now contains information on 243,938 patients who received 1,822,605 blood products. Distributions of transfusion patient characteristics show similar patterns for hospitals of the same category. The age distribution of academic hospitals shows a large peak for newborns. In both academic and general hospitals most of the blood goes to elderly patients. Most of the red blood cells (RBCs) given in academic hospitals are transfused to patients with neoplasms and circulatory diseases. Even numbers of RBCs are given more often than odd numbers, so RBCs are mostly given in pairs. The post-transfusion survival rate is higher in general hospitals than in academic hospitals. Survival of women who are transfused around child delivery does not differ significantly from survival of the general population. Recipient survival in terms of blood use and underlying disease was analyzed. Censuring the survival rates proved to be complex and a model for proper calculation of the survival was performed in collaboration with the Department of Applied Mathematics of the Technical University Delft. The PROTON study provides quantitative information on various characteristics of blood transfusion recipients. The similarities in distribution patterns of patient characteristics in hospitals of the same category suggest that the (randomly

sampled) hospitals included in the study can be used to extrapolate these distributions to national level. Identifying differences between hospitals on their use of blood can be used for the optimization and of blood usage. The PROTON data are essential for cost-utility analyses on new safety interventions in the blood supply.

Identifying relevant parameters for prediction of national blood demand

Changes in the characteristics of the national population will affect changes in the populations of blood recipients and donors. The Executive Board of Sanquin has asked the TTA group to identify key parameters in the PROTON study relevant for the prediction of possible changes in the distribution and demand of blood components.

Cost-Utility of Blood In Transfused patients (CUBiT-study)

The incremental cost-effectiveness ratio (ICER) of new blood safety measures may show poor cost-effectiveness, as the effects of recent measures are modest in comparison to the already achieved blood safety level. Reporting such unfavorable ICER's of new safety measures such as NAT in the medical literature, may generate biased opinions as to the value of blood transfusion in itself. The Executive Board of Sanquin has asked the TTA group to start a study, based on the PROTON data, to establish the cost-utility of blood as a therapy in different transfused patient categories.

Modeling emerging Infections in the Transfusion Chain (MITCH-study)

Recent collaboration initiatives of the Julius Center and the TTA group with scientists and Prof RA Coutinho of the Center for Infectious Disease Control (Cib) of the National Institute for Public Health and Environment (RIVM), strengthens the knowledge base for infectious disease modeling in the transfusion chain. Factors such as global climate change, increased traffic and intensive agricultural methods generated increasing concern about recent outbreaks of emerging infections. Rational decisions for blood safety based on quantitative risk assessments are needed. The Executive Board of Sanquin has asked the TTA group to develop risk models for emerging Transfusion Transmitted Infections (TTI) in the transfusion

chain. As emerging infections can be unpredictable by nature, a generic TTI model in the transfusion chain will be developed encompassing all relevant model parameters for biological and epidemiological characteristics of example TTI's, representative of different possibly emerging TTI's. This model will allow *ad hoc* introduction of actual emerging infectious disease parameters into the model, thereby allowing timely assessment of the quantitative risk to the blood supply. The risk analysis and decision processes include structured expert opinion elicitation and decision frameworks. Such strategies are common in other fields like industrial risk management, water management and environment, however they are not yet practiced in the blood transfusion chain.

Viral risks of plasma-derived medicinal products

New European legislation (EMEA guideline CPMP/BWP/5180/03) requires a viral risk assessment for HBV, HCV, HIV, Parvo B19 and HAV for all new market applications of plasma products. A risk model was developed for Sanquin Plasma Products on the basis of viral and test characteristics, donor epidemiology and the Dept of Virus Safety Studies (VSS) inactivation data. The model has been discussed at confidential meetings of the International Plasma Fractionation Association (IPFA) with risk assessors of the Biotechnology Products Laboratory (BPL) of the United Kingdom. The results of model sensitivity analyses show that the residual risk is mainly determined by the viral incidence rate, screening test sensitivity, viral reduction capacity and the product yield. The production pool size and type of donation (apheresis or whole blood donation) have low impact on the residual risk. Increasing the inventory hold period has a modest impact on the residual risk, only 0.5 logs for 1 year increase in hold period. The results show that there is large dispersion in the residual risk estimates (2 to 6 logs) depending on type of virus. Monte-Carlo (probabilistic) simulations are essential when estimating residual risks of blood products. This approach in contrast to traditional risk estimation allows incorporation of complex process specific decision strategies into the risk model. It also allows modeling of uncertain model parameters, like incubation time, duration of the window phase or viral load of an infected donation. Counter-intuitive findings were that production pool size and type of donation e.g. apheresis or whole blood

donation only have a limited impact on the residual risk. The detailed results of the study remain proprietary of Sanquin, and are to be submitted to the European Medicines Evaluation Agency (EMEA), however the methodology of the risk assessment, which is first in this field, has been presented at several conferences and has recently been published.

Experimental design and analysis of Viral Validation Studies

A study was performed to evaluate the effectiveness of the design and analysis of the robustness of virus validation studies. The aim is to improve on the output of these expensive experiments through evaluation of the current experimental design process and by applying advanced statistical techniques for analyzing the results. This work is performed in collaboration with the Department of Applied Mathematics of the Technical University Delft. It was found that indeed more information on relevant process parameters can be obtained by applying more advanced modeling techniques.

Determining the frequency of positive test results of additionally tested manufacturing pools

A study was performed to predict the frequency of positively NAT tested manufacturing pools for a plasma product. In this case the pool size of minipool NAT testing approaches the order of magnitude of the pool size of the manufacturing pools. It was found that given the donor epidemiology, additional NAT testing of the manufacturing pool can improve the safety of these plasma products, however at considerable loss of manufacturing pools. An alternative strategy may be to reduce the pool size of minipool NAT testing. The results of the study remain proprietary of Sanquin.

vCJD risk of plasma-derived medicinal products

A risk model was developed for Sanquin on the basis of expert opinion on variant Creutzfeldt-Jakob Disease (vCJD) and estimates on donor epidemiology and production process inactivation data. Monte-Carlo simulations were used for estimating the contamination risk of blood components and plasma products.

Model outcomes were discussed at confidential meetings with the Medical Advisory Board of Sanquin. The methodology has been discussed at the international risk assessment meetings of the International Expert Advisory Group of Health Canada workshops on Iterative Risk Assessment Processes for Policy Development Under Conditions of Uncertainty and Emerging Infectious Diseases. The results of the study remain proprietary of Sanquin.

Monitoring of viral infection incidence rates among blood donors in the Netherlands

Presently EMEA requires manufacturers of plasma products to report on the prevalence and incidence rates of HIV, HBV and HCV in donor populations. A proprietary report is written for Sanquin. An important measure of residual blood safety is the incidence rate. The goal of the project is to develop a monitoring tool, enabling the detection of significant deviations in incidence rates in repeat tested donors. First a developed monitoring tool is used to check changes in the incidence rates on the national level. Second, incidence rates variability is explored and regional differences within the Netherlands are examined. Furthermore, statistical tests are used to evaluate and thus enable controling the infection rates in the repeat tested donor population. For this purpose, two tests were developed. As there is a dependency between donation frequency of the infected donors and the estimated HBV incidence rate, an improved estimation process is proposed. To this end the correction of the observed inter-donation interval is made. Advice was provided on adjusting the present reporting on confirmed positive donors. The report remains proprietary of Sanquin and the results will be discussed at EMEA.

Cost-effectiveness of (HBV-) NAT testing in The Netherlands

Sanquin is to replace its HIV / HCV NAT testing program in 2008, as NAT laboratories will be further centralized and technology needs replacement. In addition NAT for Parvovirus B19 is performed for plasma products and NAT for HBV and HAV is considered. Based on the data of the infectious disease epidemiology among donors of Sanquin, the different NAT test characteristics available for donor screening (especially for HBV) and the recipient survival characteristics of the TTA transfusion chain model, the (incremental) cost-effectiveness ratio's (CER) of the

different NAT options will be estimated with priority on the decisions regarding addition of HBV NAT to the test algorithms. For the modeling the HBV infection, collaboration with the National Institute for Public Health and Environment (RIVM) was established.

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

The annual reporting on the collection, testing and use of blood and blood components in the Council of Europe Member States is an assignment of the Council of Europe to the TTA group since 2001. As the 2004 data have been communicated, the 2005 and 2006 data are to be included in a Trend Analysis on Blood Transfusions in Europe since 2001. The robustness of the data can become apparent by consistency over time. A draft was submitted for review. The reports are to be published by the Council of Europe, EDQM Dept of Biological Standardization, OMCL Network and Healthcare, Strassbourg.

Key publications

Janssen MP, Over J, Van der Poel CL, Cuijpers HT, Van Hout BA. A probabilistic model for analyzing viral risks of plasma-derived medicinal products. Transfusion 2008; 48:153-162.

Van Geloven, N. Statistical evaluation and design of virus validation robustness studies. Masters Thesis, Technical University Delft, May 2007.

Van der Bij AK, Coutinho RA, Van der Poel CL. Surveillance of risk profiles among new and repeat blood donors with transfusion-transmissible infections from 1995 through 2003 in the Netherlands. Transfusion 2006; 46:1729-1736.

Janssen MP, Van der Poel CL, Buskens E, Bonneux L, Bonsel GJ, Van Hout BA. Costs and benefits of bacterial culturing and pathogen reduction in the Netherlands. Transfusion 2006; 46:956-965.

Quality, safety and efficiency

Pathogen detection and inactivation

Detection of bacterial contamination of blood products by 16S rDNA PCR

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In 2007, the real-time PCR assay based on the 16S rRNA gene was optimized for detection of a broad range of bacteria in plasma and platelet concentrates (PC). A lambda phage internal control was constructed and implemented in the assay, which made the assay suitable for diagnostic use. Spiking studies in plasma and PCs were performed to determine the analytical sensitivity of the assay. Thirty three colony forming units (CFU)/ml of E. coli and 72 CFU/ml of *Staphylococcus epidermidis* could be detected in plasma, and 97 CFU/ml of *S. epidermidis* in PCs. The assay detected all bacteria relevant for bacterial contamination of PCs. The short turn around time of the assay makes it a candidate for testing PCs for bacterial contamination prior to transfusion.

Key publication

Pietersz RNI, Engelfriet CP, Reesink RW, Wood EM, Winzar S, Keller AJ, Wilson JT, Henn G, Mayr WR, Ramírez-Arcos S, Goldman M, Georgsen J, Morel P, Herve P, Andeu G, Assal A, Seifried E, Schmidt M, Foley M, Doherty C, Coakley P, Salami A, Cadden E, Murphy WG, Satake , De Korte D, Bosnes V, Kjeldsen-Kragh J, McDonald C, Brecher ME, Yomtovian R AuBuchon JP. Detection of bacterial contamination of platelet concentrates. International Forum. Vox Sanguinis 2007; 93:260-77.

Validation of pathogen inactivation by UV-C irradiation in platelet concentrates

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Pathogen contamination, causing transfusion-transmitted diseases, is an ongoing concern in transfusion of cellular blood products. Thus, there is a strong need for in-process steps with broad pathogen-inactivating capacity without compromising the quality of cellular blood products. In an explorative study, the pathogen inactivating capacity of UVC irradiation in platelet concentrates was investigated. The dose dependencies of inactivation of several viruses and bacteria were compared with the effect on platelet quality. A range of lipid-enveloped (LE), and non-lipid-enveloped viruses (NLE), and bacteria were studied. LE viruses were bovine viral diarrhoea virus (BVDV), human immunodeficiency virus (HIV), pseudorabies virus (PRV), transmissible gastroenteritis virus (TGEV), and vesicular stomatitis virus.

NLE viruses were canine parvovirus (CPV) and simian virus 40 (SV40). Bacteria were *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*. After spiking and irradiation, samples were tested for residual infectivity and reduction factors were calculated. Furthermore, the effect of UVC irradiation on platelet quality was determined by measuring in vitro quality parameters.
A UVC dose of 500 J/m^2 resulted in acceptable platelet quality (as measured by pH, lactate production, CD62P expression, and exposure of phosphatidylserine) and high reduction factors (>4 log10) for CPV, TGEV, VSV, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Escherichia coli*. Intermediate reduction factors (about 3 log10) were observed for BVDV, PRV, and *Bacillus cereus*. Low reduction factors (about 1 log10) were found for HIV and SV40. No differences in virus reduction were observed between cell-free and cell-associated virus, indicating that the presence of infected cells is not influencing the efficacy of virus inactivation. It was concluded that UVC irradiation is a promising pathogen reducing technique in platelet concentrates, inactivating bacteria and a broad range of viruses under conditions that have limited effects on platelet quality. However, further optimization of the UVC procedure is necessary to deal with blood-borne viruses like HIV.

Key publication

Terpstra FG, Van 't Wout AB, Schuitemaker H, Van Engelenburg FA, Dekkers DW, Verhaar R, De Korte D, Verhoeven AJ. Potential and limitation of UVC irradiation for the inactivation of pathogens in platelet concentrates. Transfusion 2008; 48(2):304-13.

Pathogen inactivation by UV-C treatment

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In studies reported above, it was found that UV-C irradiation is an effective approach for inactivation of pathogens in platelet concentrates with the exception of HIV. However, preliminary results indicated that UV-C irradiation of platelets can induce platelet aggregation. We therefore investigated the mechanism underlying this phenomenon in more detail.
Irradiation of platelets with UV-C light (1500 J/m^2, i.e. 3-fold higher than required for inactivation of most pathogens) caused platelet aggregation, which was caused by activation of the integrin αIIbβ3 (GPIIb-IIIa). Once activated, this integrin

mediates the binding of fibrinogen and thereby platelet aggregation. UV-C induced activation of αIIbβ3 occurred despite treatment with several known inhibitors of platelet activation. UV-C also induced activation of recombinant αIIbβ3 in CHO cells, an environment in which physiological agonists fail to activate. Activation of αIIbβ3 by platelet agonists requires talin binding to the β3 tail, yet αIIbβ3-Δ724 (lacking the talin binding site) was activated by UV-C light, excluding a requirement for talin binding. The UV-C effect appears to be general for integrins, because β1 and β2 integrins present on leukocytes were also activated by UV-C.
To explain these findings, we investigated the possibility of UV-C induced photolysis of disulphide bonds, in analogy with the activating effect of DTT on integrins. Indeed, UV-C induced a great increase in free thiol groups on platelet surface proteins, which included the integrin αIIbβ3.
These results show that UV-C appears to activate αIIbβ3 not by affecting intracellular signal transduction, but by reduction of disulphide bonds regulating the integrin conformation. This side effect may limit the application of UV-C for pathogen reduction in platelet concentrates, unless an efficient strategy can be developed to prevent it.

Key publications

Dekkers DW, De Cuyper IM, Van der Meer PF, Verhoeven AJ, De Korte D. Influence of pH on stored human platelets. Transfusion 2007; 47(10):1889-95.

Terpstra FG, Van 't Wout AB, Schuitemaker H, Van Engelenburg FA, Dekkers DW, Verhaar R, De Korte D, Verhoeven AJ. Potential and limitation of UVC irradiation for the inactivation of pathogens in platelet concentrates. Transfusion 2008; 48(2):304-13.

Viral safety of human milk

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In the United States, concerns over the transmission of infectious diseases have led to donor human milk generally being subjected to pasteurization prior to distribution and use. The standard method used by North American milk banks is Holder pasteurization (63°C for 30 minutes). An alternative for this Holder process is a high-temperature short-time (HTST) pasteurization process (72°C for 16 seconds) and the efficacy of this HTST process was investigated.

The process step was studied with duplicate spiking experiments using three lipid-enveloped (LE) and two non-lipid-enveloped (NLE) viruses, and three bacteria. The LE viruses used were bovine viral diarrhoea virus (BVDV), human immunodeficiency virus (HIV) and pseudorabies virus (PRV); the NLE viruses used were porcine parvovirus (PPV) and hepatitis A virus (HAV). The bacteria used were Escherichia coli, Staphylococcus aureus and Streptococcus agalactiae.
After spiking, kinetic samples were collected and tested for residual infectivity, and the reduction factors were calculated.
For the HTST step complete reduction was demonstrated for all LE viruses tested, resulting in >5.4 log10 for BVDV, >7.3 log10 for HIV and >7.3 log10 for PSR. For the NLE viruses no complete reduction was observed; for HAV the reduction was approximately 2 log10 and for PPV no reduction was observed.
The bacteria tested in the HTST step were all inactivated after 16 seconds resulting in calculated reductions of >32 log10 for *Escherichia coli*, >15 log10 for *Staphylococcus aureus* and >26 log10 for *Streptococcus agalactiae*.
It was concluded that HTST treatment may be an alternative for the Holder pasteurization; however, a major drawback of HTST is that it is extremely expensive.

Key publication

Terpstra FG, Rechtman DJ, Lee ML, Hoeij KV, Berg H, Van Engelenberg FA, Van't Wout AB. Antimicrobial and antiviral effect of high-temperature short-time (HTST) pasteurization applied to human milk. Breastfeed Med 2007; 2(1):27-33.

Viral safety of antivenoms

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Antivenoms are manufactured by the fractionation of animal plasma which may possibly be contaminated by infectious agents pathogenic to humans. A study was carried out to determine whether pre-existing antivenom production steps may reduce viral risks. The results of two typical downscaled manufacturing steps are presented, a pH 3.3 pepsin digestion of diluted plasma at 30°C for 1 hour and a caprylic acid treatment of a purified F(ab')2 fragment fraction at 18°C for 1 hour. Three lipid-enveloped (LE) and one non-lipid-enveloped (NLE) virus were applied. The LE viruses used were bovine viral diarrhoea virus (BVDV), pseudorabies virus

(PSR) and vesicular stomatitis virus (VSV). BVDV was chosen as a model for West Nile virus and Eastern, Western, and Venezuelan Equine Encephalitis togaviruses, PSR as a model for pathogenic equine herpesviruses and VSV as relevant horse plasma-borne virus. Encephalomyocarditis virus (EMC), a picornavirus, was chosen as a general model for NLE viruses.

After spiking, samples were collected and tested for residual infectivity, and the reduction factors were calculated.

The pH 3.3 pepsin digestion step resulted in complete reduction of PSR (>7 log10) and in almost complete reduction of VSV (>4.5 but ≤6.4 log10). For BVDV and EMC a limited inactivation was found resulting in 1.7 log10 and (≥2.5 but ≤5.7 log10, respectively.

The caprylic acid step resulted in complete inactivation of the three LE viruses tested, >6.6 log10 for BVDV and PSR, and >7.0 log10 for VSV. For EMC no significant reduction was obtained (0.7 log10).

The overall cumulative virus-reducing capacities were > 13.6 log10, > 11.5 log10, > 8.3 log10and ≥ 2.5 log10 for PSR, VSV, BVDV and EMC, respectively. It was concluded that the manufacturing process of antivenoms includes high viral inactivation of LE viruses and moderate inactivation of the NLE virus tested.

Key publication

Burnouf T, Terpstra F, Habib G, Seddik S. Assessment of viral inactivation during pH 3.3 pepsin digestion and caprylic acid treatment of antivenoms. Biologicals 2007; 35(4):329-34.

Improving materials and methods for storage of blood components

Red cells

Improved erythrocyte storage solution

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Recently, we developed an improved additive solution for red cell concentrates (RCC) allowing maintenance of high 2,3-DPG levels during 35 days of cold storage without concurrent ATP decline. This solution is based on the 'chloride-shift' principle demonstrated about 15 years ago by Meryman, resulting in a more alkaline cytosol favoring 2,3-DPG formation.

In 2007, we investigated methods to overcome the problem that the new solution can not be sterilized in an autoclave (due to caramelization of the glucose at the alkaline pH of the solution). It was established that splicing the solution in an acidic part with glucose and an alkaline part with the other constituents could solve the problem, because these two separate solutions can each be autoclaved. When the red cells were mixed with the alkaline part and afterwards the acidic glucose was added, the results for *in vitro* quality parameters during storage were similar to those obtained with the original solution.

Key publication

De Korte D, Kleine M, Korsten HGH, Verhoeven AJ. Prolonged maintenance of 2,3-DPG and ATP in red blood cells during storage. Transfusion (in press).

Effect of cooling rate and time of storage at room temperature before cooling on red cell concentrates *in vitro* quality

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Whole blood is routinely stored at room temperature before it is processed into components. During various processing steps, red cell concentrate may be stored at room temperature or at 4°C. This affects red cell quality. Also, the cooling speed can differ depending on the conditions. Therefore, experiments were done where units were immediately after preparation rapidly (<2 hours till 4°C) cooled, slowly cooled (>10 hours till 4°C) or were stored for 6 hours before slow cooling. The ATP-content, predictive of *in vivo* red cell survival, showed a beneficial effect of 6 hour storage and slow cooling

during the first weeks of storage. No adverse effects were seen induced by the cooling rate. At the completion of storage, any effect of holding or cooling had leveled off. These data confirm that current routine practices do not affect red cell quality.

Hematopoietic stem cells

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Hematopoietic stem cell (HSC) transplants are considered to be the best treatment option for many patients with (hematological) malignancies. In case of autologous transplantation and cord blood transplantation, it is necessary to cryopreserve the stem cells. Although the clinical results show that cryopreservation is successful in maintaining HSC viability, at least to a certain extent, it is generally accepted that cell loss/cell death occurs during cryopreservation. Improved cryopreservation protocols most likely result in decreased cell death and improved engraftment kinetics. This will decrease the costs and morbidity of autologous transplantation.
To improve the freezing protocol we used a new theoretical model, developed by Dr H Woelders (Animal Sciences Group, Wageningen UR) for the osmotic events occurring during cryopreservation. This model is mainly based on the permeability of blood cells for water and cryoprotectant. With literature values of cell characteristics and membrane permeability parameters for hematopoietic progenitor cells, simulations were performed using various concentrations of DMSO, resulting in prediction of optimal freezing protocols. The optimal cooling rates depend on the concentration of DMSO. The calculated freezing curves for 5% and 10% DMSO were applied in a programmable controlled-rate freezer and used for freezing hematopoietic progenitor cells CD34+ selected and unselected PBSCs were cryopreserved using the current or the new freezing curves. Post-thaw quality was evaluated by cell viability, colony formation and megakaryocyte outgrowth. With 10% DMSO, the use of the predicted optimal freezing curve resulted in increased post thaw viability of CD34+ cells, colony formation and megakaryocyte outgrowth. Lowering the DMSO concentration to 5% resulted in improved post-thaw viability and funcitionality, which was not further improved by using the theoretically optimized freezing curve.
Our results indicate that the current cryopreservation method for PBSCTs can be improved by either lowering the DMSO concentration to 5% or by using the

theoretically optimized freezing curve. Infusion of less DMSO and more viable cells might improve the outcome of PBSCT.

Key publication

Tijssen MR, Woelders H, De Vries-van Rossen A, Van der Schoot CE, Voermans C, Lagerberg JWM. Improved post-thaw viability and in vitro functionality of peripheral blood hematopoietic progenitor cells after cryopreservation with a theoretically optimized freezing curve. Transfusion 2008; Epub ahead of print

Platelets

Extension of storage from 5 to 7 days of split leuko-reduced apheresis platelet concentrates in plasma for pediatric transfusion

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Leuko-reduced apheresis platelets in plasma are divided into 4 units for pediatric transfusion. The whole apheresis unit is stored in a platelet storage bag with a volume between 1 and 1,5 L depending on the manufacturer. The split units are stored in small (600 mL) bags in plasma up to 5 days. On the market are bags with a nominal volume of 600 mL made from polyolefin or PVC-BTHC (butyl-trihexyl-citrate), but these bags allows only 5-day storage of the split products. A paired comparison of various newly developed bags in cooperation with one bag manufacturer revealed one suitable bag for storage of split apheresis concentrates, with maintenance of in vitro quality during at least 7 days. In 2008 this bag will be validated for routine application in Sanquin, which will result in uniform shelf-life for standard platelet concentrates (apheresis or buffy coat, full unit or split unit) in the Sanquin product portfolio.

Storage of hyperconcentrated platelet products

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There is an increasing request from hospitals for hyperconcentrated platelet products. In this procedure, a platelet concentrate of about 350 mL is reduced in volume to about 20 mL. Currently these products are made in a syringe and have a shelf life of only 3 hours. To improve quality and facilitate logistics, a system was developed where the hyperconcentrate is stored in a small gas-permeable bag and thereafter can be aspirated in a syringe. An additional advantage of this system is

that the hospital can decide if the hyperconcentrate is administered intravenously via syringe and needle or by coupling the bag to an infusion set. Studies showed that for platelet concentrates in plasma the *in vitro* quality remained good for up to 6 hours in the gas-permeable bag, followed by 1 hour in a syringe. Successive experiments with platelet additive solutions and apheresis platelet concentrates will be conducted in 2008. The new system will be validated during 2008 in blood bank practice to allow extension of shelf life from 3 to 6 hours.

In vitro evaluation of platelet quality during storage of split buffy coat platelet concentrates

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Young children who need a platelet transfusion receive (split) apheresis platelet concentrates (A-PC) to reduce donor exposure. Sometimes, in case of shortage of A-PCs, a buffy coat-derived PC (BC-PC) is split in two units. One part of the unit is kept in the original 1,000 ml DnDP storage container and the other part is kept in a 600 ml polyolefin storage container. *In vitro* quality of these split BC-PCs is unknown. The *in vitro* quality of split BC-PCs during storage was analysed in both storage containers. We concluded that both containers are suitable for storage of split BC-derived PCs for the entire storage time (7 days) of the original platelet product.

Platelet storage lesion and mitochondria

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Platelets can be stored for 7 days in plasma prior to transfusion, but several biochemical and functional parameters do change, together constituting the 'platelet storage lesion'. In the past year, the role of mitochondria in the platelet storage lesion was investigated because of the role of mitochondrial deterioration in the apoptosis of nucleated cells.

Platelet concentrates (PC) in additive solution and 30% residual plasma were stored for 16 days at 22°C (with addition of extra glucose at day 8) to amplify storage-induced changes. In short-term experiments, mitochondrial contribution to platelet metabolism was excluded by addition of FCCP, an uncoupler of mitochondrial oxidative phosphorylation.

Platelets stored for 16 days showed only a slight decrease in the mitochondrial membrane potential (MMP) as measured with JC-1 fluorescence, and also cytosolic ATP/ADP ratios were well maintained. In contrast, PS exposure gradually increased

and CD62P expression was already maximal after 10 days of storage, as indicated by flow cytometry experiments. Changes in PS exposure and the MMP were not correlated at the single cell level as determined by a newly developed double staining protocol.
There was no Caspase-9 or -3 activation or degradation of Bcl-XL during prolonged storage, indicating no change in key regulators of apoptotic cell death. In short-term experiments with FCCP, PS exposure remained low provided the cytosolic ATP/ADP ratio could be maintained by glycolytic ATP production.
This extended storage study showed that platelet activation rather than an apoptotic stimulus derived from the mitochondria causes the deterioration of platelets during long-term storage.

Platelet Hyperconcentrates treated with Mirasol® PRT

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Mirasol® Pathogen Reduction Technology (PRT) treatment inactivates leukocytes and a wide range of bacteria and viruses in platelet concentrates. This process involves the addition of riboflavin in combination with UV light. Storage of platelets in additive solution (PAS) has become an attractive alternative to storage in plasma, because it increases the amount of plasma available for other purposes and may reduce the incidence of non-hemolytic transfusion reactions such as transfusion-related lung injury (TRALI). A protocol was developed to generate double buffy coat platelet concentrates (BCPC) in low plasma volumes that could be PRT treated as double units and subsequently stored in PAS. *In vitro* cell quality of the platelets was assessed over 7 days of storage.
Double BCPCs were generated from 10 pooled buffy coat units. The average yield of the double units was $7.6x10^{11}$ in 229 mls of plasma ($3.3x10^{9}$/ml). Units were PRT treated as double units, SSP+ (Macopharma) was added post illumination (average 280 ml) to dilute platelets to $1.4x10^{9}$/ml and the units were split into two single units for storage (average volume 250 ml and $350x10^{9}$ plt per unit). Platelet function was assessed by pH, swirl, morphology, lactate production and glucose consumption rates, CD62P expression and Annexin V staining over 7 days of storage. The effect of additional glucose (10 mM) in the storage media on cell quality was analyzed.
The pH in PRT treated units on day 7 was only slightly decreased (7.13±0.04)

compared to untreated controls (7.42±0.03). PRT treated and untreated units maintained positive swirls throughout storage. The lactate production and glucose consumption rates on day 7 in PRT treated units was about 30% elevated compared to untreated control units. CD62P expression was increased in PRT treated units (39±5 %) compared to untreated controls (12±2 %). No significant difference due to PRT treatment could be detected on morphology (Kunicki score) or by Annexin V staining. The addition of glucose to the storage medium had no effect on the in vitro cell quality parameters measured.
PRT treatment of double BCPC generates two single BCPC units that can be stored for 7 days in PAS without compromising cell quality. Elevated lactate production and glucose consumption rates and CD62P expression after PRT treatment is due to cellular activation and increased metabolism of the cells. Data from clinical studies are needed to show the relevance of increased cellular activation on in vivo performance of PRT treated platelets.

Improving materials and methods for Blood Bank processing

Apheresis monocytes for dendritic cell culturing

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Apheresis techniques to obtain a monocyte rich component were developed by dr JJ Zwaginga, Sanquin Research. The effect of multiple apheresis procedures to collect monocytes from voluntary donors was studied and results were presented on international meetings. Based on the results of the apheresis procedures performed, the new developed apheresis disposable (Haemonetics) could be CE marked. To purify and enrich the monocyte fraction, products are further processed using the ELUTRA equipment from Gambro. To optimize the procedure, new parameter settings were implemented and evaluated.
Subsequently, the product was handed over to Sanquin Research (dr Ten Brinke and dr Van Ham) where the cells were cultured to become dendritic cells under sterile and GMP conditions. The first results show that it is possible to obtain a product with very few granulocytes and a high number of monocytes, suitable for further processing under sterile and GMP conditions.

Key publication

Ten Brinke A, Karsten ML, Dieker MC, Zwaginga JJ, Vrielink H, Van Ham M. Generation of dendritic cells for immunotherapy is minimally impaired by granulocytes in the monocyte preparation. Immunobiology 2006; 211(6-8):633-40.

Apheresis granulocytes from G-SCF stimulated related donors

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The demand for granulocyte apheresis components is still increasing. Based on the Sanquin Blood Bank North West region/University Hospital Utrecht protocols, these were rewritten in co-operation with Sanquin Blood Bank South West region and LUMC, resulting in a national apheresis protocol, a national protocol on treatment of pediatric patients (SKION protocol) and a draft Sanquin guideline Therapeutic Granulocyte Transfusions ('Richtlijn Therpapeutische granulocyten transfusies') to harvest granulocytes via apheresis following G-CSF and dexamethasone stimulation of related donors. International-wide interest for the protocols and co-operation was provoked. To assess the functional characteristics and efficacy of G-CSF and dexamethasone mobilized granulocytes and their effects in transfused patients, small samples from granulocyte apheresis components were tested and results were followed after transfusion in critically ill patients suffering from life-threatening infections.

Key publications

Drewniak A, Boelens J-J, Vrielink H, Tool AJ, Bruin MCA, Van den Heuvel M, Ball L, Van de Wetering MD, Roos D, Kuijpers TW. Granulocyte concentrates: prolonged functional capacityduring storage in the presence of phenotype changes. Haematologica. Accepted for publication.

Sharon RF, Bierings M, Vrielink H, Versluys B, Boelens JJ. Pre-emptive granulocyte transfusions enable allogeneic hematopoietic stem cell transplantation in pediatric patients with chronic infections. Bone Marrow Transplant 2006; 37(3):331-3 (letter).

Granulocyte apheresis with MCS+ (Haemonetics)

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There is an increasing demand for granulocyte components in children. In practice, all procedures to collect granulocytes are performed with COBE® SpectraTM. However, this machine is not widely available at the Dutch donor centers. MCS+

(Haemonetics) equipment, however, is available at the donor centers, but no collection protocols for granulocyte apheresis are available. Therefore, in 2007, a granulocyte apheresis procedure for MCS+ was written. To achieve basic figures, the outcome of 10 granulocyte apheresis procedures in voluntary donors not stimulated with G-CSF or dexamethasone were evaluated. In these donors, we were able to collect a 70 mL product with approximately 4x10e^{9} WBCs per unit and approximately 23% of granulocytes. It is likely that collection efficiency will increase in G-CSF and dexamethasone stimulated donors and/or with the use of sedimentation agents such as HES. It is therefore expected that the procedure to collect a granulocyte component with MCS+ will conform to the European guidelines and that this procedure can be finished in two hours.

Evaluation of a 'high throughput' needle

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The donation time is determined by the flow speed of whole blood during collection. By modifying the needle inset, one manufacturer aimed to reduce sheer, thereby increasing flow speed. A comparison of blood collections with this 'High throughput' needle revealed that flow speed increased by 19%, thereby reducing collection time from on average a little over 8 minutes to less than 7 minutes. The number of donations with a collection time >12 min halved, and the number of speed alarms reduced significantly. Introduction of the new needle will increase donor-friendliness, due to faster blood collection with fewer donation alarms.

Validation studies

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In 2007 many projects were executed by the Dept of Research & Development of Sanquin North East Region.Various methods and machines were validated and implemented (Sysmex XT2000i, BCSI pH1000, Compomat G4).
Furthermore studies were done for counting very low levels of leukocytes in plasma (10^{3}-10^{6} leukocytes/unit), the settling of leukocytes in filtrated blood product during the leukocyte counting and for counting platelets using flow cytometry (CD41).
For contract partners we performed a study testing whole blood filters and a study testing platelet filters. Furthermore, we evaluated a new pH measurement device, which make it possible to measure pH of the platelet concentrate in a sterile way

without sampling at any moment, in clinic and blood bank.
We assisted in the production of fibrin glue by performing all quality control tests for fibrin glue. Beside these quality studies preparations were made to start a multi center study to investigate the effect of fibrin glue in patients that have undergone knee or hip replacement.
Five national send arounds were organized by R&D, (i) two times for counting platelets in platelet concentrates, (ii) two times for counting leukocytes in plasma, red cell concentrates and platelet concentrates and (iii) once for measuring pH in platelet concentrates. These send arounds are repeated yearly.
A post authorization surveillance (PAS) for monitoring platelet increments after platelet transfusion in three hospitals was finished at the end of 2006. The data of this PAS were analyzed in 2007 and an internal report was written.

In co-operation with Prof E Vellenga of the UMCG a project called 'Enhancing the quality and quantity of stemcells in autologous transplants', and funded by the Tekke Huizinga foundation, was started. The aim of this project is i) To characterize the stem cells defects for long-term engraftment in patients undergoing autologous SCT, ii) To search for clinically relevant ways to treat the transplant and restore the quality of stem cells.

Key publications

Dijkstra-Tiekstra MJ, Kuipers W, Setroikromo AC, De Wildt-Eggen J. Platelet counting in platelet concentrates using various automated hematology analyzers. Transfusion 2007; 47:1651-7.

Dijkstra-Tiekstra MJ, Kuipers W, Setroikromo AC, De Wildt-Eggen J. Overnight or fresh buffy coat derived platelet concentrates prepared with various platelet pooling systems. Transfusion. Accepted for publication.

Dumont LJ, Gullikssson H, Van der Meer PF, Murphy S, Nixon JG, De Wildt-Eggen J, Vandenbroeke T, Aubuchon JP. Interruption of agitation of platelets concentrates; a multicenter in vitro study by the BEST collaborative on the effects of shipping platelets. Transfusion 2007; 47:1666-73.

Badlou BA, Van der Meer PF, Akkerman JWN, Smid WM, Pietersz RNI. Metabolic energy reduction by glucose deprivation and low gas exchange preserves platelet function after 48 H storage at 4 degrees C. Vox Sang 2007; 92:311-8.

New therapies and evaluation of clinical applications

Clinical trials with products derived from human plasma and recombinant plasma products may be found under the heading Medical Department of Sanquin Plasma Products at page ##

New cellular therapies

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Cellular therapy research

Projects in this research line focus on the development of new cellular therapies. that can facilitate the hematopoietic stem cell transplantation and can facilitate tissue regeneration.

Bone marrow micro environment

The adult hematopoietic stem cells (HSC) reside in highly specialized niches within the bone marrow. Within this microenvironment, the interactions of HSC with adhesion molecules on neighboring cells and extracellular matrix components are thought to be critical for the maintenance of the HSC population. Comparative gene-expression profiling of HSC in quiescent state, during regeneration and after injury did identify new molecules which might regulate the interactions of HSC with its niche. Several adhesion molecules and extracellular matrix proteins were identified to be differentially expressed in these studies. The objective of the current project is to unravel the mechanism by which several of these proteins regulate the differentiation /self renewal of HSC under homeostatic or regenerative conditions. By Q-PCR the expression of these targets was compared on CD34+ cells isolated from bone marrow, mobilized peripheral blood or umbilical cord blood. We found a significant lower expression of ALCAM (CD166) and Endoglin (CD105) in bone marrow HSC compared to mobilized HSC, suggesting a role for these molecules in HSC mobilization. In contrast, the expression of the extracellular matrix molecule Bigh-3 (TGFBI) was found to be higher in bone marrow HSC as compared to mobilized PBSC, suggesting a role for Bigh-3 to maintain the HSC in the bone marrow. In addition we found the SDF-1-induced migration of HSC to be mediated by Bigh3 in a haptotactic manner. Overexpression and knock down studies are ongoing to determine the direct role of the several targets on HSC differentiation as well as their role in hematopoietic regeneration.

Mesenchymal stem cells are known in this light to support the efficacy of stem cell transplantation. We hypothesized that MSC have the potential to induce a proangiogenic state of the endothelium. Finding this proangiogenic effects on endothelial cells would explain marrow tissue regeneration and repair and thus enhanced MSC mediated repopulation. Furthermore, using immunohistochemistry of bone marrow sections of NOD/SCID mice, we observed significant differences between irradiated mice which received CD34+ cells and mice which received CD34+ cell in combination with MSC. Also our *in vitro* experiments clearly show in various ways (phenotype, expression profile, proliferation, survival, migration, *in vitro* angiogenesis) that MSC conditioned medium has profound angiogenic effects. Although we could demonstrate that some of these effects are partly mediated by VEGF, HGF and bFGF, an yet unknown factor present in the MSC conditioned medium is involved in this angiogenic effect as well.

As part of the Dutch Program on Tissue Engenering we studied the migratory behavior of MSC obtained from different sources.

Crucial in these processes is the presence of MSC at the site of injury, however the recruitment and migration of MSC towards their destiny is poorly understood. With respect to future cell therapy, we are studying the process of migration of various human mesenchymal stem cell sources, and hypothesize that only a subpopulation of *ex vivo* expanded mesenchymal stem cells is capable of specific homing.

For this purpose, MSC from different sources and age, i.e. fetal lung (FL), fetal bone marrow (FBM), adult bone marrow (ABM) and adult adipose tissue (AT) were derived by plastic adherence and subsequently expanded. All MSC sources were characterized as CD73+, CD90+, CD105+, CD34- and CD45-. MSC (passage 6-10) were allowed to migrate for 4h towards SDF-1αFCS, PDGF-BB or bFGF over fibronectin-coated 12 µm pore size transwell plates. FL-MSC migrated significantly better towards SDF-1α as compared to ABM-MSC or AT-MSC. This enhanced migration capacity towards SDF-1α is specific for FL-MSC since AT-MSC migrated significantly better towards 20% FCS as compared to FL-MSC. Even ABM-MSC responded better to FCS than FL-MSC. This suggests that MSC of all sources are able to migrate but require different triggers to induce migration. Interestingly, migratory MSC originating from all tissue sources maintain their proliferation and differentiation capacity and seem to express CXCR4 at a higher level than MSC that did not migrate.

To be able to migrate, cells need to rearrange their actin cytoskeleton. This actin polymerization can be initiated by various chemokines. In response to SDF-1α, strong actin polymerization was observed in FL and BM-MSC while AT-MSC hardly responded. However, AT-MSC were able to display actin polymerization in response to FCS. In general, these actin polymerization data confirm the migration results.
In order to elucidate whether the observed differences in migration potential were due to developmental stage, cultured MSC derived from fetal bone marrow were tested as well. No significant differences in migration capacity were observed between ABM- and FBM-MSC for any of the chemoattractants evaluated. Interestingly, FL-MSC had a significant increased migration capacity as compared to FBM-MSC towards SDF-1α, PDGF-BB, HGF and bFGF.
In conclusion, these results suggest that the migratory capacity of culture expanded MSC may depend on the tissue of origin rather than on their developmental stage. To further elucidate the properties of the migratory MSC subpopulation(s), a micro array experiment was performed, focussing on the differences between migrating and non-migrating MSC. Preliminary results indicate that the largest group of upregulated genes in the migrating MSC subpopulation are involved in (or related to) the G-protein coupled receptor protein signaling pathway, known to be important in cell migration.

Key publication

DiMascio L, Voermans C, Uqoezwa M, Duncan A, Lu D, Wu J, Sankar U, Reya T. Identification of adiponectin as a novel hemopoietic stem cell growth factor. J Immunol 2007; 178(6):3511-20.

Neovascularization therapy

The other focus of our cellular therapy research deals with the potential of blood or bone marrow derived cells to form the blood vessel lining endothelium. We have previously demonstrated that monocytic cells are responsible for the outgrowth in the so-called Endocult assay, a colony assay for Endothelial colony forming cells (CFU-EC). CD4+ T-cells seemed to facilitate the monocytic colony formation by a still unknown paracrine factor. Direct cell-cell contact was needed for this facilitation. This cell contact could be inhibited by CD3 or MHC-classII antibodies, and could

be replaced by activating CD3/CD28 antibodies. These findings suggest that the proangiogenic state of monocytes is mediate by T-cell help in a sterile inflammation reaction. Monocytes activated by T-cells will be tested for their revascularizing potential in the ischemic hind limb model, operational at our department.
The SCL has been cosely involved in the Hebe trial in collaboration with ICIN (Interuniversity Cardiology INstitute). In this trial patients receive intracoronary peripheral blood or bone marrow derived mononuclear cells after a myocardial infarct. The optimal processing method was validated.

Key publications

Van Beem RT, Noort WA,Voermans C, Kleijer M, Ten Brinke A, Van Ham SM, Van der Schoot CE, Zwaginga JJ. The presence of activated CD4+ T-Cells is essential for the formation of Colony Forming Unit-Endothelial Cells (CFU-EC) by CD14+ Cells. J Immunology. 2008; in press.

Van Beem RT, Hirsch A, Lommerse IM, Zwaginga, JJ , Noort WA, Biemond BJ, Piek JJ, Van der Schoot, CE, Voermans C. Recovery and functional activity of mononuclear bone marrow and peripheral blood cells after different cell isolation protocols used in clinical trials for cell therapy after acute myocardial infarction. Eurointervention. 2008; in press.

Megakaryocytopoiesis

We previously showed that stem cells can be expanded *ex vivo* into megakaryocytes by the combination of thrombopoietin (Tpo) and IL-1. Thus far, the addition of *ex vivo* generated megakaryocytes to a stem cell transplant has not shown to considerably shorten the duration of severe thrombocytopenia in patients immediately after myeloablative therapy. However, the composition of the graft might need further optimization. Therefore, the aim of this study was to assess the contribution of *ex vivo* generated megakaryocytes from human mobilized peripheral blood (MPB) CD34+ cells to *in vivo* platelet production. To this end, we have used the NOD/SCID mouse as an *in vivo* model for human hematopoiesis. MPB CD34+ cells were cultured for 7 days in the presence of Tpo (100 ng/ml) and IL-1α (10 ng/ml). Mice were transplanted with unmanipulated CD34+ cells, expanded megakaryocytes, or a combination. In mice receiving grafts containing at least 50%

expanded megakaryocytes, *in vivo* human platelet formation was detectable as soon as three days after transplantation, and the human platelets formed in this model were shown to be functional. Moreover, shortening the culture period delayed the *in vivo* platelet formation. These data indicate that the period of thrombocytopenia after intensive chemotherapy can be overcome by co-transplantation of human MPB megakaryocytes generated ex vivo according to our protocol.
Despite extensive studies we did not succeed in the in vitro production of platelets. We therefore will use the transplantation model of CD34+ cells in NOD/SCID mice to obtain human platelets and test their functionality.

Key publications

Macaulay IC*, Tijssen MR*, Thijssen-Timmer DC, Gusnanto A, Steward M, Burns P, Langford CF, Ellis P, Dudbridge F, Zwaginga JJ, Watkins NA, Van der Schoot CE, Ouwehand WH. Comparative gene expression profiling of in vitro differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. Blood 2007; 109(8):3260-9. *Both authors contributed equally.

Tijssen MR, Van Hennik PB, Di Summa F, Zwaginga JJ, Van der Schoot CE, Voermans C. Transplantation of human peripheral blood CD34-positive cells in combination with ex vivo generated megakaryocytes results in fast platelet formation in NOD/SCID mice.Leukemia. 2008; 22(1):203-8.

Hemert FJ, Thurlings R, Dohmen SE, Voermans C, Tak PP, Van Eck-Smit BL, Bennink RJ. Labeling of autologous monocytes with (99m)Tc-HMPAO at very high specific radioactivity. Nucl Med Biol 2007; 34(8):933-8.

Cord Blood

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Cord blood research at the Blood Bank South West Region is focused on increasing the applications of cord blood (CB) for stem cell (SC) transplantation as well as other purposes, e.g. red blood cell (RBC) transfusion. The clinical CB research is supported by translational research, using as a model the NOD/SCID mouse.

The use of autologous cord blood for anemia of prematurity

After validating the manufacturing of CB collections with a special separation device (Sepax; Biosafe) we showed that is possible to process and store a CB derived RBC transfusion product for up to 21 days in SAGM, which was used in a clinical study as an autologous RBC product. In collaboration with both the Leiden- and Utrecht University Medical Center a three year feasibility study, sponsored by ZonMw, ended in 2007. In this randomized clinical trial we investigated whether autologous RBCs derived from CB could be used as an alternative for allogeneic RBC transfusions in premature infants (<32 weeks of gestation). Primary endpoint was a ≥50% reduction in allogeneic transfusion needs.

In 57% of the UCB collections, the minimum volume for processing (≥15ml) was obtained. For 36% of the total study population and for 27% of the transfused infants an autologous CB red cell product was available, which could not fulfill the primary aim to reduce allogeneic transfusions by ≥50%. The availability of an autologous product was highly related to the gestational age, e.g. 17% availability at a gestation of 24 to 28 weeks, 48% availability for infants with a gestation of 28 to 30 weeks and 36% availability at a gestation of 30 to 32 weeks. The infants in the 24 to 28 weeks group had very high transfusion need (87%), thus virtually all available products would be used, while the transfusion needs in the group of 30 to 32 weeks were much lower (19%). Most efficient, for future studies, autologous CB can be collected for preterms born after a 28-30 weeks of gestation.

Simultaneously, we studied *ex vivo* expansion and differentiation of CB stem cells towards the (pro)erythroblast, which may be used as an alternative for RBC transfusions in these preterm infants. Using a limited number of GMP approved growth factors, e.g. TPO, erythropoietin (EPO) and stem cell factor (SCF), an optimal expansion protocol was developed. We observed a high expansion rate (up to 2700-fold) of pure erythroid lineage cells (95%) using a combination of SCF and EPO, without a difference in growth kinetics between term CB SC and pre-term CB SC.

Key publications

Khodabux CM, Von Lindern JS, Van Hilten JA, Scherjon S, Walther FJ, Brand A.
A clinical study on the feasibility of autologous cord blood transfusion for anemia of prematurity.
Transfusion. 2008; in press.

Hack KEA, Khodabux CM, Von Lindern JS, Brouwers HAA, Scherjon SA, Van Rijn HJM, Van Hilten JA, Brand A, Page-Christiaens GCML. Bloedtransfusiebehoefte bij prematuren in twee Nederlandse perinatologische centra. Ned Tijdschr Geneesk 2008; in press.

Double Cord Blood transplantation and other graft enhancing research

Data from US centers showed that clinical outcome of double CB transplant is superior to single CB tranplantation allowing its application in adult patients. Clinical data using DNA chimerism showed that 4-8 weeks after transplantation only one of the 2 CB established stable engraftment. The mechanisms of either quantitative or qualitative CB characteristics or immunological effects between the 2 cord bloods, are unknown. In collaboration with the Erasmus Medical Center Rotterdam, a study to evaluate clinical results and the mechanisms of double CB transplantation in adults was started in 2007. By using monospecific anti-human HLA monoclonal antibodies, we can in many recipient/donors combinations, separate patient and the 2 donor cell populations. This may reveal the sequence of events of engraftment of different hematological lineages and whether donor lymphocytes play a role in rejecting the co-transplanted graft.

In the NOD/SCID model the effects of double CB transplantation is further unraveled and compared to other approaches to improve engraftment such as cotransplantation with mesenchymal stem cells (MSC) and *ex vivo* expansion with thrombopoietin (TPO).
In this NOD/SCID model we observed that *ex vivo* expansion, but not MSC co-transplantation accelerates platelet recovery. On the other hand, long-term human chimerism in bone marrow and blood in the NOD/SCID is only increased after co-transplantation with MSC. Experiments must demonstrate that both TPO expansion and co-transplantation with MSCs preserves the advantages of both

methods. Similar to humans, double CB transplantations in NOD/SCID mice increased engraftment to a higher degree as explained by the additive effect of two CB's, however, in mice dominance of one of the CB over the other was not always observed. Our results thus far are not conclusive on the role of SC or of non-SC accessory cells in this.

Double transplantation as well as *ex vivo* expansion using growth factors may alter the function of the immune cells present in the graft, which can have implications for graft rejection or graft versus host/graft versus leukemia potentials. TPO alone or the TPO/SCF/Flt3/IL-3/IL-6 cocktail alter the phenotype distribution as well as the function of T cells and antigen presenting cells dependent on the cytokine cocktail used. In double cord combinations differential graft versus graft potential may be the cause of rejection of one of the grafts.

Key publication

Schipper LF, Van Hensbergen Y, Fibbe WE, Brand A. A sensitive quantitative single-platform flow cytometry protocol to measure human platelets in mouse peripheral blood. Transfusion 2007; 47:2305-14.

Quality aspects of cord blood

The inconsistent quality of CB and the lack of reliable tests to monitor this among different centers are worrying. We conducted a series of international send-arounds for CB SC enumeration and HPC-growth in collaboration with the BEST Collaborative (working party cellular therapy). We revealed huge inter-laboratory differences in the number, viability and growth capacity of the CB SC. We will continue to improve this inter-laboratory standardization of CB analysis protocols and address other quality questions, e.g. on the role of nucleated red blood cells (NRBC) in CB, on the effect of cryopreservation and subsequent CB expansion and engraftment and whether washing of CB prior to transplantation is (dis) advantageous. Regarding the role of NRBC in CB we excluded a contribution of NRBC to HPC growth, but rather identified NRBC as a confounder for higher SC concentrations.

Key publications

Brand A, Eichler H, Szczepiorkowski ZM, Hess JR, Kekomaki R, McKenna DH, Pamphilon D, Reems J, Sacher RA, Takahashi TA, Van de Watering LM. Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Viability does not necessarily reflect the hematopoietic progenitor cell potency of a cord blood unit: results of an interlaboratory exercize. Transfusion 2007; Dec 7. Epub ahead of print.

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Clinical Research on Cellular Blood Products

Reduction of blood transfusions in Orthopedic surgery

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In orthopedic surgery several new approaches to reduce blood transfusions and to improve wound healing are available. In order to make evidence based choices on the usage of bloodsparing drugs, e.g. epoietin or several forms of autologous shed blood re-infusions, large multi-arm and multi-center studies are needed and that a strict uniform transfusion protocol is applied. In 2004 we conducted in close collaboration with Leiden University Medical Center, a multi-center study was designed on integrated blood sparing approaches (Optimal Blood Management (Transfusie Op Maat study – TOMaat –). Four participating hospitals included a total of 1841 patient at the end of 2007. In December 2007 an interim analysis was performed which was approved by the Data Safety Monitoring Board for continuation without adjustments. By October 2008, all 2500 patients will be included.

Key publication

So-Osman C, Nelissen RGHM, Brand R, Brand A. Pre-operative risk factors for a red blood cell transfusion in elective orthopaedic surgery. Vox Sang 2007; 93; suppl. 1:267-8 (abstract) ISBT June 23-27, 2007.

Anemia and quality of life

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There are several circumstances when the appropriate transfusion trigger is not known and the Dutch consensus report is not sufficiently specific. In particular this regards extreme situations such as elderly patients, very low birth weight patients and bleeding after pregnancy. In elderly patients after hip fracture the postoperative hemoglobin level is associated with length of hospital stay and speed of mobilization. As part of the aforementioned TOMaat study, mobilization and functional and health quality questionnaires will be related to the hemoglobin level.

After moderate post-partum hemorrhage the decision to transfuse is often initiated by presumed impairment of performance and quality of life of the mother rather than on medical indications based on hypoxia. This dilemma is currently investigated in a randomized study in 13 participating hospitals in the South West Region of the Netherlands. In this study patients are randomly assigned to (non) transfusion after fluxus and Hb level between 3-4.9 mmol/l. Quality of life at regular intervals up to 6 weeks is the endpoint. Currently 200 of 400 patients have been included.

Key publications

Jansen AJG, Duvekot JJ, Hop WCJ, Essink-Bot ML, Beckers EAM, Karsdorp VHM, Scherjon SA, Steegers EAP, Van Rhenen DJ. New insights into fatique and health quality of life after delivery. Acta Obstet Gynecol 2007; 86:579-84.

Jansen AJG, Le Noble P, Steegers EAP, Van Rhenen DJ, Duvekot JJ. The relationship between Hb change and blood loss after delivery. Brit J Obstet Gynecol 2007; 114(5):647.

Jansen AJG, Essink-Bot ML, Duvekot JJ, Van Rhenen DJ. Psychometric evaluation of health related quality of life measures in women after different types of delivery. J Psychosom Res 2007; 63(3):275-81.

Clinical complications of transfusions during tissue trauma and critical illness

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Perioperative blood transfusions enhance the chance for postoperative complications.The deleterious role of leukocytes in perioperative transfusions on postoperative complications was shown in 2 RCTs in cardiac surgery and in one RCT in miscellaneous surgery.
The collection of data on the follow-up of these patients with the aim to also report on the 10 year follow-up in cardiac surgery and 5 year follow-up in surgery for gastrointestinal oncology, were completed in 2007 and are currently analyzed.

To explore the pathophysiology of postoperative complications such as infections and multi-organ failure (MODS) the role of cytokines and complement is investigated. In collaboration with the Leiden University Medical Center, we recently revealed that the complement activation pathway by MBL (mannin-binding lectin) was not affected by leukocytes in blood products, although low MBL levels protected against MODS after cardiac surgery.
The feasibility of cytokine gene expression (GE) as parameter for post transfusion immune modulation by transfusion in different patient populations (premature infants, various types of surgery) was tested by a broad cytokine and chemokine screening panel with an array technique, determining the expression of 114 cytokine genes.

Key publications

Bilgin YM, Van de Watering LM, Eijsman L, Versteegh MI, Van Oers MH, Brand A. Is increased mortality associated with post-operative infections after leukocytes containing red blood cell transfusions in cardiac surgery? An extended analysis. Transf Med 2007; 17:304-11.

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Van Hilten JA, Sitnyakowski L, Van de Watering LMG, Brand A, A monitoring approach on gene expression to understand the clinical impact of transfusion and disease. Transfus Medi 2007; 17 (suppl.) SI31:21-22 (abstract BBTS2007, Glasgow).

Bilgin YM, Brand A, Berger SP, Daha MR, Roos A. Mannose-binding lectin is involved in multiple-organ-dysfunction-syndrome after cardiac surgery: effects of blood transfusion. Transfusion in press.

TRALI

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The incidence, patient and product epidemiology of TRALI in the Netherlands is investigated and coupled to criteria for optimal clinical and laboratory diagnostic work-up. In 2007 a look-back study of plasma products implicated in TRALI was completed. Because since July 2007 all Dutch plasma products are derived from male donors, th incidence of TRALI before and after this intervention is included in the epidemiological TRALI survey which ends dec 2008.

Key publications

Wendel S, Biagini S, Trigo F, et al. Measures to prevent TRALI. Vox Sang 2007; 92(3):258-77.

Lamboo M, Poland DCW, Eikenboom JCJ, Harvey MS, Brand A, De Vries RRP. Coagulation parameters of thawed fresh-frozen plasma during storage at different temperatures. Transf Med 2007; 17(3):182-6.

Kapiteijn E, Brand A, Kroep J, Gelderblom H. Sinutinib induced hypertension, thrombotic microangiopathy and reversible posterior leukencephalopathy syndrome. Ann Oncol 2007; 18(10):1745-7.

Epidemiological studies to test the hypothesis that TRALI is caused by leukocyte antibodies

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Several blood establishments have decided to exclude plasma from female donors who have been pregnant, in order to avoid the risk of TRALI in plasma recipients. This measure is based on the current dogma that TRALI is caused by leukocyte antibodies. The objective of this project is to examine the scientific basis of this policy.

A systematic review of the literature shows that only a tiny proportion of the published studies on the prevalence of leukocyte antibodies in donors of TRALI patients have been designed in a way that allows for a quantitative estimate of

the relative risk associated with these antibodies. Based on 14 out of 82 papers we calculate that the risk to get TRALI is 15-fold higher in recipients of antibody containing blood products than in recipients of products without antibodies. However, there seems to be a considerable publication bias favoring the role of antibodies. In an international collaborative study of patients who received only products from donors of the same sex, we found that recipients of products from male donors had the same risk to get TRALI as recipients of products from female donors. This finding does not support the antibody hypothesis for the pathogenesis of TRALI.

Key publication

Middelburg RA, Van Stein D, Briët E, Van der Bom JG. The role of donor antibodies in the pathogenesis of Transfusion-related acute lung injury. Transfusion 2008; in press.

Studies of the clinical efficacy of platelet products

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The use of platelet concentrates (PC) for the prevention and treatment of bleeding complications in patients with thrombocytopenia, due to cytotoxic therapy or malignancies of the bone marrow, is generally accepted.
Several new platelet products are implemented or under development, although clinical studies for an appropriate selection for one of these products are lacking. Moreover, observed differences of *in vitro* quality parameters are inconsistent with clinical efficacy, while a striking effect of several patient factors on clinical efficacy is present, annihilating product related factors.
Two important issues are driving the current direction of PC development: prolongation of storage time, maintaining or even increasing safety in regard to contamination with pathogens. A clinical trial concerning these issues has started in the beginning of 2007. In this trial platelets stored in an additive solution (PAS-3) treated with or without a photochemical pathogen reduction procedure (PR-PAS-3), stored up to 7 days, will be compared to platelets stored in plasma. Hospitals in three Sanquin regions are participating and currently 147 (of expected 300) patients were included. The results of this study will be used to design new quality monitoring criteria for the testing of PCs before transfusion. An *in vitro* comparison

between several different platelet concentrates has shown remarkable differences and a scoring system is being developed encompassing both metabolic (lactate), as well as flowcytometric markers (P-selectin, Annexin V). At the end of 2007 a pilot study was started testing markers of endothelial damage in relation to platelet transfusion effects. A clinical trial of several platelet products, most likely plasma stored PCs, hyperconcentrated platelets and additive solution stored PCs in pediatric patients is scheduled for 2008.

Key publications

Brand A. RDP Based platelet supply in the Netherlands. Transfusion Today 2007; 70:7.

Kerkhoffs JLH, Novotny VMJ. Klinische toepassing van synthetische bewaarvloeistoffen en fotochemische pathogen reductie bij plaatjesconcentraten bij hemato-oncologische patienten. Ned Tijdschr Hematol 2007; 4:23-5.

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Therapeutic erythrocytapheresis as treatment for Hereditary Hemochromatosis patients

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Hereditary Hemochromatosis (HH) is a genetic disorder of iron metabolism resulting in excessive iron overload Phlebotomy (P) is currently the standard therapy for HH patients and typically consists of removal of 500 ml whole blood weekly. The target is to reduce the serum ferritin levels to 50µg/L and /or transferrin saturation bellow 50%. Depending on the initial ferritin levels this requires 20 - 100 P's over a period of 6 to 24 month. Thereafter P's are reduced to 3 to 6 times a year. More recently TE has become a new therapeutic modality. With TE, up to 1000 ml

erythrocytes per procedure can be removed, compared to 250 ml erythrocytes per P. Thus TE potentially offers a more efficient method to remove iron overload with fewer procedures in a shorter time period. The results from our pilot study, in which 6 patients treated with TE were compared to a historical control group of 6 HH patients treated with P, showed a reduction of almost 70% in both the total number of procedures and the duration of treatment in the TE group. Although the procedure costs compared on the basis of a single TE session were higher, the total costs for the whole treatment period were at least comparable but probably cheaper with the use of TE (1).

In collaboration with Atrium Medical Center and the University Hospitals of Maastricht and Nijmegen, a randomized clinical trial was started in 2005, among up to 38 new diagnosed HH patients (homozygote for C282Y) to compare the two modalities of iron overload reduction (Phlebotomy and Therapeutic Erythrocytapheresis), based on average changes per single procedure. Primary outcome measures are treatment duration and number of treatments to reach ferritin levels below 50 µg/L. Secondary outcome measures are decline in hemoglobin levels, restitution of liver functions, patient discomfort and costs. Statistic analysis: preliminary results Student t-test, RCT Sequential analysis.

Data from the first 26 included patients were available for analysis, with in total 429 treatments. A statistical significant reduction per single TE procedure versus P was seen in decline of Hb, serum Fe, ferritin, and amount of removed iron. Significant decline of ALAT was seen in TE. Removed volumes per procedure were comparable (481 ml whole blood with a mean Ht of 43% versus 533 ml of erythrocytes volume with a mean Ht of 86%). Plasma protein levels were significant better conserved by use of TE. A clear reduction in side effects was seen in TE (1/87) versus P (9/342).

TE seems to be an effective and safe method to remove iron overload in patients with HH. Per single TE procedure 2,26 times more iron can be removed than per single P procedure. Also the decline in serum ferritin level is 2,7 times higher per single TE procedure then per single P procedure. On top of that a large reduction in the total number of procedures by use of TE compared to P seems attainable.

Key publications

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Donor studies

Systematic recruitment and retention of donors

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The results of the studies into the determinants of blood donation intentions showed that affective attitude, subjective norm, descriptive norm, self-efficacy, and personal norm are the most important predictors of blood donation. Based on these results we decided to focus on 2 strategies for improving donor recruitment. The first strategy is improving the Sanquin recruitment leaflet. A content analysis showed that these leaflets are more focused on raising knowledge instead of recruitment. Information targeting affective attitude, self-efficacy, and personal norm was barely included in the leaflet. A subsequent experiment among university students showed that the leaflet was not effective in motivating non-donor students to become blood donors. To improve recruitment, we want to add information targeting affective attitude, self-efficacy, and personal norm.
The second strategy to improve donor recruitment focuses on 'donors recruiting new donors'. A study into the determinants of 'donors recruiting new donors' was conducted to see if donors were willing to participate in this and to study what factors influence this willingness. Donors received a questionnaire measuring this either at home or at the blood bank. The results showed that 53% of the donors at home had a positive intention to participate, compared to 59% of the donors at the blood bank. For donors at home, the most important predictors of the intention to participate were self-efficacy, cognitive attitude, and having positive experiences at the blood bank. For donors at the blood bank the most important predictor also was self-efficacy, followed by affective attitude, a personal norm to donate blood, and feeling responsible to help donor recruitment.
Based on these results we designed an information leaflet for donors asking them to participate in a 'donors recruiting new donors' campaign and targeting self-efficacy, affective attitude, and cognitive attitude (the three most important predictors). 'Donors recruiting new donors' postcards are designed to be distributed among potential donors (by participating blood donors) and can be filled out to register as a blood donor. Participants in this study will be assigned to 1 of 3 conditions, depending upon their arrival at one of the participating blood centers. At the registration desk they either receive an envelop with the 'donors recruiting new donors' information leaflet and the postcards, or an envelop with the postcards

only, or nothing (standard blood bank practice). All blood donors receive three questionnaires. The first questionnaire is send one week prior to donation, the second questionnaire one week after donation, and the third questionnaire 6 weeks after donation. The questionnaires measure the determinants of intention and intention to participate. The last questionnaire measures whether donors have recruited new donors among their family and friends (self-reported behavior).

Key publications

Lemmens KPH, Abraham C, Hoekstra T, Ruiter RAC, De Kort WLAM, Brug J, Schaalma HP. Why don't young people volunteer to give blood? An investigation of the correlates of donation intentions among young adults. Transfusion 2005; 45:945-55.

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Lemmens KPH, Abraham C, Ruiter RAC, Veldhuizen IJT, Schaalma HP. Can we ask more of donors than just giving blood? Paper presented at the 20th Annual Conference of the European Health Psychology Society, 30 Aug-2 Sept 2006. Warschau, Poland.

Development and evaluation of theoretically founded interventions aimed at donor retention

Retention of new blood donors: a study into motivation of new blood donors

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Each year 5% of all donors withdraw from donating blood for unknown voluntary reasons. The majority of these donors lapse before they have made their 5th donation. Recently, we have started a longitudinal study to find out which psychological determinants are related to voluntary withdrawal of new donors. In this study, new

donors will be followed for the first 2 years of their donor 'career'. They will be asked to fill in one questionnaire on motivation before their first medical screening, one after the first donation and one after they have been registered as a donor for a year. These psychological data will be combined with donation data from the blood bank data management system (e)Progesa. This way we will be able to assess the impact of psychological factors like motivation and attitude on donation behavior. We will also assess the impact of external factors, such as physical reactions after donating blood, on intention to donate again. With this information, a new study will be designed to assess the effect of interventions aimed at retention of new blood donors.

Lapsed donors and their intention to return

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The results of a study into the intention to return as a blood donor amongst 800 lapsed donors showed that there are lapsed blood donors who would be willing to donate again. Of the 241 respondents that were included in the analysis, 61% of the respondents indicate that they would return as a blood donor if asked by Sanquin. Attitude towards returning as a blood donor is the main determinant that predicts intention to return. Attitude, in turn, can be predicted by moral norm and the experienced physical reactions during or after donation.

In addition to intention to return, we studied differences between donors with a short donor career and donors with a long donor career. It has been argued that early in the donor career, when individuals just started donating blood, they have different motivations for retention or voluntary withdrawal than when they have already donated blood a few times.
Differences between donors that withdrew as a blood donor after 1 to 4 donations, and donors that withdrew as a blood donor after 5 or more donations indicate that the length of the donor career is mainly dependent on the experienced physical reactions. Donors with a short donor career experienced a higher frequency of experienced physical reactions than donors with a long donor career. In addition, donors with a long donor career appreciate a free health check more, whilst donors with a short donor career report more problems with integrating donation into their daily life. Thus, recruitment amongst lapsed blood donors could pay off if organized properly.

Interventions should aim at raising attitude towards donation, by focusing on donors' moral norm and on coping strategies to deal with negative physical reactions. Donors who withdrew from blood donation after only a few donations may be more motivated to return when interventions focus on coping with physical reactions and time-management. Donors who made more donations before withdrawal may be more tempted to return when appeals focus on the free health check.

Donor cohort studies

Donor InSight Study

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Donors form the essential starting point in the transfusion chain from donor to patient. Although much research has been done on blood and blood products, few studies have focussed their attention on the donor. Donor research is essential for establishing and maintaining a high qualitative blood supply. The main goal of the Donor InSight study is therefore to know more about our donors. The objectives are to gain insight into (1) the characteristics of the donor population and (2) into the efficiency of different processes in the blood bank that involve donors. With respect to donor characteristics, research will be aimed at investigating aspects such as the socio-economic background of donors, lifestyle, nutrition, medical history, donor motivation and physical activity. Concerning the efficiency of the blood bank donor processes, research questions will address the dynamics of the donor population. What are the processes that play a part in becoming a regular donor? How many donors do become regular donors and for what reasons? Why do people resign? What is the overall main deferral rate, and does it differ between various donor groups?
In order to gain insight into the above stated questions and research lines, a dynamic cohort containing approximately 10% of the national blood donor population will be formed. In this cohort study, whole blood and plasma donors will be followed in time using both questionnaires and routinely gathered blood bank data.
The majority of our research activities in 2007 consisted of data collection by distributing questionnaires. For a decent handling of the data, students were recruited to assist in the data entry.

In total, 17,675 questionnaires were sent out. A response rate of 63% was achieved. Eleven percent of the invited donors indicated by means of a reply card, telephone, or email that they were not willing to participate in the Donor InSight study. Twenty-six percent did not respond at all. Currently, initial analyses are being carried out on the data collected so far. Preliminary results are expected in 2008.

Donor management

DOMAINE – DOnor MAnagement IN Europe

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Blood components are vital to modern medicine. It is estimated that 5 to 10 out of 1,000 persons need a blood transfusion each year. Within this population, there is a large group of multi-gallon recipients across Europe that is depending on long-term transfusion therapy. While the European population is ageing, the demand for blood components is growing. However, the number of potential blood donors is reducing, aggravated by the introduction of additional safety measures, considered necessary to protect patients from emerging diseases. The supply-demand balance is subject to fluctuation because of the variable nature of supply and demand itself. However, shortages already do occur and are more likely to occur in the future. Some countries or regions may be more affected than others. The shortfall of blood components in the future is a Europe-wide problem, further enhanced by the free movement of European citizens. Therefore, a European solution is required, involving blood establishments that are responsible for a safe and sufficient blood supply in each country.

The most efficient and secure way of creating a safe and sufficient donor population in Europe, is the development and adoption of European good practice and cooperation between blood establishments and professionals at the European level. The DOMAINE project aims to achieve good practice. DOMAINE will produce a Manual containing European good practice in donor management and a Training Program for European blood collection professionals to ensure adoption of good practice at a local level.
DOMAINE is carried out by 14 blood establishments from 14 Member States and the patient-driven organization Thalassemia International Federation. The project

is supported by an Advisory Board, consisting of professionals from the sector. DOMAINE receives co-funding from the European Union, within the framework of the Public Health Work Program 2007.

DOMAINE will promote good blood donor management (collection, processing, distribution and use) in Europe through several work packages:

- Performing a Survey on current practice in European blood establishments, including practices geared to promoting voluntary unpaid donations.
- Developing a Donor Management Manual in several European languages, identifying and recommending good European practice for donor management with respect to: i) donor recruitment, ii) donor retention, iii) donation procedures, including deferral policy, and iv) patients requiring long-term transfusion therapy.
- Developing a Training Program which will assist blood establishments within each Member State to implement Good Donor Management.

By developing a Manual for European good practice, this Project provides an added value at a Europe-wide level in the field of public health. The Manual will allow a consistency of good donor management which will be beneficial for all European citizens. The Manual also ensures good practice reproducibility and transferability. The project will stimulate collaboration between EU member states with respect to sharing knowledge and experience in blood establishments, and will focus on the importance of identifying performance indicators and guidelines.
The constitution of the consortium guarantees a good coverage with respect to differences in cultural context as well as policy making bodies. This will ensure acceptance of the Manual by all European countries. The Manual will be accompanied by a training program, the scope of which is to train key persons in blood establishments in correctly using the Manual recommendations.

Key publications

Aessopos A, Kati M, Meletis J.Thalassemia intermedia today: should patients regularly receive transfusions? Transfusion 2007; 47(5):792-800.

Cobain TJ, Vamvakas EC, Wells A, Titlestad K. A survey of the demographics of blood use. Transfus Med 2007; 17(1):1-15.

Seitz R, von Auer F, Blümel J, Burger R, Buschmann A, Dietz K, Heiden M, Hitzler WE, Klamm H, Kreil T, Kretzschmar H, Nübling M, Offergeld R, Pauli G, Schottstedt V, Volkers P, Zerr I. Impact of vCJD on blood supply. Biologicals. 2007; 35(2):79-97.

Prediction of future Hemoglobin levels

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The aim of the proposed study is to predict future hemoglobin levels using a multivariable approach. Correct prediction of future Hb levels in whole blood and plasma donors will diminish the number of Hb deferrals. It will also help in maintaining a healthy donor population with respect to its iron stores and Hb levels, which is important in preserving (new) regular donors. The present study focuses on the following research objectives:

i) To assess and construct a prognostic model for future hemoglobin levels in whole blood and plasma donors. In particular, the study will investigate to what extent donor and donation information or variables already obtained in the blood bank information system provide prognostic knowledge for Hb level outcomes.

ii) To investigate the added value of erythrocyte-zinc protoporphyrin (ZPP) measurements in the prognostic model.

iii) To derive a simple prediction rule that will be applicable and easy to use in everyday blood bank practice.

Donor Management related consultancy projects

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Within Sanquin regularly questions are raised regarding donor management issues. The questions address policy decisions and can require preliminary investigations as well as more extensive studies. The answer is generally not straightforward and requires a projectwise approach, based on solid methodology. The Executive Board of Sanquin has appointed the Department of Research and Education of Sanquin Blood Bank South East Region as the department that prepares answers to such policy questions pertaining to donor management. This department has the personnel and equipment to carry out this consultancy role adequately. The results are not published in scientific journals, but are generally written in reports for practical internal use. The consultancy projects frequently lead to new research ideas, such as 'turbid plasma' as an important reason for product deferral.

In 2007 the following consultancy questions were addressed:

- How satisfied are both employees and donors with the new Mobile Blood Collection Center (Mobiele Afname Locatie).Topics included are for example privacy, donation setting and workload.
- What do readers of the blood donor magazine 'Bloedverwant' think of both its content and lay-out?
- Are donors who filed a complaint in 2007 satisfied with the way Sanquin has addressed their complaints?
- How satisfied are current donors with aspects pertaining to their donorship and the donation process itself? A bi-annual evaluation of a random sample of our current donors.
- How satisfied are donors in the North West Region with the current opening hours and waiting times and are they prepared to travel to another donation center?
- How long do donors have to wait for their bleeding procedure in the donation centers in the South East Region? Measuring donor waiting and processing times.
- How effective is the in-corporate training 'Back to the Heart of the Matter'(Terug naar de Kern), from the Unit Production South East Region, in heightening product awareness and work satisfaction?

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  - Dimers in intravenous immunoglobulin (IVIg) 71
  - Structural and functional properties of human IgG4 71

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QUALITY, SAFETY AND EFFICIENCY

- Improving materials and methods for Blood Bank processing
  - Validation studies

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Research lines

BLOOD TRANSMITTED INFECTIONS

- Virological aspects of AIDS
  - Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection. 82
  - Susceptibility of recently transmitted subtype B human immunodeficiency virus type 1 variants to broadly neutralizing antibodies. 82
  - The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection. 84

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- Fetomaternal and transfusion induced alloimmunization
  - Red cell alloimmunization 42

NEW THERAPIES AND EVALUATION OF CLINICAL APPLICATIONS

- Clinical research on cellular blood products
  - Anemia and quality of life 117
  - TRALI 119
  - Studies on the clinical efficacy of platelet products 120

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  - Red cells
    - Improved erythrocyte storage solution 98
  - Haematopoietic stem cells 99
  - Platelets
    - Platelet storage lesion and mitochondria 101
    - Platelet Hyperconcentrates treated with Mirasol® PRT 102

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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.

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Development work on a manufacturing process for holotransferrin was started in close collaboration with L von Bonsdorff, Sanquin Oy, Finland.
After finalizing the development work, a large scale installation was engineered and three batches on large scale were manufactured. The resulting product was characterized and virus validation studies were performed with excellent results. Holotransferrin will be used for a new drug product, Oxaliplatin-encapsulated transferrin-conjugated PEG-liposomes for targeting Oxaliplatin (I-OHP) to colon cancer cells via cancer manifesting transferrin receptors. Clinical studies using this new product are planned for 2008.
As spin-off of the development of holotransferrin, a project was started to develop an apotransferrin product. Apotransferrin can be manufactured using the manufacturing installation for holotransferrin as well. The first large scale batch was manufactured and characterization studies are started, including virus validation studies.

Project leader: M Kleijn (m.kleijn@sanquin.nl)

To enhance the virus safety of Cetor®, a high-purity C1-inhibitor product, a 15 nm Planova-filtration step was developed and implemented in the manufacturing process for a next generation C1-inhibitor product. Feasibility to use USA plasma as source material for a 15 nm Planova-filtered C1-inhibitor-N product was shown and three large scale batches with this improved process were manufactured.
A characterization program was developed to study impurities profiles of the intermediate products. Clinical studies in The Netherlands and USA with this virus

safe C1-inhibitor-N were started in 2005 and finalized in 2007.
In close collaboration with Sanquin Research, a project was started to characterize C1-inhibitor products. With Sanquin Virus Safety Services prion removal studies were performed for the new 15 nm Planova filtration step with good results.

Project leader: GJ Derksen (g.derksen@sanquin.nl)

A project was started to develop a second intermediate pure FVIII product besides Aafact in close collaboration with Dr. R. Laub from CAF-DCF (Brussels, Belgium).
To guarantee the virus safety, studies on the feasibility of the use of two new virus reducing techniques (20 nm filtration and UVC technology) are started.

Manager: A Koenderman (a.koenderman@sanquin.nl).

In collaboration with the Dept of Immunopathology, studies on IgG products are ongoing to study dimer formation and polymerization of IgG and its significance in the occurrence of adverse events in patients and the role of sialylation of IgG.

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The CAF-DCF Product Development Division 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 and environmental pollutants.

The paradigm of plasma derivative safety is approached by a combination of different approaches: NAT screening, the 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). This year saw the launch of a study covering the therapeutic protein contents of plasma pools from unpaid European versus paid US donors.

Focusing on therapeutic proteins (IVIG, albumin, AGP, FVIII) and their excipients in plasma or concentrates, the Division develops both immunological methods (e.g. epitope identification, specific neutralizing antibodies) and biochemical techniques (such as intrinsic and extrinsic protein fluorescence) and exploits them in industrial applications or as tools for monitoring IVIG infusion.

In 2007 began a pilot clinical study in which the effects of IVIG infusion are studied on total and serotype-specific anti-pneumococcal antibodies in pediatric patients with primary immunodeficiency and after bone marrow transplantation.

Total and serotype-specific anti-pneumococcus antibodies as a tool for ensuring plasma pool quality and for monitoring IVIG infusion

Use of three different standards to determine levels of antibodies against major pneumococcus serotypes in single donations, plasma pools from paid and unpaid donors, and intravenous immunoglobulins

Immunity against *Streptococcus pneumoniae*, a worldwide cause of morbidity and mortality, relies on a sufficient level of antibodies against capsular polysaccharides. The available international standard 89-SF and QC sera are pools from small sets of vaccinated individuals. PPS is an in-house standard made with >5000 donations and available in large amount. Our results demonstrate that PPS is suitable for use as a working standard for total and serotype-specific anti-pneumococcus antibodies.

In contrast to plasma pools, individual donations were found with the overall assay (Elizen, Zentech, Liège) to contain low, normal, and high levels of APb. The serotype-specific assays also revealed low, medium, and high levels of serotype-specific APb, with wide dispersion. Differences were found between plasma pools from paid US and unpaid European donors. Specific anti-APb were found in IVIG at substantial levels, suitable for protecting IVIG-treated patients against most serotypes.

Therapeutic protein contents in plasma pools made with donations from unpaid European donors and paid US donors

National and international bodies, including WHO, recommend that blood and its products be obtained from unpaid donors. Yet because of the difficulty of obtaining sufficient quantities of plasma though voluntary donations, most of the world's present supply of polyvalent intravenous immunoglobulins (IVIG), albumin, and clotting factor concentrates are made with donations from paid donors. We analyzed individual cryoprecipitate-depleted plasma pools made with more than 1000 donations collected from unpaid donors in 4 European countries and from paid donors in 6 different US blood transfusion centres. The US plasma pools showed reduced amounts of total protein, albumin, total immunoglobulin, and specific anti-pneumococcus antibodies. In contrast, all plasma pools showed similar levels of alpha-1 glycoprotein (AGP or orosomucoid). The study will be confirmed and extended.

Use of a new in-house standard to monitor total anti-pneumococcal antibodies in 13 healthy children and 18 pediatric bone marrow transplant patients treated with IVIG: a clinical study

A first study shows that detection of total anti-pneumococcus antibodies can be used successfully to monitor IVIG infusion in bone marrow transplant children between 1 and 22 years of age. The children were infused with IVIG. Peak and trough were determined for total IgG, IgG2, and total anti-pneumococcus antibodies (Elizen, ZenTech, Liège, Belgium). The results show that the total anti-pneumococcus antibody concentration rose as much as 4-fold, reaching levels comparable to those measured in healthy children.

Ongoing clinical study: monitoring the effects of IVIG infusion on total and serotype-specific anti-pneumococcal antibodies in pediatric patients with primary immunodeficiency or having undergone bone marrow transplantation

In 2007 began a prospective multicenter non-interventional pilot clinical study to monitor the effects of IVIG infusion on total and 14 single-serotype-specific anti-pneumococcal antibodies in pediatric patients with primary immunodeficiency and patients having undergone bone marrow transplantation (excluding patients with GVDH). Fifty subjects will be enrolled in 9 different university centres. To date, 18 patients have already been enrolled.

Analysis of physiological functions of different pharmaceutical albumin preparations

Human serum albumin (HSA) is used clinically to maintain colloidal osmotic pressure in critically ill patients. HSA also functions as a specific carrier of small molecules and has a considerable antioxidant capacity and an esterase-like activity. A recently developed concept in albumin dialysis, exploited in the so-called MARS technology, is based on the principle that HSA may bind toxic compounds passing through a hemodialyser. Ten different commercial HSA preparations, purified by the ethanol fractionation procedure adopted by each manufacturer and stabilized with different stabilizers, were analyzed. Significant differences were observed between preparations. A drastic modification was found with recombinant HSA. The presence of stabilizers was found to reduce significantly the binding capacity of Sudlow's site II (ibuprofen, benzodiazepine binding site). The esterase-like and antioxidant properties were also reduced to some extent in comparison to the stabilizer-free non-pasteurized control. When considering the benefits of albumin administration, one should take into account the different functions and properties of the albumin preparation used. Product 5 (CAF-DCF) shows a good performance index in all tests.

Specific virus–inactivation treatments applied to plasma proteins: solvent-detergent and UVC irradiation

Solvent-detergent treatment is not a universal solution combining enveloped virus inactivation and preserving protein function: the case of alpha-1-glycoprotein (AGP or orosomucoid)

Solvent-detergent treatment is known as the main virus-inactivation procedure, very effective against enveloped viruses and widely used in plasma fractionation, as it preserves protein activity. We have focused on AGP, an immunomodulatory and pro-inflammatory molecule that also acts in drug transport. As a carrier of small drugs, AGP is envisaged for clinical use in cases of intoxication or in liver transplantation. In 2007, therefore, the division purified AGP to a high level (>99%) and, to increase the safety of the product, treated it with solvent-detergent (Tween 80/TNBP or Triton X100/TNBP). Intrinsic and synchronous fluorescence studies and drug-binding competition experiments indicated that solvent-detergent does not induce AGP polymerization but reduces its specific binding activity and the number of sites while slightly modifying its tertiary structure.

UVC irradiation as an efficient virus-inactivation technique for plasma derivatives: the cases of AGP and FVIII-SD

UVC irradiation is a useful virus-inactivation technique, effective against both non-enveloped and single-stranded enveloped viruses. It is reliable and inexpensive, does not require addition of a dye or a scavenger, and results in low product loss. UVC-treated AGP maintains its functional and structural properties, as shown by intrinsic and synchronous fluorescence experiments targeting tryptophan and tyrosine (known to be extremely sensitive to UVC), SEC, and drug-binding and disulfur bond integrity tests.

Concentrates of FVIII SD-UVC were produced previously by CAF-DCF from 10 kg cryoprecipitate (3 consistency batches). All the studied parameters (FVIII, vWF activities, thrombin activation, FIX activation, lipid binding), neoantigen detection in a rabbit model, and virus validation studies indicate that the UVC irradiation step enhances the safety of FVIII concentrates. This project continued in 2007 thanks to the collaboration of GJ Derksen and A Koenderman (Amsterdam), focusing on different sources of cryoprecipitate.

B19 infectivity in hepatocarcinoma cells

Longitudinal investigation of 19 B19-positive donors for specific anti-B19 antibodies and B19 infectivity

Seventeen donors (12 males and 5 females) with an initial B19 level above 105 IU/ml were monitored for 1 year. Two were first-time donors and 15 were repeat donors. Every donor was interviewed for clinical symptoms. The study started in 2006. Samples were collected and screened for B19 DNA and specific IgM and IgG antibodies. All donors actively developed anti-VP antibodies (IgG and even IgM) specific to linear and conformational capsid epitopes. Despite the presence of abundant anti-B19 IgG antibodies, persistent B19 infectivity was demonstrated in several cases by infectivity testing in a hepatocarcinoma cell model. Results showed that live B19 virus persisting for more than one year is not a rare event. Progenies were still infectious, since the infecting virus was able to multiply in successive subcultures. In one patient we were able to use diagnostic kits to demonstrate the presence of specific antibodies that failed to neutralize B19.

Neutralizing B19 infectivity towards hepatocarcinoma cells with IVIG concentrates

We have shown that hepatocarcinoma-derived cells behave as permissive cells for Erythrovirus (parvovirus) B19, yielding infective progeny through virion amplification. Our infectivity test based on this model has proved readily adaptable to routine use, in contrast to previous ones described in the literature: it is easy to handle, produces up to 107 infectious particles, and does not require erythropoietin. Therapeutic IVIG concentrates produced by different manufacturers were tested for their capacity to neutralize B19. First results show that the neutralization capacity depends not only on the anti-B19 titer (determined in a specific ELISA) but also on the specific production procedure.

Collaborations with: Caillet-Fauquet P[1], Craciun L[2], De Launoit Y[1,4], Dupont E[2], Draps ML[1], Goldman M[2], Op De Beeck A[1], Roth WK[3], Schmidt M[3], Seifried E[3], Themann A[3], Derksen G-J[5], Koenderman A[5], Tuerlinckx D[6] and working group.

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Key publications

Di Giambattista M, Branckaert Th, Hougardy V, Kemball-Cook G, Laub R. In silico prediction of FVIII epitopes recognized by natural autoantibodies in polyvalent immunoglobulin concentrates. Molecular Immunology 2007; 44:1913-23.

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The Medical Department is responsible for the design and conduct of clinical trials with (recently developed) plasma products in order to obtain marketing authorization or new indication(s). Therefore the Medical Department closely cooperates with clinical investigators in the Netherlands e.g. the Dutch Inter-University Working Party on the Study of Immune Deficiencies and the Hemophilia Treatment Centres, and abroad.

To ensure safety of the products the Medical Department is obliged to have implemented an appropriate system of pharmacovigilance to collect, collate and evaluate information about suspected adverse reactions of medicinal products. Pharmacovigilance is performed both passively based on received reports on adverse events and actively by performing post authorization safety studies (PASS) in ad random patient groups. Periodic Safety Update Reports (PSUR's) provide the authorities pharmacovigilance data. PSUR's were prepared for GammaQuin® and RhedQuin® for a fourth-and-a-half year review period.
The 5th PSUR for Nanogam® was compiled for a one year review period.

Medical information and advice is provided to medical specialists, physicians, nurses and pharmacists on the optimal use of plasma products. Furthermore, the Medical Department assists in the recruitment of new plasmapheresis donors and performs for the Sanquin Blood Banks the selection of specific units of erythrocytes for immunization in order to obtain specific source plasma for the fractionation of anti-rhesus (D) immunoglobulin.

Clinical trials ongoing in 2007

Nonafact®
The safety of the usage of Nonafact® in regular patient treatment is being assessed in a multi-center clinical trial, entitled 'Post marketing study in hemophilia B patients using Nonafact® 100 IU/ml powder and solvent for solution for injection (human coagulation factor IX) (human plasma derived factor IX product, freeze dried)', in five Hemophilia Treatment Centers in the Netherlands. The clinical study report will be finalized in 2008.

Nanogam®

A multi-centre, controlled, cross-over clinical study, 'Treatment in patients with recurrent infections and IgG Subclass Deficiency, and/or Deficient Anti-polysaccharide Antibody Response' was started in cooperation with the Dutch Inter-University Working Party on the Study of Immune Deficiencies. The aim of the study is to compare the efficacy of Nanogam® with that of antibiotics in the treatment of recurrent (upper respiratory tract) infections in patients with IgG-subclass deficiency or a deficient anti-polysaccharide antibody response. The study was divided into two parts: treatment of adult patient and treatment of children (≥5 years). The study in adult patients was started in seven centres in 2007. The children part will be initiated in 2008.

An investigator-initiated study, 'Desensitization of highly pre-sensitized dialysis patients waiting for kidney transplantation by rituximab, IVIG-L and rescue Plasmapheresis' (the DRIP-study), has started in order to assess the efficacy of treatment with rituximab in combination with Nanogam® to reduce allo-antibody levels in patients with high anti-HLA antibody titres, awaiting renal transplantation. The primary objective of the study is to achieve a negative cross-match and transplantability. Furthermore, patient and graftsurvival and graft function will be assessed. The study was initiated in two Dutch renal transplant centres.

MBL

A clinical trial with Mannan Binding Lectin, (MBL, a product from Statens Serum Institut (SSI), Copenhagen, Denmark), entitled 'Phase II study on Mannan Binding Lectin (MBL) substitution in MBL-deficient children with chemotherapy-induced neutropenia', was performed. The objective was to investigate the pharmacokinetics and the clinical and biological effects of MBL replacement therapy in MBL-deficient children during chemotherapy-induced neutropenia. In the Academic Medical Center in Amsterdam 12 patients were included who received a total of 20 treatments. In December 2006 the study was completed. The clinical study report has been finalized.
From this study it was concluded that MBL SSI is a well-tolerated and safe product

in children. The pharmacokinetics of MBL in MBL-deficient children are comparable to adults after correction for body weight. The half-life of MBL was estimated to be about 36 h for a child of 25 kg. The used dosage in the study leads to adequate substitution of MBL. Moreover, MBL SSI has biological activity, MBL-mediated opsonization and C3/C4 activity, in MBL-deficient children.

Nanofiltered Cetor®

To optimize viral safety, a double 15 nm filtration was introduced in the production process of Cetor®, a highly purified C1-esterase inhibitor concentrate. This improvement in the manufacturing was submitted as a variation for marketing authorization. Therefore, a multi-center study 'Pharmacokinetics, clinical efficacy and safety of C1 inhibitor concentrate (C1-esteraseremmer-N) for the treatment of hereditary (and acquired) angioedema' was set up in collaboration with the Academic Medical Center Amsterdam, Erasmus Medical Center Rotterdam, University Medical Center Groningen, University Medical Center St Radboud Nijmegen, and Haga Hospital The Hague. The study comprised three parts, pharmacokinetics (part A, phase II), treatment of attacks of angioedema (part B, phase III) and prophylactic use of C1 inhibitor (part C, phase III). Part B of the study provided data on the efficacy and safety of C1-esteraseremmer-N in the treatment of angioedema attacks. Part C of the study is still ongoing and will provide data on both the safety and efficacy to prevent angioedema attacks. Part A and B of the study were completed and the study reports have been finalized. The results of part A have provided evidence that the pharmacokinetic properties of C1-esterase inhibitor and Cetor® are similar. In part B of the study the results clearly demonstrate that C1-esteraseremmer-N is highly effective and safe in the treatment of acute angioedema attacks.

Key publications

Hofstra JJ, Choi CW, Strengers P, Marcar JJ, Levi MM, Cuperus RA. Pharmacokinetics, clinical efficacy and safety of C1 inhibitor concentrate (C1-esteraseremmer-N) for treatment of hereditary and (acquired) angioedema. Book of Abstracts, 5th C1INH Deficiency Workshop, Budapest 2007: 43, (abstract).

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Kramer CM, Van Twuyver E, Kleine Budde I, Koenderman A, Over J and Strengers PFW. Sanquin Cetor®: past, present and future. Book of Abstracts, 1st International Leadership Conference, Frankfurt 2007:31 (abstract).

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Sanquin Reagents has developed a broad range of blood grouping and immunology reagents for laboratories, including several innovative products for diagnostic use and for fundamental and clinical research. Sanquin reagents are available worldwide through a network of distributors, and bulk reagents for manufacturing are supplied directly form Amsterdam. Sanquin Reagents is ISO 9001 and ISO 13485 certified. Sanquin Reagents 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.

R&D projects

The project portfolio in 2007 consisted of ongoing projects in the fields of blood grouping and immunology reagents. The development of a new assay for *in vitro* diagnostic (IVD) use will typically take 2-4 years, depending of the complexity of the project and time needed to obtain regulatory approval.

The following development projects were continued or started in 2007:

(i) assays to detect free, human immunoglobulin light chains (kappa, lambda) in blood.
(ii) latex-based assays to quantify human IgG subclass species in blood for the Immage instrument.
(iii) a blood donor bloodgrouping chip based on DNA genotyping of red cell antigens.
(iv) a kit to exchange peptides in MHC multimers (class I) using UV-cleavable peptides, and an ELISA to check for the efficacy of the peptide exchange
(v) an ELISA for quantifying human perforin in plasma.
(vi) a manufacturing line to automate the labeling, filling and closing of Cellbind cards.
(vii) QualiCard, a calibration tool for Magister/Cellbind.

Products

New products & services

The following new products were commercially introduced in 2007:

(i) Magister, a fully automated instrument for Cellbind gelcard testing was launched in several countries outside Italy (second wave of introduction).

(ii) PeliControl, a whole blood control.

(iii) Pelicase Quality Survey (external QC Sanquin).

(iv) New PeliPIP software program (red cell panel interpretation program).

(v) Various white label reagents (as spin-off from Sanquin Research).

Quality system

Two ISO certificates were renewed in 2007 (ISO 9001 & ISO 13485).

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Sanquin Pharmaceutical Services (SPS) is a business unit specialized in a broad array of pharmaceutical services aiming at the development 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.

Contract production

SPS has ample experience in designing production strategies and scaling up of production in compliance with EU and FDA guidelines. For this purpose SPS holds a GMP-license for the production of clinical grade pharmaceuticals including large scale fermentation, purification and sterile filling. The use of a specially developed serum-free culture medium in fermentation, guarantees a process free of concerns related to the transmission of pathogens such as prions and mammalian viruses. Several generic purification schemes for different types of proteins are available, allowing SPS to provide their clients already in an early stage of development with a validated process. In their multipurpose plant several projects can be handled simultaneously, allowing for fast turn around times.

Biosafety testing

SPS is also experienced in conducting a broad array of biosafety tests required for the pharmaceutical release of biotech products in compliance with both EU and FDA guidelines. For this purpose, all assays have been GCLP (Good Control Laboratory Practice) accredited. Other QC services such as protein characterization, stability test programs, formulation studies and process validation (for demonstrating the reduction of (model) viruses or DNA during purification) as well as the validation of client dedicated assays are part of their dedicated activities.

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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 Dutch Health Care Institutions and commercial companies. 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 collaboration with Sanquin Research.

Developments in 2007

A number of new tests and services were introduced in 2007:

- Mutation analysis JAK2 Exon 12
- Mutation analysis bcr-abl transcripts in Imatinib resistant CML
- A one-day blood group antigen DNA typing service (Fy, Jk and Ss)
- Non-invasive foetal DNA typing for K, Rhc, RhE and HPA-1a
- A granulocyte agglutination technique with improved sensitivity for detection of HNA-3a antibodies
- An immunoprecipitation test for detection of autoantibodies to aquaporine-4 (AQP4) in Neuromyelitis optica
- Determination of antibodies to thyroid antigens was automated
- Assays for determination of autoantibodies to ovarian tissue and antibodies to C1q were introduced
- Measurement of C1-esterase inhibitor and of complement factor 2 (C2) was automated
- Measurement of Band 3 expression (EMA test), quantitative membrane protein analysis by SDS/PAGE followed by densitometry and flow cytometric measurement of CD47 (as marker for erythrocyte aging) were introduced
- Anti-beta2-glycoprotein I antibody ELISA for diagnosing patients suffering from

the antiphospholipid syndrome
- Quality of testing of cadaveric blood samples for microbial safety for tissues is improved by the introduction of immuno-assays based on magnetic beads (Architect Abbott).
- The test for Parvo B19 DNA is improved to include genotype 1-3 detection
- Sanquin National Screening Lab (NSS) has implemented a new blood group typing platform (Magister)
- Mechanization of NSS laboratory is improved by introduction of uncapping and sorting robots (ProV robots)

In close collaboration with all involved departments, a specialized committee of the Sanquin Diagnostic Services Division, dedicated to innovation of diagnostic services (called DC-I), was active in 2007 in the following fields:
- Use of array technologies for diagnostic purposes;
- Development of a single nucleotide polymorphism (SNP) platform.

In the following paragraphs, the above-mentioned developments will be described in more detail, ordered according to department.

Immunocytology

In 2006, Sanquin Diagnostic Services introduced routine diagnostic testing for JAK2 mutation in myeloproliferative diseases. In 2007 the incidence of this mutation in other diseases and in healthy persons was extensively studied to determine the specificity of the assay. A slightly increased incidence in patients with deep venous thrombosis was found, suggesting that DVT might be the first manifestation of MPD. Both for JAK2 and for bcr-abl mutation analysis was introduced on research basis in 2007, once these assays were validated they will be introduced in routine diagnostics.

In 2007 we have validated PHOX2B as the first neuroblastoma specific target for the detection of minimal residual disease in neuroblastoma. The clinical relevance of the PHOX2B-PCR is now being tested in a prospective clinical trial. Sanquin will not only test Dutch neuroblastoma samples but also all German samples. For the cytogenetic

classification of B-CLL patients, a test based on Multiplex Ligation-dependent Probe Amplification (MPLA) was developed in 2007 and will be validated in 2008.
In collaboration with the European Study Group on minimal residual disease in acute lymphoid leukemia standard guidelines for the interpretation of RQ-PCR results were established.

Immunohematology

The focus of the department of immunohematology is to function as an (inter) national reference laboratory for erythrocyte, platelet and granulocyte serology, encompassing:

- identification of alloantibodies in transfusion and pregnancy
- serologic examination in alloimmune cytopenia
- serologic studies in TRALI and other immunological transfusion reactions
- laboratory monitoring of pregnancies at risk for hematological disease of the foetus and newborn or neonatal alloimmune thrombocytopenia
- screening of Rhesus D negative pregnant women on the presence of antibodies to erythrocytes during week 30 of the pregnancy

During 2007, assays were developed and implemented in several of the above mentioned fields of interest:

- A one-day blood group antigen DNA typing service (Fy, Jk and Ss)
- Non-invasive foetal DNA typing for K, Rhc, RhE and HPA-1a. The diagnostic accuracy of these tests was evaluated in 2007, which will be continued in 2008
- Development of a granulocyte agglutination technique with improved sensitivity for detection of HNA-3a antibodies
- Development of an autoantibody adsorption technique facilitating the exclusion of the presence of alloantibody in sensitive LISS-based column techniques

Autoimmune Diseases

Neuromyelitis optica (Devic's disease) is an inflammatory demyelinating disease that is characterized by severe attacks of optic neuritis and myelitis. In 2005, a specific autoantibody was discovered against the CNS water channel aquaporin-4 (AQP4)

in the sera of up to 70% of NMO patients, but in none of the sera of MS patients. For the detection of autoantibodies to aquaporine-4 (AQP4) in Neuromyelitis optica, we have adopted a method that involves transient co-transfection of GFP-AQP4 in human HEK293 cells. The high levels of expression make it possible to detect the antibodies in immunoprecipitation, in which bound antigen is measured as the fluorescence of the GFP moiety in a fluorimeter. This assay was validated in close collaboration with dr R Hintzen (Erasmus MC, Rotterdam) and will be used for routine diagnostics in the near future.
During 2007 the determination of antibodies to thyroid antigens was automated. The list of assays for autoantibody determination was extended with antibodies to ovarian tissue and antibodies to C1q.

Immunochemistry/Allergy

In the course of 2007 the automated measurement of C1-esterase inhibitor on a TECAN 'Freedom Evo' ELISA processor was taken to a point where testing for patient samples is possible. Assaying for samples from Sanquin Plasma Products will follow in due course. In addition, an automated ELISA test on the Genesis TECAN robot for determination of complement factor 2 (C2) was introduced in the routine diagnostic package.
Testing for ethanercept (Enbrel®) levels in patient serum was implemented on a Ortho Summit ELISA robot. Promising results for antibody testing against infliximab and adalimumab using biotinylated F(ab2) fragments were obtained, which may make it possible to use one single radiolabel (125I-streptavidin) for all monoclonal therapeutical antibody tests in the near future.

Red Blood Cell Diagnostics/Blood Cell chemistry

In 2007, research was devoted to extend our standard test package to explain erythrocyte membrane defects. Sofar, we offer the measurement of spectrin and of osmotic resistance (AGLT-test), but we now also measure the expression of Band 3 (EMA test) and perform quantitative membrane protein analysis by SDS/PAGE followed by densitometry. New targets are the flow cytometric measurement of CD47 (as marker for erythrocyte aging) and a computer-aided morphological analysis of patient RBC.

After introduction of a multiplex PCR to detect different forms of alpha thalassemia in a routine setting, we now apply Multiplex Ligation-dependent Amplification (MLPA) techniques to analyze rare patients with either alpha- or beta-thalassemia. Mutation analysis is performed to further characterize these patients. In collaboration with dr Richard van Wijk (Dept of Hematology of the Utrecht Medical Centre) mutation analysis is also performed on patients with Band 3 defects.

Blood Coagulation

The antiphospholipid syndrome is characterized by vascular thrombosis and/or specific pregnancy morbidity. Antiphospholipid antibodies are a heterogeneous population of antibodies recognizing different phospholipids-binding proteins. It is now generally accepted that antibodies with affinity for beta2-glycoprotein I are best correlated with clinical symptoms related to the antiphospholipid syndrome. Therefore we have introduced the anti-beta2-glycoprotein I antibody ELISA at our department. In addition, it was shown that antibodies directed against the first domain of beta2-glycoprotein I are better correlated with thrombosis than antibodies directed against the other domains of beta2-glycoprotein I (De Laat et al. Blood 2005;105:1540-5). This would be a great opportunity to increase the specificity of the anti-beta2-glycoprotein I antibody ELISA dramatically.
Regarding protein S, we are exploring the possibility of designing an assay based on the inhibitory properties of monoclonal antibody CLB-PS13, which was proven to block protein S function at several levels (Hackeng et al. Blood 2004; 104:3624-30). This offers excellent possibilities to develop a functional protein S test, based on the action of a unique monoclonal antibody.

Infectious Diseases (blood donor screening)/Viral Diagnostics

In 2007, the HIV combo-, HBsAg-, anti-HBc-, anti-HBs-, anti-HCV- and anti-Syfilis- on the Architect robot were validated for cadaveric blood samples for microbial safety testing for tissues. Application of magnetic bead particles (Architect) instead of the latex particles/glass fibre technology (AxSYM) improves the specificity of the immuno-assays on cadaveric samples, especially for the antigen tests. The Architect tests for microbial safety of tissues were implemented in 2007.

HBsAg and anti-HIV viral marker tests on plasma pools for fractionation were validated according to the new EMEA guidelines. Special attention is paid to prevention of antigen antibody complex formation in case of HBsAg detection with anti-HBs present as result of vaccination of donors. The impact of the anti-HBs containing matrix is assessed in titration experiments of HBsAg positive samples in anti-HBs positive and anti-HBs negative plasma. SOP's are defined for optimal preparation of samples, storage and assay execution.

In 2007, the concentration of the screening activities of the blood donations for microbial safety in the National Sanquin Screening lab (NSS) was materialized for 70 %. It is foreseen that the concentration of the screening activities will be completed in June 2008. A new platform for the NAT testing of donations covering HBV, HCV and HIV detection for the cellular blood components and fresh frozen plasma will be selected by scientific evaluation of commercially available assays and systems. A tender to select the most appropriate and cost effective offer was submitted. The viral detection will be performed in small test pools making sensitive detection of HBV-DNA possible for prevention of HBV infection by donations in the pre sero-conversion- and in the 'occult' phase of infection. The selected platform and the assays will probably be implemented in the second half of 2008.

The unforeseen announcement by BioMerieux of discontinuation of instruments and reagents for automated nucleic acid extraction in Spring 2009 made it necessary to purchase a new platform for extraction of nucleic acid from pooled plasma and intermediates for manufacturing of plasma derivatives. A survey of commercially available systems potentially appropriate for this application revealed that none of the offered systems is directly suitable for this purpose when used according to the Instruction for Use. It is concluded from comparison experiments between different systems that the EasyMag system of BioMerieux based on magnetic silica extraction from plasma samples is most fitting for extraction of high volumes of plasma (up to 2 ml). This system offers efficient nucleic acid recovery and is suitability for automation. Special 'in house' protocols were developed to prevent agglutination of the magnetic particles during the extraction procedure. A protocol on the

Genesis pipetting station (Tecan) was developed for the EasyMag procedure with complete bar code tracking in the LIMS in order to prevent interchange of samples. The application of the pipetting station also prevents cross contamination between samples. The validation for suitability of the extraction protocols for the markers Parvovirus B19 DNA (PV-B19), HAV-RNA, HCV-RNA and HIV-RNA on pooled plasma and on intermediates was completed successfully. The validation of these assays according to European Pharmacopoeia- and EMEA guidelines are underway.
The test for quantitative PV-B19 DNA detection was further developed. Since October 2006, a combination of two amplification tests on the same nucleic acid sample was officially introduced in screening and 'in process' testing to close the deficiency in detection of the molecular variants genotype 2 and 3 as was observed with the Roche LightCycler test. Since December 2005 this combination was already used to collect epidemiological data on the distribution of these variants in the Netherlands and Belgium. Screening of 3.2 million donations revealed three genotype 2- isolates and no genotype 3 with a load above the exclusion limit for plasma units. Because of delay of commercially available quantitative assays for PV-B19 DNA (postponed to mid 2010), it was decided mid 2007 to develop an 'in house' test kit, based on the already available test for genotype 1-3 detection. This assay was supplemented with internal control detection. Procedures for manufacturing of the kits and feasibility studies for use in screening and 'in process' testing for PV B19 DNA are completed successfully. The validation of the kit according to the CE in vitro diagnostic- and OMCL guidelines is underway and will be completed May 2008.

HLA diagnostics and Paternity testing

The Luminex technology for HLA antibody screening and defining HLA antibody specificity was investigated. The validation was finalized and this technology was implemented in the early part of 2007. The validation study clearly revealed that although solid phase based HLA antibody screening methods are less laborious and makes life much easier, the clinical relevance of some of these antibodies remains unclear.
The pcr-ssp based Killer Immunoglobuline like Receptor (KIR) genotyping analysis for allogeneic bone marrow transplantation was implemented for research purposes.

At the moment a collaborative study to investigate the relevance of activating KIR genes in the selection of the most suitable HSCT donor is being undertaken. From the reactions of the participating hematologist (our customers), a need for such analysis is clearly present.
During 2007, the following innovative projects were further investigated:

- High resolution typing package: validation and implementation of the high resolution typing systems for HLA-DQB1, HLA-A and HLA-B;
- Information and Communication Technology (ICT): the evaluation of the possibilities to convert the so called 'Eurotransplant data base' in an Oracle based application were finalized and the expectation is that the conversion will take place in Q1 of 2008.

New platforms

Studies to change the current department structure of Sanquin Diagnostic Services into a structure more based on employed technologies were continued. Apart from a DNA (isolation) platform, an ELISA platform was started. Evaluation will be continued during 2008.

TRIX

TRIX (Tranfusion Register on Irregular antibodies and X (cross), a computer-based register for irregular erythrocyte antibodies test problems) has extensively been tested and validated in 2006 and nationally implemented in May 2007. This register will serve as a tool to prevent (delayed) transfusion reactions in blood recipients, because also only previously detectable irregular erythrocyte antibodies (IEA) will be taken into account by the selection of donor blood.

Key publications

Van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, Flohr T, Sutton R, Cave H, Madsen HO, Cayuela JM, Trka J, Eckert C, Foroni L, Zur Stadt U, Beldjord K, Raff T, Van der Schoot CE, Van Dongen JJ; European Study Group on MRD detection in ALL (ESG-MRD-ALL). Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia 2007; 21(4):604-11.

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Beunis MH, Smeenk RJT, Vossen RCRM, Willekens FLA. Implementatie van TRIX in 2007. NVB Bulletin 2007, 1:12-9

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Virus Safety Services (VSS) is a virology group dedicated to conducting virus validation studies of plasma-derived products and other biologicals. We offer a range of virus systems, which meet with the latest requirements of national and international regulatory bodies. VSS has more than twenty years of experience in the field of virus validation. VSS is familiar with blood safety issues being part of a blood-product producing organization.

Virus validation studies

We have extensive experience in validation of various process steps, including the more delicate ones, like column and nanofiltration steps. We have state-of-the art BSL3 facilities, including strict separation between virus negative and virus positive areas. In 1998 full accreditation was granted by the Dutch Council for Accreditation, which participates in the European Cooperation for Accreditation of Laboratories (EAL). An Endorsement of Compliance with the OECD principles of GLP based on assessments performed according to the Netherlands GLP Compliance Monitoring Program and according to Directive 2004/9/EC was granted in 2005. VSS provides tailor-made solutions for virus validation problems. Detailed information on the virus reducing capacity of process steps is provided. Furthermore efficient experimental designs are used for demonstrating robustness of process steps and overall accurate insight into viral safety of your product is achieved.

Virus systems available

Appreciating requirements from relevant guidelines, for performance of virus validation studies VSS can offer the following relevant or model virus systems.

- HIV (Human immunodeficiency virus), a relevant virus for products of human origin
- HAV (Hepatitis A virus), a relevant virus for products of human origin
- Human Parvovirus B19, a relevant virus for products of human origin
- BPV (Bovine parvovirus), a specific model virus for Parvovirus B19
- BVDV (Bovine viral diarrhoea virus), a specific model virus for hepatitis C virus
- CPV (Canine parvovirus), a specific model virus for Parvovirus B19
- EMC (Encephalomyocarditis virus), a specific model virus for hepatitis A virus

- PPV (Porcine parvovirus), a specific model virus for Parvovirus B19
- PSR (Pseudorabies virus), a general model virus for lipid enveloped DNA viruses (e.g. hepatitis B virus)
- SV40 (Simian virus 40), a general model virus for non-enveloped DNA viruses
- TGEV (Transmissible gastroenteritis virus), a specific model virus for SARS (severe acute respiratory syndrome)
- VSV (Vesicular stomatitis virus), a general model for lipid enveloped RNA viruses

Research lines

- Quality, safety and efficiency
  - Pathogen detection and inactivation
    - Validation of pathogen inactivation by UV-C irradiation in platelet concentrates 93
    - Pathogen inactivation by UV-C treatment 94
    - Viral safety of human milk 95
    - Viral safety of antivenoms 96

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

Objectives are

(I) to support restricted economy countries in developing safe, efficacious and sustainable blood supply systems based on current quality principles,

(II) to provide modular training programs on transfusion medicine for restricted economy countries focused on the managerial and quality aspects of the transfusion chain, and

(III) to extend and strengthen the training and consultative potential within the Sanquin organization.

In close collaboration with the Academic Institute for International Development of Transfusion Medicine (IDTM) educational programs and applied research in health sciences related to the field of transfusion medicine were developed. Existing educational and scientific collaborations of the Academic Institute IDTM and SCS at the University of Groningen contribute to the development of safe, efficacious and sustainable blood supply systems based on current quality principles.

Educational programs supported by the Academic Institute IDTM, University of Groningen:

1. Noordelijke Hogeschool Leeuwarden (NHL)

a) Two BBA students from China worked on the development of an SCS and IDTM brochure and websites (SCS Communication plan).

2. Vrije Universiteit (VU) Amsterdam, Dept Biology and Society,Faculty of Earth and Life Sciences in collaboration with the Dept of Social Pharmacy, Pharmaco-Epidemiology and Pharmacotherapy, Faculty of Pharmacy, RUG.

a) Graduation student worked on Safety of the blood supply; an attempt to predict the value of the current routine TTI marker screening in Kampala, Uganda.

3. Groningen University (RUG), Faculty of Mathematics and Natural Sciences and Dept of Social Pharmacy, Pharmaco-Epidemiology and Pharmacotherapy, Faculty of Pharmacy.

a) Graduation student in Sciences, Policy and Business, worked on Blood transfusion policy and practice at Mulago Hospital in Kampala, Uganda.

Collaborative scientific projects of the Academic Institute IDTM, University of Groningen and Sanquin Consulting Services:

1. University of Amsterdam (UvA), Faculty of Economy and Econometry, Dept of Operations Research.

a) PhD fellow from Amsterdam, working on a Formal approach for practical optimization of blood platelet production.

2. Groningen University (RUG), Dept of Social Pharmacy, Pharmaco-Epidemiology and Pharmacotherapy, Faculty of Pharmacy.

a) PhD fellow from Groningen, working on Health Economics of blood transfusion safety in developing countries.

3. Groningen University (RUG), Faculty of Medical Sciences and Makerere University, Faculty of Medicine, Kampala, Uganda.

a) PhD fellow from Kampala, Uganda working on 'Appropriate use of the limited supplies of homologous blood in Uganda.

Key publications

Smit Sibinga CTh. Detecting and Monitoring Reactions in the Developing World. In: Popovsky MA (ed). Transfusion Reactions. 3rd edition AABB Press, Bethesda, MD, USA, 2007:449-65.

Haijema R, Van der Wal J, Van Dijk NM. Blood Platelet production: Optimization by Dynamic Programming and Simulation. Comput Operat Res. 2007; 34:760-79.

Patent port folio and licensing

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Besides results of research projects published in scientific and professional publications, Sanquin also disseminates knowledge in the form of patents and other forms of Intellectual Property. As Sanquin is not only a research institute, but first and foremost a blood supply organization, product and process innovation are also very important. This relates to Sanquin activities itself, and to biotech, pharmaceutical and diagnostic and devices companies. In 2007 several patents were filed.

In the table below you will find an overview of the valorization status of Sanquin Research patents and hybridoma's of the last five years. Most patents/hybridomas listed have a primary therapeutic application.

Sanquin patents 2002-2007	Status
MHC Multimers*	Open for licensing
Diagnostic methods involving determining gene copy numbers*	Open for licensing
DCs maturation	Open for licensing
Anti-FVIII	Open for licensing
FVIII mutants	Open forw licensing
Mabs for intact hemostatic proteins	Open for licensing
C1-est inhibitor in AMI	3rd party licensed
CD 97	3rd party assigned
Trombose PCR/FV Leiden	3rd party licensed

Sanquin hybridoma's 2002-2007	Status
Anti CD 19*	Open for licensing
Anti c-1q / Anti c3-2/ 2C8	Open for licensing
4-7B	3rd party licensed
Anti CD70*	3rd party licensed
Anti IL 6*	3rd party licensed

* In recent years it has become apparent that monoclonal antibodies, as therapeutic biologicals, seem to offer new possibilities for patients that were difficult to treat otherwise; for instance patients with autoimmune diseases or cancer.

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 AMC-Sanquin Landsteiner Laboratory for Blood Transfusion Research, housed mainly in Sanquin's premises in Amsterdam.

2nd source of funding

Dutch Medical Research Council (ZON/MW)
Netherlands Genomics Initiative (NROG)
Netherlands Organization for Scientific Research (NWO)
European Commission (FP7; PHEA)

3rd source of funding (Charities, private funding Organizations, non-Dutch Research councils)

Dutch AIDS Fund (SAF)
Dutch Cancer Fund /KWF
Dutch Heart Foundation
Dutch Thrombosis Foundation
Foundation for Pediatric Cancer Research
Friends of Research on MS
Gratama Stichting
Joghem van Loghem Foundation
Landsteiner Foundation for Blood Research (LSBR)
Leiden University Fund
Ministry of Public Health, Welfare and Sport
National AIDS Therapy Evaluation Center
SENTER/Novem
Tekke Huizinga Foundation

4th source of funding: Contract and codevelopment partners

Ablynx
Academic Hospital, University of Maastricht
Academic Medical Center, University of Amsterdam
Adenbrooks Hospital
American Red Cross
Amcell Corporation
Amgen
ASAC
A-Viral ASA
Baxter BioScience
Baxter Health Care
Baxter Oncology
BCSI
Berna Biotech
Biogen
BioMérieux Nederland
BioSafe
Biotest Pharma GmbH
Cardiovascular Research Institute Maastricht (CARIM)
Cerus Corporation
Crucell
Diaclone
DSM Biologics

Finnish Red Cross
Fresenius HemoCare
Gambro BCT
Genmab
GlaxoSmithKline
Guava Technologies
Haemonetics
HAL/Madaus
Innogenetics
Jan van Breemen Institute
Kamada
Laboratoire Français du Fractionnement et des Biotechnologies
Leiden University Medical Center
LevPharma
Macopharma
Microsafe BV
Miltenyi Biotec
Morphosis AG
Natal Bioproducts Institute
Navigant Bonville
NIZO laboratories
Numico
OncoMab
Organon/Schering Plough
Ortho-Clinical Diagnostics Pharming
Philips
PhotoBioChem
ProLacta
Région de Bruxelles-Capitale
RIVM, National Institute for Public Health and the Environment
Roche Diagnostics
Roche Pharmaceuticals
Schering Corporation
Seattle Genetics
Slotervaart Hospital
Staten Serum Institute
Synaps BV
University Medical Center Utrecht
Vitaleech Bioscience
Vrije Universiteit Medical Center, Amsterdam
Wageningen University and Research Center
Zentech s.a.
Zentral Laborator Bern

Other sources of funding

Ministry of Economic Affairs (WBSO)

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