

# Emerging viruses in organ transplant recipients

Immune responses to H1N1/09 influenza  
vaccine and hepatitis E virus infection

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Cover illustration: “A cat with the little invisible cat” by Joel Sundberg (when he was 5 years old). Illustrates the larger influenza virus and the “unknown” hepatitis E virus

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Ineko AB

To all my friends at work



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Immune responses to H1N1/09 influenza vaccine and hepatitis E virus infection

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### ABSTRACT

Solid organ transplant (SOT) recipients run the risk of serious infections. The pandemic influenza A H1N1/09 had unknown severity, so large-scale vaccination was needed. The AS03-adjuvanted vaccine (Pandemrix®) had unknown effects among SOT recipients. We aimed to explore the influenza-antibody (ab) response, ab persistence 1 year later and response to the seasonal influenza vaccine (TIV/10) among adult SOT recipients. Reports of narcolepsy and possible allo-sensitisation following the H1N1/09 vaccination necessitated an analysis of HLA abs and further follow-up. 80% of SOT recipients and 100% of controls had seroprotective H1N1/09 titre levels after 2 vaccine doses ( $p=0.003$ ). A significant loss of protection after 1 year was seen in all subjects. TIV/10 boosted a rise in seroprotection from 47% to 71% in the SOT group and 63% to 100% in controls. Non-responders were more often on triple immunosuppression and had lower renal function. No SOT recipient developed *de novo* HLA abs, but HLA abs with new specificities were detected in some patients. No acute rejection was seen within 2 years after vaccination. Two had chronic rejection within 1 year but a lower and mixed DSA response to the vaccine. The 4th study aimed to investigate the prevalence of hepatitis E (HEV) IgG, IgM and HEV infection, as chronic infection has been reported among SOT recipients. At transplantation, the anti-HEV IgG prevalence was significantly higher in SOT patients compared with blood donors, 30.6% and 16.8% respectively ( $p<0.0001$ ). The patients appeared to have been infected at an earlier age. Two cases of *de novo* and 2 chronic HEV infection were suspected but could not be verified by HEV-RNA.

**To summarise**, the AS03-adjuvanted H1N1/09 influenza vaccine was effective among SOT recipients but significantly less compared with controls. One third of all subjects lost their seroprotection after one year, but TIV/10 reproduced some of the former protection. No patient developed *de novo* HLA abs. The unexpected high prevalence of anti-HEV IgG among the Swedish SOT recipients highlights the possibility of hepatitis E as a new opportunistic infection in the immune compromised host.

**Keywords:** Solid organ transplant, SOT, Hepatitis E, Influenza, H1N1, AS03 adjuvant, HLA antibodies, DSA, rejection

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# SAMMANFATTNING PÅ SVENSKA

Den organtransplanterade patienten med livslång immundämpande medicinering löper risk att drabbas av allvarliga infektionskomplikationer. Två aspekter av detta har studerats i denna avhandling.

Influensavirus A H1N1/09 sommaren 2009 fick en pandemisk spridning över världen. Sverige beslöt att massvaccinera befolkningen. Ett nytt, relativt prövat vaccin valdes (Pandemrix<sup>®</sup>), innehållande en tillsats för starkare vaccinations svar. Vaccinet var ej testat på transplanterade varför effektivitet och ev. följdverkningar var okända. Vi följde 82 patienter och 28 friska kontrollpersoner som samtliga fick 2 doser med 1 månads mellanrum av H1N1/09-vaccinet. Blodprov togs månad 0,1 och 2. Nivån av antikroppar mot H1N1/09 bestämdes med s.k. hemagglutinations-inhibitionstest (HAI). Ett år senare gavs en dos säsongsinfluensavaccin med en komponent av H1N1/09-viruset (TIV/10) till 49 patienter och 11 friska. Nivån H1N1/09-antikroppar månad 0 och 1 mättes. Andras fynd att vaccinet hos ett fåtal givit upphov till nya vävnadsantikroppar (HLA-antikroppar) påkallade test av blodprov tagna månad 0 och 2 efter H1N1/09-vaccinationen avseende HLA-antikroppar. Samtidigt gjordes 5-årsuppföljning via granskning av journaler.

80% av de transplanterade och 100% av de friska nådde infektions-skyddande antikropps nivå efter 2 doser H1N1/09-vaccin ( $p=0,003$ ). Antikropps nivå 1 år senare var 47% respektive 63% i de båda grupperna. TIV/10 ökade immunsvaret till 71% respektive 100% hos patienter respektive friska. De transplanterade som ej fick skyddseffekt av vaccinet var i högre grad behandlade med fler mediciner mot avstötning och hade lägre njurfunktion. Ingen av de som saknade HLA-antikroppar före vaccinationen utvecklade HLA-antikroppar och ingen fick akut avstötning under 2 år från H1N1/09-vaccinationen. Av de 26 som hade HLA-antikroppar före vaccinationen fick 7 nya dito men de var ej riktade mot det transplanterade organet (donorspecifika antikroppar; DSA). Av de 15 med DSA vid vaccinationen fick ingen nya DSA men full utvärdering var ej möjlig pga. äldre donatorsdata. Två diagnosticerades med kronisk avstötning inom ett år från vaccinationen men hade lägre respektive blandad styrka på DSA varför samband med vaccinationen är mindre trolig. De njur-transplanterade med DSA vid studiestart hade också lägre njurfunktion och sju av nio hade förlorat sina njurar vid 5 år. Ingen transplanterad drabbades av narkolepsi, ny autoimmun

sjukdom men en person fick plötslig dövhet ena örat efter första vaccinationsdosen.

Hepatit E virus (HEV) orsakar en infektion i levern, diarré och feber liknande Hepatit A. HEV har ansetts ofarlig då infektionen går över av sig själv och endast ett fåtal fall rapporteras per år i Sverige. Bättre analysmetoder har nyligen visat att HEV är vanligare än vi trott; 16,8% av svenska blodgivare har antikroppar mot HEV. Nya rapporter om att HEV kan ge kronisk leverinflammation hos organtransplanterade, gjorde att vi ville kartlägga förekomsten av HEV i vår patientpopulation.

196 organtransplanterade 2008–2009 lämnade blod- och urinprov från transplantationen och regelbundet under 2 års uppföljning. Vid transplantationen hade 30,6% av patienterna antikroppar mot HEV som tecken på genomgången infektion jämfört med 16,8% av blodgivarna ( $p < 0,0001$ ). Ålderssambandet var starkt, ju äldre desto fler hade HEV antikroppar. I åldersgruppen under 50 år hade 10 transplanterade patienter HEV-antikroppar vilket procentuellt var fler jämfört med blodgivarna ( $p=0,04$ ) men i åldersgruppen över 50 år var det ingen skillnad. I åldrarna >60 år hade 47% av de transplanterade och 54% av blodgivarna genomgången HEV. Under uppföljningen efter transplantationen fann vi utifrån HEV-antikroppsmönstret 2 misstänkta fall med kronisk HEV och 2 som troligen smittades under uppföljningen men inga av dem hade symtom och vi kunde ej påvisa viruset med genetisk diagnostik (qPCR HEV-RNA).

Sammanfattningsvis var H1N1/09 vaccinationen effektiv hos de transplanterade men signifikant sämre jämfört med friska. Efter 1 år hade en tredjedel i båda grupperna tappat sin skyddande antikropps nivå men TIV/10 ökade nivån något bland de transplanterade. Patienter som ej hade HLA-antikroppar påverkades ej av H1N1/09 vaccinationen. HEV var vanligare bland organtransplanterade jämfört med friska, vid tiden för transplantationen hade nästan en tredjedel haft hepatit E. Då möjligheten finns att HEV kan ge kronisk infektion med sekundär organpåverkan bör alla nya fall med okänd leverinfektion och ev. även okänd njursjukdom testas för HEV.



# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Felldin M, Studahl M, Svennerholm B, Friman V.  
The antibody response to pandemic H1N1 2009 influenza vaccine in adult organ transplant patients.  
Transplant Int. 2012;25(2):166-71
- II. Felldin M, Andersson B, Studahl M, Svennerholm B, Friman V.  
Antibody persistence one year after pandemic H1N1 2009 influenza vaccination and immunogenicity of subsequent seasonal influenza vaccine among adult organ transplant patients.  
Transplant Int. 2014;27(2):197-203.
- III. Felldin M, Johansson S, Holgersson J, Friman V.  
HLA antibody responses in adult solid organ transplant recipients after AS03-adjuvanted influenza A (H1N1) vaccination.  
In manuscript.
- IV. Felldin M, Friman V, Lindh M, Norder H.  
High prevalence of anti-HEV IgG in a Swedish solid organ transplant population.  
Submitted.

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# ABBREVIATIONS

ALF	Acute liver failure
AMR	Antibody mediated rejection
APC	Antigen presenting cell
AS03	Name of vaccine adjuvant
ATG	Anti thymocyte globulin
BKV	BK virus
C4d	Complement fragment number 4d
CD	Cluster of differentiation
CDC	Complement dependent cytotoxic
CMV	Cytomegalovirus
CNI	Calcineurin inhibitors
CS	Corticosteroids
CSF	Cerebrospinal fluid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSA	Donor specific antibody
eGFR	Estimated glomerular filtration rate
Fab	Fragment antigen binding
Fc	Fragment crystallisable
FC/FACS	Flow cytometry
GBS	Guillain-Barré syndrome
H1N1/09	Influenza A(H1N1)pdm09
HA or H	Haemagglutinin
HAI	Haemagglutination-inhibiting antibodies
HEV	Hepatitis E virus
HLA	Human leucocyte antigen
HPV	Human papilloma virus
ICU	Intensive care unit
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
KDOQI	Kidney disease outcome quality initiative
MDRD	Modification of diet in renal disease-equation
MF59	Name of vaccine adjuvant
MFI	Mean fluorescence intensity
mGFR	Measured glomerular filtration rate

MHC	Major histocompatibility complex
MPA	Mycophenolic acid
mTOR	Mammalian target of rapamycin
NA or N	Neuraminidase
NK cells	Natural killer cells
PCR	Polymerase chain reaction
PRA	Panel reactive antibody/ies
qPCR	Quantitative PCR
RNA	Ribonucleic acid
SOT	Solid organ transplant
TCMR	T-cell mediated rejection
TCR	T-cell receptor
TG	Transplant glomerulopathy
Th	T helper cells
TIV	Trivalent influenza vaccine
TNF	Tumour necrosis factor
Treg	T regulatory cell
WHO	World Health Organisation

# 1 INTRODUCTION

The success of solid organ transplantation (SOT) has been hampered by serious infectious complications. The knowledge and treatment of opportunistic infections, together with the development of new immunosuppressive medications, has led to improvements in early graft survival, decade after decade. Vaccination before transplantation against specific bacterial and viral agents, effective prophylaxis against opportunistic infections in the early phase after transplantation and the opportunity to monitor viruses, in particular using PCR, have opened the door to the finer tuning of the immunosuppressive treatment.

For many years, yearly vaccination against seasonal influenza has been part of the recommended treatment for all SOT recipients, but, upon the arrival of the influenza A(H1N1) pandemic in the summer of 2009 (named by the WHO as “influenza A(H1N1)pdm09”) large-scale vaccination was also needed in the general population. With only a little antigen available, a new adjuvanted vaccine was used in Sweden, as well as in other countries. The efficacy and side-effects of this vaccine among SOT recipients were unknown at the start of the vaccination, why we decided to conduct a study of vaccine response and side-effects in our patient cohort. As a spin-off from this primary study, we conducted a second study one year later, examining the remaining serological memory of the pandemic vaccination and the subsequent booster effect of the seasonal influenza vaccine in 2010. Due to both the alarming reports of narcolepsy in children and young adults following the AS03-adjuvanted vaccine and the publication of studies indicating a higher risk of rejection than expected after this vaccine, we decided to analyse whether our cohort of SOT recipients developed HLA antibodies due to the vaccination.

Hepatitis E virus (HEV) causes an infection, with symptoms resembling hepatitis A. It has been regarded as a harmless, self-limiting hepatitis in the industrialised world, with very few cases reported on a yearly basis. However, following the development of diagnostic tools with high specificity and sensitivity has revealed that HEV infection is far more common than previously thought. Moreover, chronic fatal HEV infections have been reported among immunocompromised patients, in particular SOT recipients in other European countries. We decided to study the magnitude of HEV infection in our SOT population.

# 1.1 Organ transplantation

The clinical transplantation of tissue and organs started more than a century ago with a thyroid tissue transplantation in 1883 by the Swiss surgeon Theodor Kocher, who thereby restored lost organ function. Other hallmarks were when Alexis Carrel from France developed the surgical technique of vascular anastomosis in 1902 and Joseph Murray and his team at Peter Bent Brigham Hospital in Boston, USA, conducted the first successful kidney transplantation in 1954 between a pair of identical twins [1]. Kidney transplantation became the pathfinder in the history of organ transplantation due to the development of the lifesaving dialysis therapy against uraemia, the opportunity to use optimal kidneys from live donors and also the simplicity of monitoring organ function [2]. As a result, most of the pioneering work in humans began in the field of kidney transplantation.

## 1.1.1 History of transplant immunology

It was evident in the early years that the presence of an immunological barrier, studied by the British scientists Medawar and Billingham, among others, using skin grafts in animals [3]. They found that, the more genetically alike, the less the rejection and, furthermore, grafts between monozygotic twin animals were not rejected at all.

Of the two main immunological barriers against transplantation, the blood group antigen, ABO system, was discovered back in 1901 by Karl Landsteiner, but the importance of respecting the blood group barrier in organ transplantation to avoid hyper-acute rejection was first described in 1964 by Thomas E Starzl [4].

The human leukocyte antigen (HLA) system has been discovered stepwise by different scientists starting in 1958 and, in the same year, anti-human leukocyte antibodies were discovered. One important step was the development of the micro cytotoxic test enabling the detection of HLA antigens and antibodies by Paul Terasaki in 1964 [5]. In 1969, Patel and Terasaki showed that a positive CDC crossmatch before transplantation led to rejection within two days in 80% of the recipients compared with only 4% if the crossmatch was negative [6]. Since then, a positive CDC crossmatch between donor lymphocytes and recipient sera is a contraindication to transplantation in all organ transplantation except liver transplantation. It was also found that, the better the HLA match, the better the graft survival and the transplant community



prioritised a full HLA match. However, in 1971, when Terasaki reported that there was no great difference in graft survival between one or more HLA mismatches [7], strict HLA matching in organ transplantation was slowly abandoned. Only HLA-identical siblings did better.

### **1.1.2 History of immunosuppression**

In 1951, Billingham and Medawar found the prolonged survival of skin grafts when the animals were treated with cortisone [8]. However, the first immunosuppression used in clinical transplantation between dizygotic twins or more distantly related persons was sub-lethal total body irradiation, but this had to be abandoned due to the development of bone marrow aplasia and death from severe infections. In the late 1950s, cytotoxic drugs were developed to treat malignancies, but 6-mercaptopurine and its analogue, azathioprine, were also introduced in organ transplantation in 1960, but graft and patient survival were still very poor. A milestone was reached when Thomas Starzl et al. used concomitant prednisone therapy, resulting in prolonged kidney graft survival in 1963 [9]. This led to the start of several new kidney transplantation centres in the western world. By the end of the 1970s, the lesson of using steroids in lower doses was learned and the one-year graft and patient survival was 60% and 90% respectively after kidney transplantation. The first successful liver transplantation was performed in 1967, but multiple technical and physiological difficulties resulted in a high mortality rate. During the pioneering years, it was evident that the liver is a more tolerogenic organ compared with other whole organs, as a number of recipients survived without immunosuppression [2]. The first heart transplant was performed in South Africa by Christian Bernard in 1967 and it attracted enormous publicity worldwide, while the first successful lung transplant took place in 1981. In the late 1970s, a new drug was developed that would move the field of organ transplantation another step forward – cyclosporine A. This drug, when used in lesser nephrotoxic doses, led to significantly better graft survival in all organ transplantation and heralded the start of other whole organ transplantation programmes. In the early 1990s, tacrolimus was introduced, followed some years later by mycophenolic acid. Together with steroids, these drugs are still the cornerstones of organ transplantation.

### **1.1.3 Organ transplantation in Sweden and Gothenburg**

In Sweden, the first kidney transplantations were performed in Stockholm in 1964. The year after, Lars-Erik “Charlie” Gelin started our unit at Sahlgrenska University Hospital. Kidney transplantation was soon also established in Uppsala and Malmö and together we currently perform 400-450 kidney transplantations every year. Here in Gothenburg, we perform almost 40% of Swedish renal transplantations and, in 2016, we celebrated kidney number 6,000, while in Sweden a total of 15,062 kidney transplantations had been performed at the end of 2016. Liver transplantation programmes are running in Stockholm (since 1984) and Gothenburg (since 1985) with about equal numbers of transplantations every year, increasing steadily towards a total number of almost 200 a year. The first Swedish heart transplantation was performed in Gothenburg in 1984 and, today, together with Lund, about 60 heart and 60 lung transplantations are performed on a yearly basis. The results for all organ transplantations have gradually improved, in particular during the first year after transplantation. For example, after renal transplantation in Gothenburg, as in most centres, the current one-year graft survival is 97%. Organ transplantation has thus developed during the last 50 years to become an important part of the treatment of end-stage organ failure. As the results have improved, we have been able to widen the indications and transplant individuals with higher burdens of co-morbidity. The number of different transplants performed at Sahlgrenska University Hospital is shown in Fig 1.

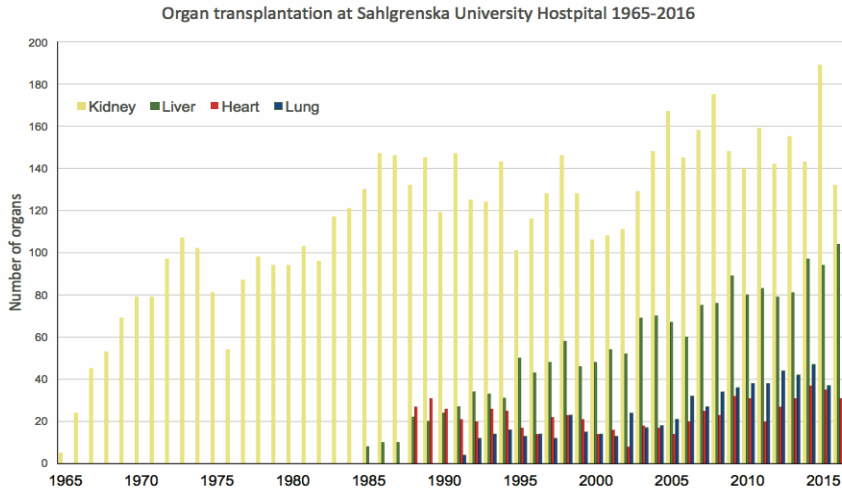


Figure 1. Solid organ transplantation at Sahlgrenska University Hospital since the start in 1965. In yellow: kidney (total no. 6,029), green: liver (1,745), red: heart (676), blue: lung (648).

## 1.2 Immune defence

Our defence against foreign organisms and substances is composed of three major defence levels [10]. The first are the mechanical and chemical barriers, such as the skin, the acidity in the stomach and the mucus layer in the respiratory and gastrointestinal tract. The second level is the *innate immunity* which reacts immediately to microbes with an inflammatory response made up of mainly white blood cells, such as macrophages and neutrophils, which, together with chemical agents, act on phagocytosis and the destruction of microbes. The third level of defence is the *adaptive immunity*, consisting mainly of T and B lymphocytes which are activated more slowly and, after a maturation process, take part in the elimination of the foreign cells or substances (Fig 2).

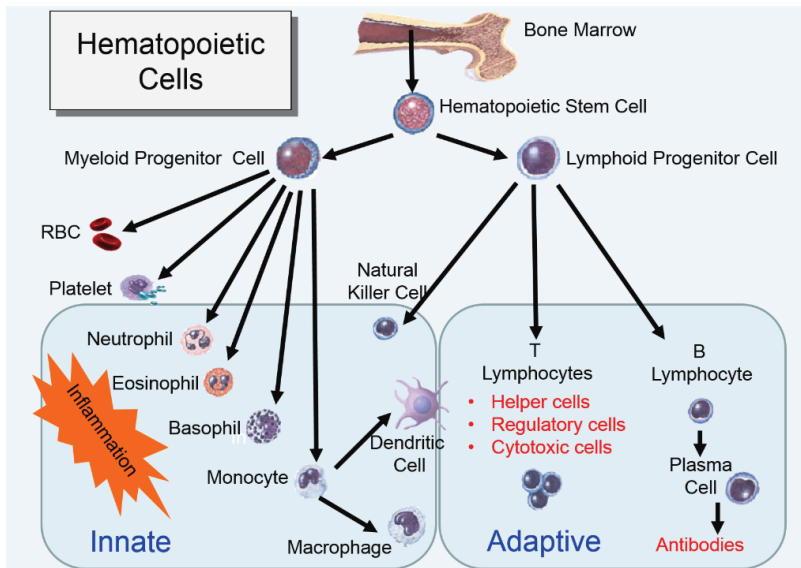


Figure 2. The innate and adaptive immune cells. By permission of prof Wayne LaMorte <http://sphweb.bumc.bu>.

## 1.2.1 Innate immunity

This immediate defence reacts to danger signals such as microbes or tissue damage. Pattern recognition receptors (PRRs) on macrophages and their like recognise a repertoire of bacterial or viral molecules (called pathogen-associated molecular patterns – PAMPs) or molecules from dying host cells (called damage-/danger-associated molecular patterns – DAMPs) [11], leading to the production of a number of pro-inflammatory mediators, such as the cytokines IL1 and TNF $\alpha$ . Complement is activated and this helps the recruitment of more inflammatory cells such as neutrophil granulocytes and macrophages in the acute phase. Prostaglandins promote vessel dilatation and increase vessel permeability, thereby facilitating the movement of inflammatory cells to the site of inflammation. The microbes are then phagocytosed. By opsonisation, i.e. the binding of immunoglobulin (IgG) and complement on the surfaces of microbes, phagocytosis is facilitated. The dendritic cell (DC) is less competent in phagocytosis compared with macrophages, but it plays an important role as a presenter of foreign pathogens in the form of degraded peptides on its cell surface. The dendritic cell is the

most important “antigen presenting cell” (APC) and it is a link between the innate and adaptive immune systems. The dendritic cell engulfs the foreign pathogen and presents small parts/peptides on its HLA surface, enabling the activation of the adaptive immune system.

## 1.2.2 Adaptive immunity

The adaptive (or acquired) immune system is developed and differentiated during life, depending on the antigens we encounter. It consists mainly of lymphocytes of the T- and B-cell type, all of which have a unique and specific receptor on their surface recognising a specific antigen. The activation of this highly specific immune defence takes one to two weeks the first time it encounters an antigen, but a proportion of cells and antibodies remain as an immunological memory and, when the immune system meets the same antigen again, the activation is faster.

Antigen presentation is mediated by Major Histocompatibility Complex (MHC) molecules found on cells surfaces. In humans, these structures are called human leukocyte antigen (HLA). They are a key component in the host immune defence, as foreign antigens (for example, viral peptides) are presented to T-cells by these molecules, enabling the host immune cells to recognise the intruder. Moreover, in the case of a transplanted organ, the host’s immune cells recognise the foreign donor HLA and this will also activate the immune system. As a result, the HLA system is responsible for the host’s ability to recognise cells as “self” as opposed to “non-self”. (*The HLA system is further described in Section 1.2.7.*)

### T-cells

The T-cell receptor (TCR) consists of two proteins which have high variability, leading to a broad repertoire of specificities. In the thymus, T-cells mature through a selection process where those T-cells with non-functional TCR and those with TCR that is able to react to self-antigens are sorted out. The thymus thus teaches the immune system what is self and non-self. The T-cell harbours a protein cluster called CD3 (“cluster of differentiation”) in all differentiation stages and the mature T-cell expresses it on the surface of the cell and forms a part of the T-cell receptor. CD3 is used as a marker in histopathology to identify T-cells of all types. There are two major types of T-cell, CD8+ and CD4+, with different binding preferences to the APC and different effects.

**CD4+ T-cells:** These cells bind to cells expressing HLA Class II and they therefore only bind to APC. The CD4+ cells mature into two basic groups of cells; T-helper cells (Th) or T-regulatory cells (Treg). However, there appear to be a number of subsets of T-cells within each of these two main characters, each subset derived under the influence of different cytokines with somewhat different cytokine production from a mature T-cell. Activated Th cells perform three major actions. **Firstly**, the Th cell produces cytokines like interferon gamma ( $\text{INF}\gamma$ ) which help the macrophage to enhance its capacity to kill the pathogen. **Secondly**, T-helper cells are essential in the B-cell proliferation process to mature into antibody-producing plasma cells. **Thirdly**, T-helper cells are needed to activate the CD8+, cytotoxic, T-cells. The T-helper cell therefore plays a central role in the activation and effect of the adaptive immune system. Tregs are able to control and down-regulate the adaptive immune response. They are recognised by the expression of the transcription factor, FOXP3. They suppress T-helper cell activation and also the APCs. Tregs are thereby able to induce tolerance towards the antigen. However, the circumstances under which the Treg effect will dominate the immune response is not known.

**CD8+ T-cells:** Naïve CD8+ T-cells mature into cytotoxic T-cells and recognise antigens presented on HLA Class I molecules which are present on all nuclei-containing cells in the body. For example, a virus-infected cell will produce viral protein, and the cell will decompose the protein into peptides, as the cell does with its own proteins. The viral peptides will be presented on the HLA Class I molecule on its surface. CD8+ T-cells, randomly formed in the thymus with the specificity of the viral peptide, will recognise the peptide and will thereby be partly activated. The cytotoxic T-cell requires further help from an also activated CD4+ T-helper cell in the environment of the cytokines IL2 and  $\text{INF}\gamma$ . A fully activated cytotoxic T-cell is able to kill all the cells presenting the antigen for which it has specificity.

## **B-cells**

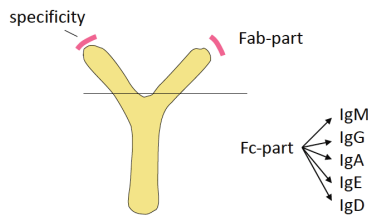
There are different subsets of B-cells. B1 spontaneously secrete IgG and IgM antibodies with low affinity directed towards various carbohydrates as expressed by bacteria. B2 are the “conventional” B-cells which can be activated to become immunoglobulin-producing plasma cells. This activation requires a number of interactions with the T-helper cell. The B-cell surface has membrane-bound immunoglobulins and, when they meet their specific

antigen, the activation process starts and they develop receptors for the cytokines IL4, IL6 and IL10. These cytokines are produced by activated T-helper cells which have met APCs presenting the same antigen. The B-cell is able to internalise the antigen and present a part of the antigen as a peptide on its HLA Class II surface. A T-helper cell with the specificity of this antigen interacts with the B-cell. At the same time, the T- and B-cells interact via co-stimulatory molecules; for example, the CD40 ligand of the T-cells binds to the CD40 of the B-cells which enhances the activation of the B-cell. The result is effective B-cell stimulation to proliferate into mature, long-lived, plasma cells. B-cell maturation without Th-stimulation results in short-lived plasma cells [12].

The interaction between the TCR - antigen –MHC – has been named “signal one”. To fully activate lymphocytes, co-stimulation is needed, named “signal two” (exemplified for B-cells above). T-cells stimulated without co-stimulation can result in T-cell anergy or apoptosis. Naïve T-cells express only few co-stimulatory receptors as CD28 and CD27 but they can be either up-regulatory or inhibitory. A large diversity of receptors and ligands are present on APC, lymphocytes and also non-hematopoietic cells.

### Immunoglobulins (Ig)

An antibody consists of two parts, the specific antigen-binding, called “Fab” (F=fragment, ab= antigen binding), and the “Fc” (F=fragment c= crystallisable) part defining the immunoglobulin type and biological action. There are four different types of immunoglobulin; IgM, IgG, IgA, IgE and IgD (Fig 3).



*Figure 3. One unit of an immunoglobulin and its principal parts. “Fab”: the antigen specific binding site and the Fc part defining the isotype of immunoglobulin; IgG, IgM, IgA, IgE and IgD. Reproduced and modified by permission. Johan Mölne and Agnes Wold, “Inflammation” 2007 Liber förlag.*

Monomeric IgM is the membrane-bound antibody on all naive B-cells, but, when secreted, it consists of five units of immunoglobulins held together by a J-chain. There are thought to be two types of IgM, natural/innate IgM and immune/adaptive IgM. Natural IgM is produced by a subtype of B-cells without having encountered a specific pathogen and constitutes the majority of circulating IgM in serum. These IgM are polyreactive and bind antigens with low affinity, compensated for by the 10 binding sites of antigens (Fig. 4). They are also complement binding and as a whole they are very effective in the clearance of infectious agents. The immune/adaptive IgM is produced by B-cells in the spleen and lymph nodes after antigen exposure. Specific IgM is detectable five to 10 days after the start of an infection and it has been used as a marker of these events. IgM production usually subsides after six weeks when the B-cell matures, but specific IgM production sometimes lasts for five to six months [13]. Recently in mice, specific IgM production have been seen even two years after infection [12].

IgG is secreted by long-lived plasma cells, usually at a constant rate. It is antigen specific and it binds its antigens with high affinity. There are four subtypes of IgG; IgG1-IgG4. They differ in their complement-binding capacity; IgG3 > IgG1 > IgG2 and IgG4 do not bind complement at all. IgG production can be measured one to six weeks after the debut of illness and lasts for years, lifelong if the virus remains latent.

IgA is present in serum as a monomer and is non-complement binding. IgA plays an important role in mucosal immunity. Plasma cells in the mucosa produce a dimeric IgA, two units of immunoglobulin, connected with a J-chain and a secretory component enabling “secretory IgA” to pass the epithelium into the lumen of the organ where IgA is able to neutralise infectious agents.



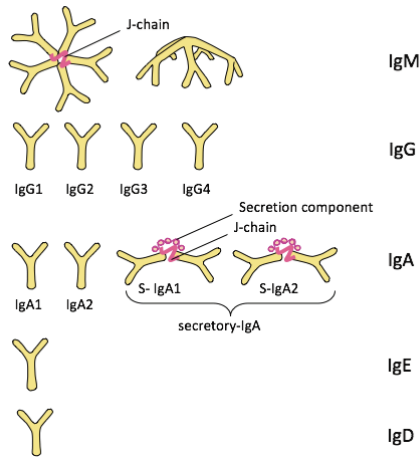


Figure 4. The different sorts of immunoglobulin molecules. IgG with 4 subtypes, IgM forming a pentamere and IgA forming secretory IgA, a dimer. Reproduced and modified by permission. Johan Mølne and Agnes Wold, "Inflammation" 2007 Liber förlag

## Immunological memory

The hallmark of the adaptive immune system is its memory. There are both memory T- and B-cells, as well as long-lived plasma cells. The latter is the source of constant antibody (IgG) production. These memory cells reside primarily in lymphoid tissue and in the bone marrow, but they can quickly be recruited in conjunction with danger signals. They are re-activated at lower levels of their particular antigen exposure, sometimes without the need for co-stimulation, resulting in a faster and stronger response.

### 1.2.3 Viral immunology

Viruses are small particles, with diameters ranging from 20 to 300 nanometres, and consist of genetic material (DNA or RNA) surrounded by a protein shell called a capsid. They depend on the host cells for their metabolism and proliferation and overrule the normal metabolism of the host cells in favour of producing new viral particles. The new viruses are released by either host cell death or exocytosis (non-enveloped viruses) or by "budding", where the viral particle uses a part of the host cell membrane to form an envelope, usually with the incorporation of viral receptors (enveloped virus). Some viruses are capable of a very rapid infectious cycle, within hours, resulting in the

production of  $10^{11}$  new virions within a day – an enormous amount, considering that humans are composed of about  $10^{14}$  cells [14].

The immune response to a viral infection is a combination of the innate and adaptive immune system. First, the innate system is triggered by the viral presence leading to the release of interferons which inhibit (or “interfere” with) the virus replication as  $\text{INF}\beta$ ,  $\text{INF}\alpha$  or IL1. INF, in turn, activates “natural killer cells” (NK cells).

NK cells are classified as lymphocytes, but they lack the T-cell receptor-CD3 complex and are members of the innate immunity (Fig. 2). Activated NK cells produce cytokines, mainly  $\text{INF}\gamma$ , but they are also cytotoxic. They target cells lacking the MHC complex, which explains why NK cells selectively kill stressed cells with down-regulated MHC, such as virus-infected or mutated/malignant cells. When the INF/cytokine level becomes high, this triggers an acute-phase reaction with corresponding clinical symptoms, such as fever and others depending on the site of infection and, at this stage, the liver has shifted production from serum albumin to c-reactive protein and other acute-phase reactants. Dendritic cells in the lymphoid tissue in the mucosa and lymph nodes present viral antigen to T-lymphocytes, starting the activation of the adaptive immune system. B-lymphocytes are activated and mucosal IgA, then IgM and finally IgG production is started. As a memory of the viral infection, IgG persists, often lifelong, but sometimes also both  $\text{T}_{\text{CD}8+}$  and  $\text{T}_{\text{CD}4+}$  cells.

## 1.2.4 History of vaccination

The first attempts at vaccination appear to have taken place in China more than 1,000 years ago. Small droplets from a smallpox virus (variola virus) pustule were rubbed into small scars in the skin of a healthy person to cause an intentional yet low-grade infection with the aim of protecting this person from subsequent lethal disease [14]. The technique was called “variolation”. Although some individuals nonetheless developed generalised disease, variolation slowly spread through the world. In 1796, Edward Jenner found that persons infected with cowpox virus developed a similar yet milder infection compared with smallpox and were subsequently immune to smallpox infection. When he used cowpox virus in variolation, the method turned out to be much safer, with a good protection rate. This was the first vaccine, the term coming from the Latin word *vacca* = cow.

## 1.2.5 Viral vaccines and their immunology

Today, two major types of vaccine are used; live-attenuated vaccines, such as measles, mumps and rubella vaccine, where there is a possibility of viral replication, particularly in the immune-compromised host. For this reason, these are not used after organ transplantation. Secondly, inactivated vaccines with no potential replication, such as the first generation of influenza vaccine. More recently, subunit vaccines (trivalent influenza, hepatitis A and B vaccines) and virus-like particles (like HPV vaccine) have been developed and other techniques are in the pipeline [14].

The immunological reaction after vaccination depends on many factors, such as the virus properties, choice of antigen and route of administration. Most vaccines today are used as prophylaxis against acute viral infections which entails a risk of severe complications or long-term complications (such as cancer). The purpose of these vaccinations is to evoke a good immunological memory, if possible both humoral and cellular, and/or mucosal immunity. The protection should be long lasting, at best lifelong. The vaccination must have minimal side-effects and no negative long-term effects. It should preferably also involve a simple administration regimen and cost effectiveness [15].

### Vaccine-induced memory

Specific antibodies against the virus are the key effector mechanism in viral disease protection by neutralising the viruses. This has been shown by the protection effect of passively administered antiviral antibodies, exemplified by the prophylactic treatment to prevent hepatitis B [14]. The vaccine antigen stimulates B-cells to produce antigen specific antibodies as a primary response to the vaccine. The serum concentration will gradually rise reaching a maximum at about 14 days. Gradually the immunoglobulin concentration will fall but memory B-cells will remain dormant long term. Repeated vaccine doses produce stimulation towards a faster and more specific IgG production with higher affinity (Fig5). Vaccines capable of stimulating  $\text{INF}\gamma$  production will increase the immunoglobulin production of subclasses IgG1 and IgG3 which are complement binding, further promoting the neutralising capacity. Hence, these repeated immunisations (booster doses) can improve the breadth of the immunisation by recruiting antibodies against different regions of the viral structure, for example, regular influenza vaccination.

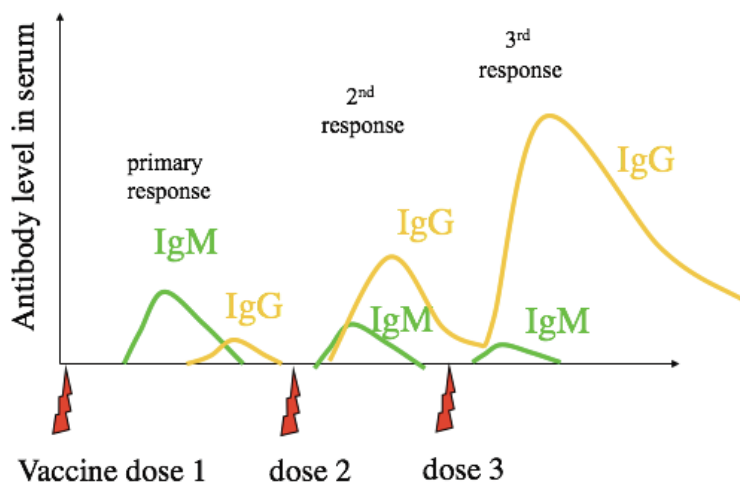


Figure 5. Serological response after repeated vaccination doses favouring a robust IgG secretion long term. Reproduced and modified by permission. Johan Mölne and Agnes Wold, "Inflammation" 2007 Liber förlag

Virus-specific IgG are, however, seldom sufficient by themselves to provide full protection from the viral disease. Vaccination also induces an accompanying T-cell memory, both T-helper cells and cytotoxic T-cells. The cell-mediated memory is needed for better protection of the host from future infections [16].

## Adjuvants

Adjuvants (from Latin "adjuvare" = to help) are compounds able to enhance or modify the immune effect of a vaccine antigen. The more refined the antigens used (compared with live-attenuated vaccines), the more concomitant antigen stimulation is needed to induce an immunological memory. Moreover, if fewer antigens are available or there is a need for rapid immunisation, adjuvant is added to the vaccine. The immunological effect of commonly used adjuvants has not been fully elucidated and the first adjuvants in vaccines were empirically derived. In the 1920s, it was recognised that, if a local inflammation was inflicted at the injection site, the protection provided by the vaccine dose was better. Further research led to the development of the first adjuvant vaccine used in humans containing aluminium salts ("alum") in 1932. Not until 1997 was another adjuvant approved for use, "MF59", in an influenza

vaccine formulation. MF59 is an oil-in-water emulsion composed of squalene oil, polysorbate and sorbitan trioleate. Squalene is a synthetic precursor of cholesterol and steroid hormones with normal endogenous production in humans of about 1g/day and it is therefore fully metabolised in the body. The two latter compounds are both surfactants and these three compounds form small oil droplets. The antigen does not adhere to the droplets, but the oil emulsion stimulates the innate immune cells to phagocytose and, as a result, antigen uptake is more efficient [17]. The enhanced protection rate among risk groups with tolerable side-effects was demonstrated in randomised, controlled studies in the late 1990s [18].

**AS03**, the adjuvant in Pandemrix<sup>®</sup> (GlaxoSmithKline, Dresden, Germany) used in Sweden during influenza A(H1N1)pdm09, is also an oil-in-water emulsion with squalene and polysorbate (surfactant), but the third component is alpha-tocopherol (vitamin E). The mode of action is comparable with that of MF59, although an even more favourable immune stimulation effect has been attributed to the tocopherol component [19]. This adjuvant was a more novel agent compared with MF59 and was less studied when the decision on vaccine type had to be made in the summer of 2009. AS03 was developed as an adjuvant to the H5N1 avian influenza pandemic vaccine, with a first human study published in 2007 [20]. The vaccine was further used in larger cohorts with a total of > 6,000 participants, showing both excellent efficacy and safety data [21, 22].

## **1.2.6 Transplant immunology**

The main barriers to the acceptance of a transplanted organ are the main blood group type, the ABO system, and the tissue type, the HLA system.

### **ABO system**

The ABO blood group system is the most important of many blood groups. The ABO antigens are expressed not only on erythrocytes but also by many epithelial and endothelial cells, such as those in the kidneys, liver. They are composed of a core saccharide attached to either a lipid or a protein and a terminal oligosaccharide. In early childhood, natural antibodies of IgG and IgM subtypes are developed against the A and B antigens. Individuals with blood group A develop antibodies against B. Individuals with blood group B

develop antibodies against A. Persons with blood group O (“ohne” = without) lacks this blood group antigen but form antibodies against both A and B, while persons with blood group AB have no antibodies. If the blood group barrier is not respected in organ transplantation, these antibodies will cause a hyper-acute rejection. However, with immunosuppression and the removal of the preformed ABO antibodies, the blood group barrier is possible to overcome, albeit with a higher risk of early acute rejection. After a few weeks, a state of adaptation to the foreign blood group antigens on the graft occurs and the long-term graft survival equals the survival of ABO compatible grafts [23]. The adaptation mechanism is not known.

## **HLA system**

The HLA system comprises surface molecules and is essential for presentation of antigens for the adaptive immune system and, in the context of transplantation, to distinguish between cells of “self” origin or “non-self”. They are encoded from chromosome 6p21.3 containing some 224 genes and known as the most polymorphic genetic system in humans. There are two major classes of MHC/HLA; Class I and II. The Class I region is most importantly composed of the genes encoding HLA-A, -B and -C and these Class I HLA structures are found on all cell surfaces except red blood cells. The Class II region mainly contains the genes for HLA-DR, -DQ and -DP. Class II are only found on APCs. To date, 12,021 HLA Class I alleles and 4,230 Class II have been defined [24]. The genes of HLA Class I and II are closely linked and inherited as a haplotype in a Mendelian manner, with the exception of HLA-DP which has a variable inheritance. This means that children inherit one haplotype from their mother and one from their father. Among siblings, the probability of sharing one haplotype is 50%, while 25% of siblings are HLA -A, -B, -C, -DR, -DQ identical (“HLA identical sibling”) and 25% share no HLA haplotype [25].

The tissue typing technique has evolved from cellular or serological techniques to molecular biological methods, all based on polymerase chain reaction (PCR) analyses. A description of the historical development of the different techniques can be found in Fig. 6. The serological tissue typing had an error rate of up to 20% in both Class I and Class II [26]. At our centre, PCR HLA typing was first used for Class II typing in 1993 and, since 2007, all HLA typing is done by PCR. Differences in the HLA structure between donor and recipients trigger the T- and B-cell immune response, as described. If a

recipient has HLA antibodies present before transplantation, the graft has to be matched with the recipient by choosing a graft which does not express those HLA antigens against which the HLA antibodies are directed; otherwise, there is a risk of rejection; the stronger the HLA antibody, the faster and more severe the rejection. HLA antibody formation can occur during pregnancy, blood transfusion or, most frequently, previous transplantation. Recipients with HLA antibodies are termed HLA sensitised or immunised.

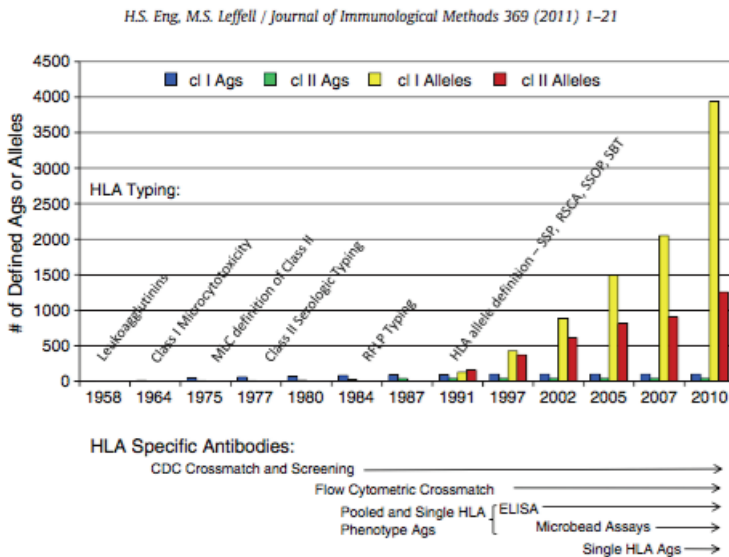


Figure 6. The development of HLA typing and the number of discovered antigens and alleles with time. Reproduced with permission, Eng HS, Leffell MS, Histocompatibility testing after fifty years of transplantation. Journal of Immunological Methods 369 (2011)1-21.

## Detecting HLA antibodies

Cell-based techniques: HLA antibodies are detected by either cell-based techniques or solid-phase techniques. Complement-dependent cytotoxic (CDC) crossmatch was the first technique developed in 1964 for the identification of HLA antibodies in the recipient [5]. By mixing donor

lymphocytes with the recipient's serum, adding rabbit complement and finally a fluorescence dye, dead lymphocytes could be visualised in the fluorescence microscope. Using this technique, complement-dependent and thereby strong, clinically significant HLA antibodies can be discovered. A positive CDC crossmatch is regarded as a contraindication to all organ transplantation (described in Section 1.2.2.), with the exception of liver transplantation [6]. However, the CDC technique will not detect antibodies which are complement independent (subtype IgG4), weaker complement activator (subtype IgG2) or antibodies present in lower levels. A more sensitive cell-based crossmatch technique was developed using flow cytometry for detection (flow, FACS or FC crossmatch). Donor lymphocytes are mixed with the recipient's sera, together with fluoresceinated anti-human globulin, enabling HLA antibody detection by flow cytometry. The use and interpretation of flow crossmatch results have been centre specific; some centres have regarded a positive result as a contraindication to kidney transplantation, while others – like our centre – have regarded a positive result as a risk factor for rejection with a need for a higher immunosuppression level. Both CDC and cell-based flow crossmatches are not specific to HLA antibodies and can be positive, due to auto-antibodies or therapeutic antibodies, such as rituximab and antithymocyte globulin (ATG).

**Solid-phase technique:** In many ways, the modern solid-phase technology for HLA antibody detection is a precise way to determine both the type and strength of the antibodies. There are a few different commercially available tests, but, as the Luminex platform from OneLamda, developed by Paul Terasaki, is the most commonly used, including at our centre, I have focused on this technique. A solid matrix, here polystyrene microbeads, is coated with purified HLA antigens and incubated with the patient's sera. The antigen-antibody binding is detected by anti-human IgG (or IgM, if chosen) marked with fluorochromes identified in the Luminex fluorometer, a flow cytometer. For screening purposes, multiple HLA antigens grouped into Class I and II are coupled with the beads and the result is positive or negative, depending on whether or not HLA antibodies are present (LABScreen Mixed<sup>®</sup>). A positive result is most often further analysed for the detection of single, specific HLA antigens. The beads are then coated with single HLA antigens, the 100 most common, enabling the detection of the specificity of the HLA antibody (single-antigen assay). The number of antibodies is measured as the “mean fluorescence intensity” (MFI). The test is semi-quantitative, evaluating the



relative antibody strength, and there is no linear relationship between the mfi value and number of antibodies. Using this technique, it is possible to detect very low numbers of HLA IgG antibodies.

However, the method is also associated with some difficulties. Different laboratories and centres have chosen different cut-off levels at which to consider an HLA antibody as clinically relevant. Our centre, like many, regards a value of  $\geq 1,000$  MFI as a positive finding, but others have chosen 500 or even 2,000 MFI as a cut-off. Rare antibodies might be missed using this technique. In broadly sensitised patients, it might be difficult to identify single antibodies. Lot-to-lot differences have been reported and, due to the somewhat complicated laboratory handling of the test, day-to-day differences have been seen, all influencing the outcome of the test [27].

**Panel-reactive antibodies (PRA):** both cell-based (CDC) and solid-phase techniques are used to screen whether the recipients have HLA antibodies before they are accepted for transplantation. With the CDC technique, T- and B-lymphocytes are tested separately. With a panel of lymphocytes derived from 20-30 previously HLA-typed healthy individuals, selected to represent a broad variety of HLA antigens, PRA is measured. The T-cells express Class I antigens and the B-cells both Class I and II antigens. The breadth of the patient's HLA antibody repertoire is given as a percentage of positive reactions of the total cell panel, but neither the titre nor the strength is measured.

**Matching:** in kidney transplantation in particular, donor and recipient matching has a proven effect on graft outcome. For many years, HLA matching has only been regarded and calculated by the HLA-A, B- and DR-loci and, as most people have two of each (if not homozygote at any loci), the maximum mismatch is 4/2 and the minimal 0/0 (Class I/Class II respectively). However, the Class I locus HLA-C and the Class II loci -DQ and -DP are currently also possible to determine with accuracy, when evaluating both a recipient and a donor. A known tissue type of the donor, together with the single antigen-determined specificity of the recipient's HLA antibodies, make a **virtual crossmatch** possible. As a result, HLA antibodies against the prospective donor (**donor-specific antibodies, DSA**) can be evaluated even before blood samples are taken and a subsequent crossmatch is made. Only DSA of HLA origin will be accounted for, non-HLA DSA will not be discovered. These antibodies are rare and will not be further discussed. Different transplant

centres and organ procurement organisations throughout the world have different policies for allocating and matching kidneys.

In Gothenburg, historically, renal recipients on the waiting list with HLA antibodies demonstrable with the CDC-PRA technique have been prioritised in allocation to crossmatch-negative donor kidneys, as it is difficult to find a crossmatch-negative kidney for an HLA-immunised patient. HLA antibodies only detectable with the solid-phase technique and also FC-crossmatch positivity have been disregarded. It is currently possible to make an extensive immunological evaluation of living and sometimes also diseased donor kidneys pre-transplant, in order to transplant at the lowest possible immunological risk by avoiding significant DSA.

### 1.2.7 Rejection

Rejection is an immunological phenomenon, involving all the mentioned components of both the innate and adaptive immune defence [25]. The rejection can be categorised depending on when it occurs after transplantation. **Hyperacute rejection** occurs minutes or hours after transplantation, due to preformed HLA or ABO antibodies against the donor organ. The antibodies are complement binding and bind to antigens on the endothelium, causing cell lysis and a clotting cascade, resulting in thrombosis and graft loss. This type of rejection is prevented by avoiding positive CDC crossmatch and respecting the ABO blood group barrier when choosing a graft for each recipient. **Acute rejection** is, by definition, seen days or weeks after transplantation, but it can occur later if the immunosuppressive treatment is suddenly lowered or if another event triggers the immune system. There is a gradual transition towards **chronic rejection**, a subsequent, slower rejection process. Nowadays, the distinction in time between acute and chronic rejection is often referred to as before or after six months post-transplantation.

The rejection nomenclature based on the histological picture in an organ biopsy has been used for many years in all organ transplantation. International working groups within the different organ communities have developed rejection classifications. In kidney, pancreas and liver transplantation, the Banff classification is the dominant classification [28, 29]. In thoracic transplantation, the rejection classification originates from consensus meetings within the International Society for Heart and Lung Transplantation (ISHLT), but discussions are also held at Banff meetings. The Banff kidney classification

is the most well developed, with grading of inflammation/structural changes in all renal tissue components. The biopsy specimens must be investigated by light microscopy, extensive immune histochemistry and sometimes also electron microscopy. The Banff grading also includes HLA-antibody test results, if DSA are present or not and, most recently, molecular diagnostics (gene transcripts) have been included [28]. Among the other organs, the biopsy characteristics for rejection are not yet as clearly defined, but, in the latest publication from Banff regarding heart transplantation, it is stated that rejection changes in myocardial biopsies are, in principal (and not surprisingly), similar to those in kidneys [30]. Expanded rejection criteria are probably to be expected for the other organs as well.

### **Cell-mediated rejection**

T-cells infiltrating the tissue, causing tubulitis and/or arteritis in the kidney, represent the most common type of rejection – “T-cell-mediated rejection” (TCMR) and this is most frequently an acute event. The process is started by direct allorecognition, where the T-cells have reacted to foreign donor HLA. In tissue samples, both T<sub>CD4+</sub> and T<sub>CD8+</sub> cells are seen, as well as macrophages (CD68+) and sometimes eosinophils. In kidney transplantation, inflammatory infiltrates are seen in the interstitial tissue, but, when T-cells infiltrate tubuli or arteries, the inflammation is scored as a relevant rejection. The amount of infiltrating lymphocytes corresponds to the severity of the rejection, according to Banff [28, 31]. TCMR of lower grades has been regarded as a reversible condition and is not correlated with graft loss. However, in a recent study, a clear relationship between previous TCMR and *de-novo* DSA was found [32].

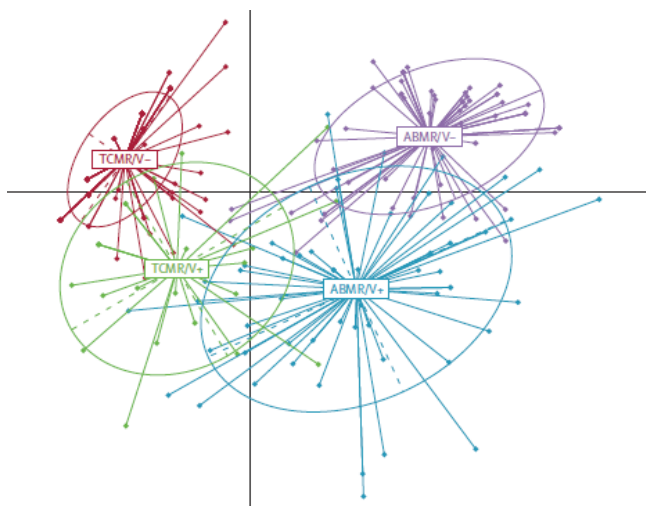
### **Antibody-mediated rejection**

Antibody-mediated rejection (AMR), also called “humoral rejection”, is a T-cell-dependent process, but with varying degrees of visible TCMR in tissue biopsies. There are three typical hallmarks of AMR, shown in bold characters. The patients have **donor-specific antibodies, DSA**. Those who have preformed DSA, or who are previously sensitised to the donor antigen, are at highest risk of early AMR. During the course of transplantation, the development of *de-novo* DSA can occur and the AMR process can begin. In the tissue, acute AMR is associated with an accumulation of neutrophils and monocytes, such as macrophages causing **acute tissue injury**, most often **microvascular inflammation**. **C4d**, a degradation product from the complement activation cascade, is often but not always present, mostly

depending on HLA IgG-subtype involvement, whether or not there is complement binding. The better the complement binding capacity, the worse the tissue injury. International consensus on the histological AMR picture has been established in kidney, pancreas and heart transplantation. Acute AMR after liver transplantation is rare and transplantations have been performed regardless of pre-transplant CDC crossmatch results, with good overall results. There are a number of theories about why the liver is an immunologically protected organ; this is perhaps due to the effective clearance of immune complexes by Kupffer cells and/or the lower expression of HLA Class II antigens or other reasons [29]. In recent years, AMR in liver has been described, after ABO-incompatible liver transplantation and among a fraction of highly immunised patients, for example. These recipients have both Class I and in particular Class II HLA antibodies with very high MFI (>10,000). In the last Banff publication on AMR in liver transplantation, the present knowledge in this field was reviewed and a grading of AMR was published [29]. In lung transplantation, there is no consensus scoring system for AMR, but there are reports of patients with a clinical picture fulfilling the three general AMR criteria; DSA, C4d and microvascular inflammation [33].

### **Acute vascular lesions in kidney grafts**

Vasculitis in renal grafts has been regarded as a cell-mediated rejection process and graded as such according to Banff. However, in recent years, isolated vasculitis can be seen in AMR and in delayed graft function as well [31]. Lefaucheur et al. found that one third of vascular rejection was due to AMR in a retrospective analysis of a large cohort of renal-transplanted recipients, when a re-evaluation of biopsies according to modern Banff criteria was made [34]. Not surprisingly, there is an overlap between cellular and humoral rejection processes, as is very clearly illustrated in Fig.7.



**Figure 7.** Four different rejection patterns with a considerable overlap; TCMR/V- (T-cell mediated rejection/no vasculitis), TCMR/V+ (with vasculitis), ABMR/V- (antibody mediated rejection/no vasculitis), ABMR/V+ (with vasculitis). Reprinted from *Lancet Vol 381* Carmen Lefaucheur, Alexandre Loupy, et al. "Antibody-mediated vascular rejection of kidney allografts: a population-based study" p313-319 (2013) with permission from Elsevier.

## Chronic rejection

In all organ transplantation, a clinical picture of slow yet inevitable graft failure is seen, even after specific causes, such as acute rejection, recurrence of primary disease, viral or bacterial infection or *de-novo* diseases, have been ruled out. Chronic rejection in lung transplantation has been called bronchiolitis obliterans syndrome (BOS), but a broader term, "Chronic Lung Allograft Dysfunction" (CLAD) is currently used, including both obstructive and restrictive patterns of progressive lung dysfunction. In heart transplantation, the clinical picture of "Chronic Allograft Vasculopathy" (CAV) with the progressive atherosclerosis of epicardial and penetrating graft vessels is seen on coronary angiograms. Liver transplant recipients run the risk of developing vanishing bile duct syndrome and, in renal transplants, interstitial fibrosis/tubular atrophy (IF/TA) and/or transplant glomerulopathy (TG) is seen [25].

Some of the patients developing chronic rejection have a history of earlier acute rejection, while others have no risk factors. Nevertheless, at some time

point, allorecognition occurs and a chronic inflammation begins. Activated macrophages produce enzymes (metalloproteinases) able to degrade tissue matrix proteins. These enzymes are dependent on nitric oxide for their activation. If the organ-specific cells in the inflammation process have lost their structure, they are not able to regenerate. Instead, fibroblasts replace them, resulting in increased collagen production. The result is the destruction of the tissue and loss of organ function [10].

The histopathological findings therefore have similarities between organs, in particular, vascular changes with intimal hyperplasia and the proliferation of smooth muscle cells leading to chronic graft vasculopathy. Importantly, a scarring process as a result of fibroblast proliferation and collagen deposition replaces the original tissue [25].

## **1.2.8 Immunosuppressive therapy**

The history of maintenance immunosuppression can be found in Section 1.1. A description of only those agents used in the studies included in this thesis, based mainly on these references, now follows [25, 35].

### **Induction therapy**

Before transplantation, often in the operating theatre before the arterial circulation of the graft is started, a high dose of immunosuppression is given – an induction therapy – to produce an immediate knock-out of the immune reaction. A bolus dose of steroids (methylprednisolone) is standard therapy throughout the world. In later years, many centres, including ours, have added the anti-interleukin-2 receptor antibody (IL-2RA) basiliximab (Simulect®) before renal and liver transplantation. In high-risk recipients, such as thoracic transplantation, highly immunised recipients and/or the risk of delayed renal graft function, anti-thymocyte globulin (ATG) is given in combination with steroids. ATG comprises purified polyclonal antibodies against human T-lymphocytes attained by immunising rabbits or horses with human T-cells, first used in 1966. Rituximab, a monoclonal antibody against CD20+ B-cells, is standard treatment as induction before ABO-incompatible transplantation, but it can also be used in highly immunised recipients in whom DSA has or has not been detected.

## Maintenance therapy

The cornerstone of immunosuppression in SOT, following the introduction of cyclosporine A in 1983, is “triple drug therapy”. It consists of one calcineurin inhibitor (CNI), one anti-metabolite, together with corticosteroids.

**Calcineurin inhibitors (CNI):** *Cyclosporine A* and *tacrolimus* are the backbone of today’s organ transplantation and, although they are different chemical substances, they have a very similar effect on the immune system and side-effects. Intracellular in T-cells, they bind to calcineurin and thereby impair the production of cytokines such as IL2, IL4,  $\text{INF}\gamma$  and  $\text{TNF}\alpha$ , resulting in the reduction of T-cell activation. Although they are still the most effective T-cell inhibitors for oral treatment, they have troublesome side-effects, the most prominent of which is nephrotoxicity (“CNI nephrotoxicity”). The mechanism is acute and reversible vasoconstriction of renal vessels, but continuous use leads to chronic changes, with the development of arterial thickening and interstitial fibrosis. This is a limiting factor in renal transplantation when it comes to long-term graft survival and promotes the development of renal failure in other organ transplant recipients. Furthermore, negative metabolic effects, such as hypertension, dyslipidaemia and diabetes, are seen.

**Antimetabolites:** *Azathioprine* is a prodrug to 6-mercaptopurine which in turn inhibits DNA synthesis and thereby cell division. Nowadays, *mycophenolic acid* (MPA; mycophenolate mofetil or enteric coated mycophenolate sodium) is more frequently used. The drug inhibits *de novo* purine synthesis which is crucial for the proliferation of lymphocytes, while other cells often have salvage systems. MPA therefore has a more selective action of preventing proliferation in T- and B-cells. These drugs are also limited by their side-effects, in particular bone marrow depression.

**Corticosteroids (CS)** have multiple effects on the immune system and inhibit both innate and adaptive immune responses. For example, the lower production of cytokines such as  $\text{TNF}\alpha$ ,  $\text{INF}\gamma$ , IL1, IL2, IL3 and IL6 is seen. Due to the multiple negative side-effects such as osteonecrosis, diabetes, hypertension, dyslipidaemia and weight gain after transplantation, many steroid-sparing protocols have been tried with a wide variety of protocols in different organs, albeit at the expense of more rejections [25].

**Inhibitors of mammalian target of rapamycin (mTOR):** *Sirolimus* and its derivate, *everolimus*, basically have a common effect pathway and side-effects. The specific inhibition of mTOR by the drug leads to the blockade of T-cell activation by arresting the cell cycle in the G1-S phase. A number of side-effects (sometimes dose dependent) limit the use of these drugs, such as worsening proteinuria and hyperlipidaemia. The mTOR inhibitors also appear to have anti-viral, anti-tumour and anti-fibrotic effects [36].

## Rejection therapy

**T-cell-mediated rejection (TCMR)** is usually a reversible process if it is found in time and treated with high-dose steroids and an elevation of the maintenance immunosuppression. If it is steroid resistant, anti-thymocyte globulin (ATG) should be added.

**Antibody-mediated rejection (AMR)** is difficult to treat and there is no consensus on what to use and how. The triad of **rituximab** to inhibit B-cell proliferation, **plasma exchange** to achieve HLA-antibody removal and **high-dose intravenous immunoglobulin** to neutralise HLA-IgG has been used by many in different dosages and combinations with positive effects, particularly in the acute setting, but randomised, controlled trials are lacking. In the case of chronic AMR, evidence of any good therapy is lacking.

## 1.3 Viral infections

Due to immunosuppressive treatment, SOT recipients run a higher risk of infection compared with healthy individuals. Historically, opportunistic infections such as cytomegalovirus (CMV) or other herpes virus infections, as well as *Pneumocystis jiroveci*, caused tremendous morbidity, as well as significant mortality, prior to the development of antiviral medication and prophylactic treatment strategies. The use of more effective maintenance immunosuppression in renal transplantation has resulted in a higher incidence of BK virus nephropathy, with reduced graft function or even graft loss.

In this thesis, the work has focused on recent, in our experience newly emerging, viral threats to the immunocompromised individual; the influenza pandemic in 2009 and the use of a not fully tested new vaccine and hepatitis E, known, but unrecognised, in our part of the world and in particular its possible effects among our SOT recipients.



*(The basic facts in the 1.3 viral infection section have been gathered from textbooks [14, 15], unless otherwise stated.)*

## 1.4 Influenza

### 1.4.1 Influenza virus characteristics

The viruses are enveloped, single-stranded, negative-sense RNA viruses with a size of 80-120 nm [37]. There are three different types; influenza A, B and C, with different protein and genomic structures, causing different properties and epidemiology. Influenza A is further subdivided depending on differences in the two major surface glycoproteins; haemagglutinin (HA) and neuraminidase (NA) (Fig. 8). There are 16 different known HA types referred to as H1-H16 and nine NA types referred to as N1-N9. The most common subtypes of influenza A in humans are H1N1, H2N2 and H3N2. A large pool of influenza A virus is found among animals such as birds (avian influenza), where the subtypes H5N1 and H9N2 are often found, and among swine, H1N1 and H3N2. Although the zoonotic influenza types share subtype designations, they are distinctly different from the human subtypes and rarely infect humans, unless there is direct contact with the animals.

Influenza A has eight different RNA segments encoding 11 proteins and it undergoes continuous genetic changes. Point mutations in the RNA for HA and NA give rise to minor changes in these surface molecules, called **antigenic drift**. Major, epidemically important drifts are seen every two to three years. Influenza A is also able to acquire new gene segments, called **antigenic shift**, resulting in marked changes in the HA antigen structure. This can occur when cells are dually infected with both a human and an animal influenza virus and it gives rise to marked changes in the surface proteins, following which a new influenza virus is born. With no immunity in the population to the new virus strain, the virus is able to cause pandemic influenza (Fig 8). Antigen shifts do not occur in influenza B and antigen drift is much less frequent and this virus only gives rise to local epidemics. Influenza C is genetically stable and causes only sporadic, mild infections, particularly in children.

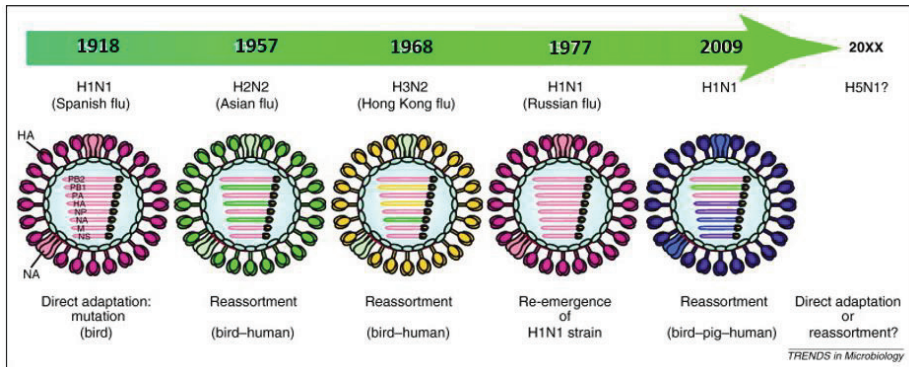


Figure 8. A schematic illustration of the Influenza A virus with its surface glycoproteins HA (haemagglutinin) and NA (neuraminidase) and 8 RNA strands. The different pandemic influenza viruses since 1918 are illustrated with the antigen shifts marked by different colours of the RNA strands. (The 1977 Influenza has not been classified as “pandemic”). Reprinted from Trends In Microbiology Vol. 20(1), Watanabe Y, Ibrahim M et al. “The changing nature of avian influenza A virus (H5N1)” p11-20, (2012) with permission from Elsevier.

## Immunity to influenza

An influenza infection gives rise to long-lived immunity to the infected virus strain. Variable cross-protection has been seen within subtype groups. Infection gives rise to antibody production against both HA and NA but also other structural proteins. The peak antibody response after infection occurs after four to seven weeks, after which it slowly declines.

The HA protein is defined by its ability to haemagglutinate red blood cells. Antibodies against HA have been shown to be protective against influenza infection. The most widely used serological assay for the determination of influenza protection detect these antibodies that are able to block haemagglutination i.e. haemagglutination-inhibiting antibody assays (HAI). There is some uncertainty about the HAI titre that corresponds to protection; 1:8-1:160 but the titre 1:40 is used since very long in vaccine studies, reducing the infection risk 40-70% among healthy, as recently confirmed [38]. The NA antibody does not neutralise virus infectivity but reduces the amount of virus leaving the infected cell. This explains why the severity of the infection becomes less. Mucosal antibody production against influenza protects the

individual from upper respiratory symptoms in particular. The cellular immune defence is less studied. CD4+ and CD8+ T-cells have been found five to 14 days after the infection [15].

## **Seasonal influenza**

Influenza infections have a typical seasonal pattern, giving rise to infection in the northern hemisphere during November and April and in the southern hemisphere between May and September. Although an influenza infection gives rise to immunity, the antigen drift will change the viral antigen and the individual will therefore become susceptible to following seasonal variants. The incubation period is short, one to five days, and the virus can have a very rapid onset and transmission in the population [14].

## **Symptoms and health burden**

The symptoms of the influenza virus are very well known, as it gives rise to a respiratory infection with fever and aching muscles. However, complications can occur, particularly among influenza infection risk groups, defined as age  $\geq 65$  years, pregnancy  $>16$  weeks, adults and children ( $>6$  months) with chronic heart-, lung-, liver- or kidney disease, diabetes mellitus or immunocompromised. Primary viral pneumonia is uncommon, but it is a very serious condition. Bacterial superinfection is well known and the largest cause of morbidity and mortality after influenza. As a result, there is mortality associated with influenza, even among individuals not belonging to any risk group. Severe influenza affects three to five million individuals worldwide every year, as estimated by the WHO, resulting in 250,000-500,000 deaths [39]. In Europe, the rates are lower and depend on seasonal and current immunity in the population to the dominant virus strain. The 2015-2016 season in Sweden was dominated by influenza A(H1N1)pdm09 (in short H1N1/09). Our country, with close to 10 million inhabitants, had 261 deaths occurring within 30 days of a laboratory-confirmed influenza diagnosis, 79% were  $\geq 65$  years, 18% 40-64 years. Among those patients with a laboratory diagnosis, the mortality was 9% if  $\geq 65$  years and 2% if 40-65 years. Of those belonging to a risk group who were in need of intensive care, only 11% had been vaccinated. Furthermore, 15 of the fatal cases did not belong to a risk group [40]. Between 1-4% of SOT recipients are infected annually [41]. Among the SOT recipients, in particular lung transplanted patients are at risk of serious infection. The

incidence during a 10 year period in Pittsburgh was among lung transplanted 41.8 cases/1000 persons year compared to liver 2.8 and kidney 4.3 [42].

### **Seasonal vaccination**

A number of reference viral laboratories around the world report their individual current influenza strain isolates and number of cases (The Global Influenza Surveillance Network) to the WHO. The WHO analyses and predicts the possible upcoming seasonal virus. The upcoming seasonal influenza vaccine is decided on the basis of these analyses. Seasonal influenza vaccination saves both lives and money. In a recent European report, 180 million persons fulfil the indication for vaccination and today about 80 million (44%) are vaccinated annually. If the vaccination adherence could be raised to 75%, the European influenza work group estimates that this would save another 9,000-14,000 lives and would result in a total health cost saving of €190-226 million [39].

The most used influenza vaccines are formalin inactivated, whole or split virus or purified surface antigen. Antigens for inactivated antigens are mass-produced in embryonated chicken eggs. Seasonal vaccine is composed of three antigens, two influenza A and one influenza B, with a minimum antigen content of 15  $\mu$ g each. This type of seasonal vaccine is called trivalent influenza vaccine (TIV).

The immunogenicity among healthy adults who have encountered former influenza strains is as follows; >85% attain a protective HAI antibody response after TIV. A serological response can be seen after 10 days and lasts for up to two to three years [14]. The safety of TIV has been established by many large studies, the largest of which comprised 250,000 vaccinated children (< 18 years of age). The most common adverse event is local tenderness at the injection site, while systemic symptoms are rare; in studies, it is equal to placebo. Data regarding transplant patients seroresponse to TIV differ. Some studies report an equal response compared with healthy [43, 44]. Most studies though, reports reduced seroprotection rates [45, 46]. The use of MPA and vaccination within six months of transplantation have been associated with diminished vaccine response [43, 45]. There are particular difficulties when analysing seasonal influenza vaccine response since the baseline seroprotection and reaction upon the vaccine may differ depending on the history of former vaccination and infections.

## **Rejection after seasonal influenza infection or TIV**

Seasonal influenza infection have in earlier years reported to coincide with the development of acute rejection [47]. If influenza vaccination also can elicit rejection has not been shown [43] although the studies mostly are small and have not been designed to address the question [46]. Few have studied the HLA reaction but TIV did not give rise to *de novo* HLA sensitisation or significant change in pre-existing HLA antibody levels among 66 stable renal transplant recipients [48].

## **Pandemic influenza**

Although ancient text indicates the occurrence of influenza epidemics since antiquity, the earliest known is the “Spanish flu” of 1918, causing the death of 50 million people (in Sweden about 35,000). This virus strain was subsequently isolated and confirmed as subtype H1N1. The next influenza pandemics were Asian flu in 1957 (H2N2), with five million deaths worldwide, and Hong Kong flu in 1968 (H3N2). The definition of pandemic influenza is not totally clear. “Simultaneous worldwide transmission” is one. A novel influenza strain with the absence of immunity in the population, combined with a worldwide spread of virus, is a summary of the WHO criteria. The severity of the disease is no longer included in the definition and this has triggered a debate [49, 50].

### **1.4.2 Influenza A(H1N1)pdm09**

In late March 2009, reports of hospitalisation and deaths among young adults in Mexico due to respiratory illness was reported to the health authorities [51]. On 21 April 2009, two cases of a novel influenza were diagnosed in California. The virus spread quickly and, on 9 June, a total of 73 countries had reported more than 26,000 laboratory-confirmed cases. On 11 June, the WHO declared the first influenza pandemic since 1969 [52].

The new virus was of the H1N1 subtype, but it had multiple antigen shifts, making it a new, never previously encountered type. It contained five RNA segments from two different and distinct swine viruses, two from birds and one from human influenza virus [53] (Fig. 8). A recent study confirms the origin as coming from the swine population in Mexico and, although new viruses have usually evolved in Asia, the Mexican source is explained by the worldwide trade in livestock [54].

## **H1N1/09 infection in healthy individuals**

The outcome among the first influenza cases in Mexico was serious. Of 899 hospitalised cases, 6.5% became critically ill and, of those, 41% died. As the influenza progressed over the world, the mortality among children, young adults and pregnant women was higher compared with that of typical seasonal influenza, but the elderly did relatively well. However, there was a substantial difference depending on regions of the world. Estimations of deaths during the pandemic have been made, but they are similar to a mild seasonal flu. However, when counting years of life lost, the H1N1/09 influenza was worse, due to the high mortality among the youngest individuals [52]. In Sweden, the first cases were diagnosed in May 2009 and, in the beginning, mostly imported cases were found. In the middle of October, there were more cases and the infection had its peak incidence in the middle of November. In Stockholm, 11% of confirmed cases were hospitalised and one (0.4%) died. Influenza disease was seen in 7% in spite of vaccination [55]. The total number of deaths in Sweden due to confirmed influenza H1N1/09 was 31, i.e. less compared to other seasons due to a lower incidence among the elderly.

## **H1N1/09 infection in SOT**

A cohort study performed in North America of SOT recipients, where 26 transplant centres reported their microbiology-confirmed cases of influenza H1N1/09 during April-December 2009, identified 237 cases, of which 71% were admitted to hospital, 16% to the ICU and 4% ( $n=10$ ) died. Almost all were treated with oseltamivir and, of those receiving the drug within 48 hours after the onset of symptoms, 8% were in need of intensive care compared with 22.4% with later introduction of the drug [56]. In a retrospective study of kidney-transplanted individuals in Brazil during the 2009 pandemic, the mortality rate was 9.1%. Since the outbreak of the infection was early during the pandemic, no one had received vaccination but almost all were treated with oseltamivir [57].

## **Pandemic vaccine**

Due to the rapid spread of the influenza pandemic and the first reports of high virulence among young people, a new vaccine had to be produced at short notice. This time not only risk groups were going to be vaccinated but also whole populations and there was therefore a shortage of antigen. In Sweden, as well as in many other European countries, the choice of vaccine fell on Pandemrix<sup>®</sup>, the monovalent, AS03-adjuvanted vaccine containing only 3.75

µg H1N1 antigen (compared with the usual 15µg in TIV), produced by GlaxoSmithKline in Dresden in Germany. Before the start of the vaccination programme in Sweden, a pilot study was conducted among healthy individuals and it revealed a 98% protection rate after only one dose of the adjuvanted vaccine [58]. It was therefore decided to give one dose to the healthy and two doses to individuals belonging to a risk group. The vaccination started in mid-October and, according to the Stockholm report, 100% of Swedish risk-group persons had been vaccinated at the beginning of December. Of Sweden's 9.4 million inhabitants in 2009, 61% were vaccinated with the pandemic vaccine [59].

Other vaccines were also used around the world; MF59 adjuvanted vaccines or monovalent, non-adjuvanted vaccines containing 15 µg of antigen.

### **Antiviral therapy against influenza**

**The M2 inhibitors** amantadine and rimantadine block the surface ion channel M2 and have proved effective against influenza A, but, starting in 2005, resistance has spread and now almost all H3N2 strains are immune to the drugs. The M2 inhibitors do not work at all against influenza B.

**Neuraminidase inhibitors** act by blocking the NA which promotes virus release from the infected cell. As a result, the medication is unable to stop the viral assault and is only able to moderate the severity. There are two drugs, zanamivir and oseltamivir, which both reduce the duration of symptoms if introduced within 36 hours after the onset of symptoms. Resistance to these drugs has also been noted [15].

### **Adverse events and narcolepsy**

Unusual syndromes, such as Guillain-Barré syndrome, have been attributed to influenza vaccine. In 1976/77, a pandemic vaccine campaign was carried out in the USA as a result of fear of a new H1N1 influenza of swine origin. Four different vaccines were used, inactivated whole or split virus, mono or bivalent. During the six weeks following vaccination, a four- to seven-fold higher risk of Guillain-Barré was noted and it was attributed to the vaccination, although no particular vaccine or component could be identified [60].

The manufacturer of AS03-adjuvanted pandemic vaccine, GSK, has pooled data from all its studies of this adjuvant (in total,  $n > 22,000$ ) and has found no

statistically significantly higher incidence of adverse events compared with non-adjuvanted influenza vaccine [61].

The possible connection between Pandemrix<sup>®</sup> vaccination and narcolepsy in children and young adults has attracted a great deal of attention. The first reports came in June 2010 from Sweden, followed shortly after by Finland and subsequently from many European countries [62].

Narcolepsy, a syndrome of hypersomnia with cataplexy (sudden loss of motor tone triggered by emotions) is caused by the selective destruction of hypocretin neurons. A genetic predisposition is seen; about 90% are carriers of the HLA-DQB1\*0602 alleles. This is, however, a very common allele (30% of Swedish and Finnish inhabitants) and an environmental trigger is needed. The suggestion is an autoimmune process, but no rise in inflammatory markers has been found. [63].

The risk of narcolepsy was increased four to nine fold in Sweden and Finland after the Pandemrix<sup>®</sup> vaccination (manufactured in Europe, 30 million doses) but not in Canada, which used Arepanrix<sup>®</sup>, the same vaccine but manufactured in Canada by the same company (6.5 million doses). The calculated number of extra cases with the diagnose in Sweden during 2009-2011 were 136. Narcolepsy after Pandemrix<sup>®</sup> vaccination was strongly correlated to the HLA-DQB1\*0602 allele and 94% of the cases had cataplexy. No other neurological disorder or autoimmune disease was overrepresented during the influenza pandemic in 2009 in Sweden. However, there was also an increase in the incidence of narcolepsy in China during the 2009 influenza pandemic, even though no vaccine was given, postulating the possibility of the H1N1 strain itself causing the disease. In fact, in an experimental study of mice lacking B- and T-cells, the mice developed a narcolepsy-like state and the virus targeted hypocretin-producing neurons [64]. For this reason, the cause of the high incidence of narcolepsy in 2009 has still not been fully elucidated.

## 1.5 Hepatitis E virus (HEV)

HEV is a small (27-32 nm), non-enveloped, single, positive-stranded RNA virus classified in a virus family of its own, the Herpeviridae. The virus is resistant to heating at 56°C for one hour but susceptible to boiling and frying for five minutes and to chlorination.



The possibility of another virus resembling hepatitis A was first recognised by dr MS Khuroo during a large epidemic in Kashmir Valley in 1978 when about 52,000 individuals developed icteric hepatitis, causing the death of 1,700 persons [65]. A faecal-oral route of transmission was established, due to contaminated water. HEV was first identified by electron microscopy in 1983 in an experiment in Moscow where a suspension of stools from nine individuals with known active non-A-hepatitis was inoculated orally into a healthy volunteer (a Russian virologist) who developed hepatitis after 35 days [66].

Today, in the light of effective vaccine against hepatitis A, HEV is considered by the WHO to be the major cause of acute hepatitis of viral origin in the world. It estimates 20 million HEV infections globally per year, 3.3 million with symptoms, and 56,000 deaths.

### **1.5.1 Epidemiology**

Four genotypes infecting humans are known; HEV1-HEV4. HEV1 and HEV2 appear in developing countries where they are endemic, causing epidemic outbreaks with a seasonal pattern. HEV1 is mostly found in Asia, Africa and Latin America, while HEV2 is found in Mexico and West Africa.

HEV3, found in industrialised countries throughout the world, and HEV4 (China, East Asia, Central Europe) cause sporadic cases. HEV3 and HEV4 have a zoonotic reservoir, as they have been found in a number of different animals such as domestic pigs, wild boar and deer which can transmit the disease to humans [67]. Other routes of transmission are via blood products [68, 69] and, in fact, via transplanted organs, as found in two case reports [70, 71] (Fig. 9).

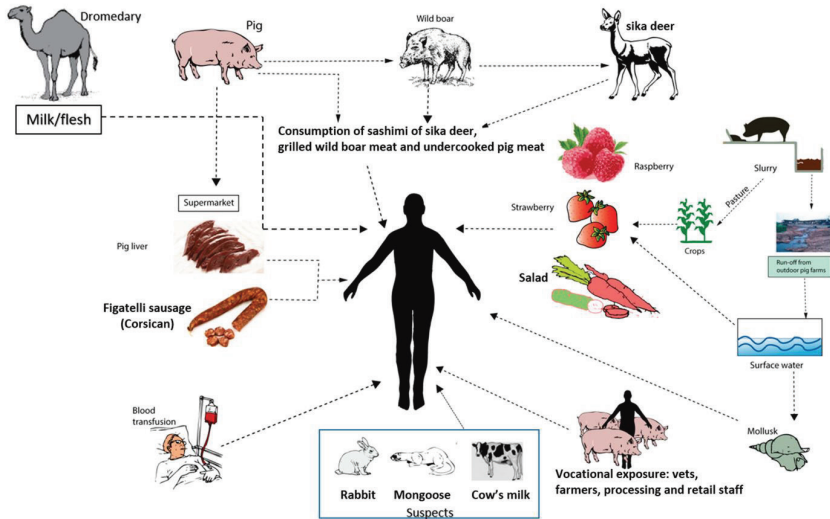


Figure 9. Routes of transmission of HEV in the industrialised world. Reprinted with permission from *Viruses* 2016 Sep 20;8(9). Khuroo MS et al. "Transmission of Hepatitis E Virus in Developing Countries".

The number of new HEV infections reported to Swedish health authorities in 2015 was 29 cases. However, it has recently been shown that HEV3 is endemic in Sweden, with a prevalence of HEV-RNA in 10% of hunted wild animals [72] and positive anti-HEV IgG among 16% of healthy blood donors [73]. In 1997, the seroprevalence in Sweden was 2-7.5%, depending on age [74], but today's higher prevalence is not due to a higher incidence but to the improvement in serological assays [73]. This was shown in the Netherlands, where the seroprevalence almost 20 years ago was 0.4%, but a re-analysis of stored sera from 1988 revealed that the true prevalence was 46% [75]. There are regional differences in HEV3 prevalence in Europe as well. HEV3 is most common in the south of France, but prevalence numbers are otherwise very difficult to interpret, as they are dependent on the diagnostic tools.

## 1.5.2 HEV diagnostic tools

**HEV serology** (IgG and IgM) has typically been used as a diagnostic tool and, today, sensitive instruments are finally available [73]. Anti-HEV IgM is a marker of acute infection, while anti-HEV IgG can be seen in both acute and chronic or healed hepatitis. Quantitative **PCR of HEV-RNA** is a definite

marker of viral replication, usually analysed in serum. A positive faecal test indicates that the individual is able to transmit disease. The presence of HEV in urine is much less well known. In one report from China, urine HEV-RNA was positive in three of eight patients with acute HEV infection [76]. In another report from France, one in 51 with acute HEV had HEV RNA in urine [77]. Fig. 10 illustrates the typical diagnostic signs of acute versus chronic HEV infection.

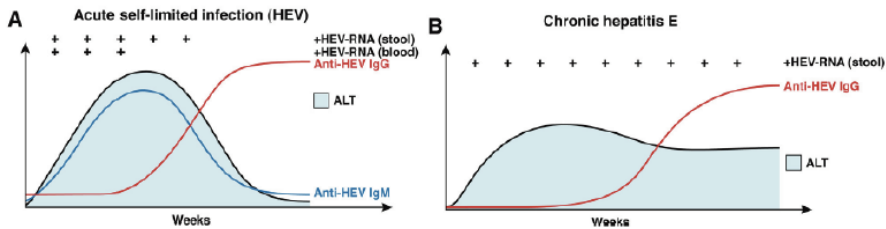


Figure 10. The typical development of HEV specific IgM- and IgG-levels and possible HEV-RNA detection during acute (A) and chronic (B) HEV infection. Reprinted from *Gastroenterology* Vol. 142, Wedemeyer H, Pischke S, Manns M. "Pathogenesis and Treatment of Hepatitis E Virus Infection". Page 1388-1397 (2012) with permission from Elsevier

### 1.5.3 HEV infection

**Acute HEV** The incubation period is usually four to six weeks, but it has been described between nine days and two months. Fever, anorexia, vomiting and jaundice then develop. A rise in liver enzymes is seen. Symptoms may last for two to four weeks. In developing countries, the disease has a mortality of 1%, if acute liver failure develops. In some of these countries a particularly high mortality rate is found among pregnant women affected by HEV2 (25%) [78]. In the industrialised world, the HEV infection is estimated to be symptomatic in less than 5% of those who seroconvert [79]. Two studies from Europe have retrospectively studied HEV-RNA in patients with acute liver failure (ALF). They found 10% and 5% respectively were HEV-RNA positive and, at the time of treatment, most cases were misdiagnosed as drug-induced ALF. Most of these patients survived, but two underwent liver transplantation and one died. [80, 81].

**Chronic HEV** In 2008, the first cases of chronic HEV infection were reported by Kamar et al. from the south of France [82]. They had identified 14 patients, liver or kidney transplanted, with acute hepatitis due to HEV. Eight of the recipients developed chronic hepatitis confirmed by liver biopsy with persistent HEV-RNA positivity and an increase in liver enzymes with a duration of > 6 months, meeting the definition of chronic disease. The same group also reported one renal recipient with liver cirrhosis due to HEV [83]. Since then, a number of case reports of chronic HEV have been published, almost all found among solid organ transplant recipients. In a retrospective study from France, three (1.45%) of 206 liver transplant recipients developed chronic hepatitis and were HEV-RNA positive. Furthermore, two of the three were HEV IgG positive before RNA detection, indicating a secondary HEV infection [84]. In terms of numbers, HEV appears to be a marginal finding. However, fatal cases are seen. In one case report, a liver transplant recipient developed liver cirrhosis 15 months post-transplantation and died of septicaemia. Retrospectively, the diagnosis of donor-derived chronic HEV infection had developed into a rapidly fibrosing liver disease [70].

**Extra hepatic manifestations** A number of reports have identified neurological manifestations secondary to HEV. The first case was reported from India; a patient with Guillain-Barré syndrome (GBS) together with acute HEV infection [85]. In the Netherlands, 5% of patients with GBS had an association with acute HEV infection, as they were IgM positive, three were also HEV-RNA positive but none had RNA in their cerebrospinal fluid (CSF) [86]. A number of case reports of encephalitis, some with RNA positivity in the CSF, are also worth noting [87]. **Renal involvement** is also described, but there are fewer cases and almost all were reported from Toulouse. In a series of 51 SOT recipients with an acute HEV infection, 57% developed a chronic HEV infection. Of them, five had a marked elevation of proteinuria, four had cryoglobulinemia and were kidney biopsied, interpreted as membranoproliferative glomerulonephritis in two. However, the proteinuria and cryoglobulinemia disappeared after treating HEV [77].

#### 1.5.4 Treatment of HEV in SOT recipients

**Reduction of the immunosuppression** is sometimes sufficient to attain viral clearance. This is in fact supported by *in-vitro* data, where HEV replication was stimulated by CNI but inhibited by MPA [88].

**Antiviral therapy** might nevertheless be needed. *Ribavirin monotherapy* is the most frequently used. In one series from Toulouse, the sustained viral response at six months after three months' treatment was 78% (n=59) and, after prolonged treatment, 85%. *Interferon* has only been used in a few cases but with 100% clearance of virus. Due to the risk of triggering rejection, interferon can only be used in liver transplant recipients [89].

## 2 AIM

The overall aim of this thesis was to study new viral infections among the immunocompromised, SOT recipient cohort and the effect of vaccination; one of our most important tools for protecting our patients from harmful viruses and other microbes.

### 2.1 H1N1 vaccine studies

The specific aims were:

- To analyse the H1N1/09 antibody response to the monovalent, AS03-adjuvanted influenza A H1N1/09 vaccine in SOT recipients compared with healthy controls
- To explore both the persistence of H1N1/09 antibodies one year after immunisation with the AS03-adjuvanted vaccine and the immune response after a booster dose with seasonal trivalent inactivated vaccine 2010 (TIV/10) containing a H1N1/09 component
- To identify adverse events due to these vaccinations using a questionnaire and chart review
- To investigate whether the influenza H1N1/09 vaccine triggers the production of *de-novo* HLA antibodies and rejection after vaccination
- To describe the graft function and incidence of rejection during the five years following the vaccination.

### 2.2 HEV study

The specific aims were:

- To investigate the prevalence of hepatitis E IgG and IgM among SOT recipients at the time of transplantation in Sweden compared with healthy blood donors
- To describe the incidence and clinical outcome of primary or secondary HEV infection in SOT recipients during the first two years after transplantation.

## 3 PATIENTS AND METHODS

All the patients were recruited at the Transplant Institute, Sahlgrenska University Hospital, Gothenburg, Sweden. The H1N1/09 vaccine cohort is studied in Papers I-III and the hepatitis E cohort in Paper IV.

### 3.1 H1N1/09 vaccine studies (*Papers I-III*)

#### 3.1.1 Subjects and study design

The vaccination programme against the H1N1/09 influenza started in mid-October 2009 and all SOT recipients were considered for vaccination, unless they were newly transplanted within the past month. A total of 82 SOT recipients from the outpatient clinic at the Transplant Institute, Sahlgrenska University Hospital, were included, as well as 28 members of the staff as healthy controls in the first study (Paper I). The subjects were vaccinated according to the Swedish guidelines with two doses of the monovalent, AS03-adjuvanted vaccine, Pandemrix<sup>®</sup>. The second dose was administered three to four weeks after the first. Blood samples were drawn before both the first and second vaccine dose and a third sample was taken one month after the second vaccination to measure the serological response to the vaccine.

The serum samples were re-analysed in those subjects who had both a baseline serum sample and a sample two months after the pandemic vaccination in order to study the HLA-antibody responses after the vaccination. Those who could be included in this analysis were 67 SOT recipients and 20 healthy controls (Paper III).

In the follow-up study, one year later (Paper II), all the participants in the primary study were asked by mail to participate. A total of 49 SOT recipients and 11 healthy controls agreed. A single dose of TIV/10, Fluarix<sup>®</sup>, containing an H1N1/09 component, was administered. Serum samples were drawn before the vaccination and one month later for the analysis of anti-H1N1/09 antibody persistence and to see whether TIV/10 boosted the H1N1/09 antibodies. The patient populations in paper I-III are presented in Fig. 11.

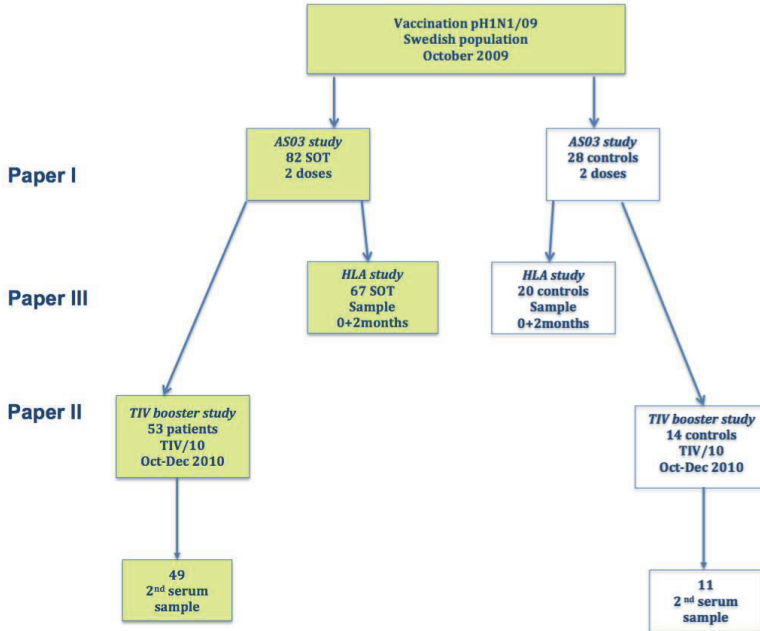


Figure 11. The patient population in Paper I-III

At the time of both the first and the second vaccine studies (Papers I and II), the patients received a questionnaire to report side-effects.

The medical charts of the SOT recipients were reviewed stepwise in Papers I-III until five years after the vaccination, with regard to influenza symptoms, patient survival, graft function, renal function (measured glomerular filtration rate; mGFR), performed organ biopsies, rejection and rejection treatment. The database at the Laboratory of Virology was searched for any positive nasopharyngeal PCR tests for H1N1/09 between October 2009 and December 2011. In Paper III, “Pro-inflammatory events”, as defined by Locke *et al.* [90] were also noted, i.e. infections, surgery, trauma or blood transfusion one month before vaccination until the last serum sample after the pandemic vaccination was drawn.



### 3.1.2 Vaccines

The influenza H1N1/09 vaccine used in Sweden was Pandemrix® (GlaxoSmithKline, Dresden, Germany), an inactivated split influenza virus vaccine, containing antigen equivalent to the A/California/07/2009 (H1N1) derived strain (NYMC X-179A): 3.75 µg and AS03 adjuvant composed of squalene (10.69 mg), DL- $\alpha$ -tocopherol (11.86 mg) and polysorbate 80 (4.86 mg). The vaccine was administered by an intramuscular injection into the deltoid muscle.

A subset of the subjects (eight SOT recipients and one control) received one dose of trivalent seasonal influenza vaccine 2009 (TIV/09) Fluarix® (GlaxoSmithKline, Brentford, United Kingdom) between the second dose of Pandemrix® and the third serum sample. Another five recipients and one control received TIV/09 after the third sample. The TIV/09 contained A/Brisbane/59/2007 (H1N1) alike strain (IVR-148): 15 µg, A/Brisbane/10/2007 (H3N2) alike strain A/Uruguay/716/2007 (NYMC X-175-C): 15µg, and B/Brisbane/60/2008 alike strain: 15 µg.

In 2010, all the participants received the trivalent inactivated influenza vaccine (TIV/10) Fluarix® (GlaxoSmithKline, Brentford, United Kingdom) containing the A/California/7/2009 A(H1N1)pdm alike strain (NYMC X-181): 15 µg, A/Perth/16/2009 (H3N2) alike strain (NYMC X-187, derived from A/Victoria/210/2009):15 µg and B/Brisbane/60/2008: 15 µg. The vaccine was administered intramuscularly into the deltoid muscle.

### 3.1.3 Haemagglutination inhibition assay (*Papers I, II*)

Pre- and post-vaccination samples were analysed simultaneously by haemagglutination-inhibiting antibody assays (HAI) as previously described [91]. Briefly, the HAI was performed with 0.5% hen erythrocytes and 4 haemagglutination (HA) units of virus (A/California/7/2009 NYMC X-179A H1N1). Sera were tested in serial twofold dilution steps at an initial dilution of 1:10 to 1:640. The HAI titre was judged as the reciprocal of the last dilution that inhibited HA. Titres of  $\geq 1:40$  were considered to be a positive antibody response and protective against influenza.

### 3.1.4 HLA typing and HLA antibody analyses (*Paper III*)

Prior to 1993 the HLA-A, -B and -DR $\beta$ 1 loci of patients and donors were typed by serology (L. Rydberg, personal communication). Then, first Class II (HLA-DR $\beta$ 1 and -DQ $\beta$ 1) and since 2007 also Class I (HLA-A and -B) of patients and potential donors were molecularly typed on genomic DNA using either polymerase chain reaction (PCR)-sequence-specific oligonucleotides (SSOs) (LABType<sup>®</sup>; One Lambda, Inc., Canoga Park, CA, USA) or PCR-sequence-specific primers (SSPs) (Olerup SSP<sup>®</sup>; Olerup SSP AB, Saltsjöbaden, Sweden), as described by the manufacturers.

**HLA antibodies** Serum samples were drawn at baseline and three to four weeks after the second H1N1/09 vaccination. All the samples were analysed simultaneously, screening for HLA ab by LABScreen Mixed<sup>®</sup> (One Lambda Inc., Canoga Park, CA, USA) detecting Class I and Class II anti-HLA antibodies. In the event of a positive screening result, pre- and post-vaccination sera were further analysed for HLA antibody specificity (HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ $\alpha$  and  $\beta$ ) using the LABScreen<sup>®</sup> Single Antigen assay (One Lambda Inc.), according to the manufacturer's instructions. The test results were analysed using HLA Fusion 2.0.0 software (One Lambda Inc.). A positive result was defined as a mean fluorescence intensity (MFI) of  $\geq 1000$ . Donor specific antibodies were defined on the antigenic level. In cases where the donor was not HLA typed on all loci, non-typed loci were when possible inferred by genetic linkage to typed loci. Also, reactivity against HLA-DQA1, HLA-DPA1 and HLA-DPB1 could not be considered if DSA or not since we were not able to retype the donors.

**Crossmatch** Before transplantation, all the patients underwent a CDC crossmatch. At our centre, all organs except livers are allocated to recipients with a negative CDC crossmatch. Liver transplantation is carried out disregarding the CDC crossmatch result, but, in this study, all liver-transplant recipients were CDC crossmatch negative at the time of transplantation.

## 3.2 HEV study (*Paper IV*)

### 3.2.1 Subjects and study design

Adult organ transplanted recipients at the Transplant Institute, Sahlgrenska University Hospital, Gothenburg, Sweden receiving an organ between 2008

and 2009 were asked to participate in a study primarily designed for the detection of DNA viruses. A total of 227 patients were included. At the transplantation samples of whole blood, serum and urine were collected and thereafter monthly during the first six months, followed by every third month until two years post transplantation. The samples were transported to Sahlgrenska from the different outpatient clinics in our region and frozen for later analysis. Clinical data were collected from the Transplant Institutes quality database and from medical chart reviews.

In a study conducted at Sahlgrenska University Hospital in 2015, 500 healthy blood donors were investigated regarding the prevalence of HEV infection [73]. These data were used in comparison with the SOT populations results analysed by the same assays

### **3.2.2 HEV antibody detection**

Anti HEV IgM and IgG were detected simultaneously by a commercial enzyme-linked immunosorbent assay (ELISA) DiaPro (Milan, Italy) according to the manufacturer's instructions. A total of 432 sera from 196 patients were tested. An OD/cut-off value for IgM of  $\geq 1.5$  and IgG of  $\geq 1.7$  was considered positive. Furthermore, IgG  $\geq 1.2$  to  $< 1.7$  and IgM  $\geq 0.8$  to  $< 1.5$  were regarded as borderline reactivity.

### **3.2.3 HEV RNA detection by real-time PCR**

All serum samples with a positive or borderline result of anti-HEV IgM and/or IgG were also analysed for HEV-RNA by real-time qPCR, all steps described in detail in Norder et.al. [73]. The RNA was extracted by using the components by NucliSENS easyMag; bioMérieux SA, France according to their instructions. Real-time PCR was performed on 20  $\mu\text{L}$  of extracted RNA in 30  $\mu\text{L}$  of master mix. Cycling conditions were performed as described in [92]. One serum sample with HEV RNA and a known amount of a plasmid with cloned HEV sequences were used as a control in each assay. Based on the results obtained for the plasmid, the sensitivity of each assay ranged from 5 to 10 copies of HEV RNA.

## **3.3 Statistical analysis** (*Papers I–IV*)

Papers I and II: Fisher's exact test was used to compare SOT recipients and controls with respect to protective antibody titres ( $\geq 1:40$ ), side-effects, gender

and age (< or >60 years) (Papers I and II). Moreover, in the SOT recipient cohort, the correlation between protective titre versus mGFR (< or > 30 ml/min and KDOQI chronic kidney disease stages 1-5) and the type of transplant was analysed using this method (Paper II). A t-test was used to compare the mean antibody titres between the two groups. Analysis of co-variance (ANCOVA) adjusted for the effects of age and gender was used to correct for these group differences (Paper I). McNemar's test was used for a comparison of the proportion of responders after the first and second H1N1/09 and TIV/10 vaccine dose (Papers I and II). Wilcoxon's two-sample test was used to compare the median titre value after the TIV/10 booster dose in SOT recipients and controls (Paper II). Pearson's correlation coefficient was used to measure correlations between titres and the different immunosuppressant (Paper II).

Papers III and IV: Fisher's exact test was used for categorical data, while continuous data were analysed with paired and unpaired t-tests. Logistic regression models were used in Paper III when analysing the renal transplant cohort to find possible factors causing graft loss (time from transplantation and vaccination, pre-vaccination rejection, HLA, DSA, mGFR, difference mfi > 1,000) and in Paper IV whether HEV IgG was dependent on age, gender or transplanted versus blood donor and type of organ transplanted. There is a need to interpret the regression analyses with caution due to the very small number of observations and possible confounding factors, particularly in Paper III.

All: p-values of < 0.05 were considered to be statistically significant. The analyses were performed using SAS software, version 9.1.

### **3.4 Ethical approval** (*Papers I–IV*)

All the studies were approved by the Regional Ethical Review Board in Gothenburg. Papers I-II: Reference number 590-10. Paper III numbers 590-10 and T368-1. Paper IV: Reference number 150-08 plus T802-16. The separate study of blood donors, already published, had reference number 737-12.

## 4 RESULTS AND DISCUSSION

### 4.1 H1N1 vaccine studies (*Papers I-III*)

#### 4.1.1 The patient cohort (*Papers I-III*)

The majority of the patients were kidney transplanted and there was a tendency towards fewer women (Papers I-III: 43%; 51%, 46% respectively). The healthy controls were younger and most of them were female (Table 1). Among the included patients, five were vaccinated within three to nine months after transplantation, but four of them still developed a protective H1N1 antibody level after the pandemic vaccination. One liver recipient was re-transplanted before immunisation with TIV/10 due to chronic rejection which began before the H1N1/09 vaccination. Another liver transplanted patient with biliary complications at the time of enrolment was re-transplanted during the month between TIV/10 administration and the follow-up sample. Both these patients responded well to the H1N1/09 vaccine, had protective titres one year later, but the latter had a lower titre after TIV/10.

*Table 1. A summary of some basic demographics of the SOT recipients and healthy controls in the vaccine studies (Paper I-III).*

Paper...	SOT	Organ transplanted % of total					Year since	SOT	Control	
	<i>n</i> =	Ktx	Ltx	Htx	Com -bi <sup>1</sup>	Other <sup>2</sup>	Tx Median	Age Median	<i>n</i> =	Age Median
Paper I	82	60	20	8.6	7.4	3.7	6	60	28	43
Paper II	49	55	26.5	8.2	10	0	7	59	11	42
Paper III	67	67	21	4.5	4.5	3	6	57	20	48

Ktx=kidney transplanted, Ltx=liver transplanted, Htx= heart transplanted. <sup>1</sup> Liver-kidney, heart-kidney, liver-lung. <sup>2</sup>Lung and limbal (corneal) stem cell transplantation. Tx = transplantation

The immunosuppressive treatment was dependent on the transplanted organ; renal transplanted patients were most often on triple immunosuppression, while two drugs were more common if the patients were liver and heart transplanted. Around 10% were on monotherapy. About 90% of the recipients were on one CNI, often in combination with MPA and/or steroids. Anti-rejection therapy was given to seven SOT recipients up to 14 months (three

within six months) before the pandemic vaccination treated with ATG and plasmapheresis in two, otherwise only a course of steroids. Four of the seven responded with protective titres. During the two-months duration of the pandemic vaccination study, the immunosuppression remained unchanged, except for one individual who was put on low-dose CNI and low-dose everolimus due to low renal function. During the following period until one month after the TIV/10 vaccination, five patients had changed immunosuppression, (three weaned off steroids, one lowered MPA dose and one due to re-transplantation liver).

Renal function was measured ( $^{51}\text{CrEDTA}$  or Iohexol) in all but five SOT recipients in whom the estimated GFR (eGFR) according to the MDRD equation was used instead. The median GFR during the pandemic vaccination was 50 ml/min/1.73m<sup>2</sup> (range 7-100) and, a year later, 47 ml/min/1.73m<sup>2</sup> (range 9-110). A GFR of less than 30 ml/min/1.73m<sup>2</sup> was found in 23% and 20% in Paper I and II respectively. In Paper III, when focusing on the different organ transplants, mGFR was 39 (range 7-100) among the renal transplanted patients and, among the other organ transplanted patients, the median was 60 (18-100) ml/min/1.73m<sup>2</sup> (the latter including five recipients with eGFR).

#### **4.1.2 H1N1/09 pandemic vaccination (*Paper I*)**

The AS03-adjuvanted vaccine was extremely effective in the healthy control group, where 96% reached a protective titre after one dose and 100% after two doses (Fig.12). This protection rate is in line with the findings in a population-based study from Stockholm which, also among one million vaccinated individuals, found only 25 persons with vaccine failure [55]. The high protection rate among healthy persons has been reported by many [93, 94]. A Norwegian study of the seroresponse among 200 health-care workers found that 78% had protective titres after just one week and 98% three weeks after one dose of AS03-adjuvanted vaccine [95].

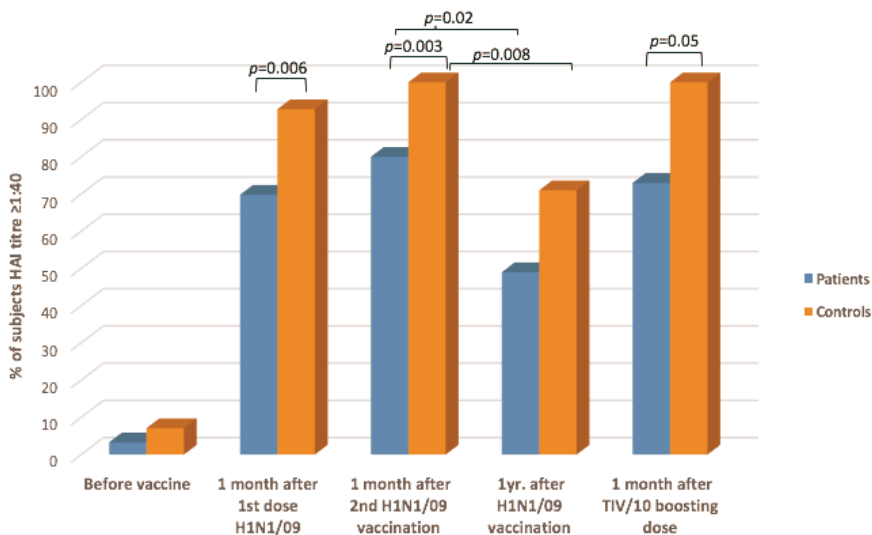


Figure 12. The percentage of Sot recipients/healthy controls with a protective titre against H1N1/09 after vaccination with the monovalent AS03-adjuvanted influenza vaccine 2009 ( $n=82/28$ ), serostatus one year later and the subsequent reaction after a booster dose of TIV/10 ( $n=49/11$ ). Only significant titre differences are noted in the figure.

Among the organ transplanted recipients, the responsiveness was lower compared with the healthy individuals, 69% after the first dose, while, after the second vaccine dose, 80% reached a protective titre ( $p=0.006$  and  $p=0.003$  respectively) (Fig. 12). There are four other studies of the AS03-adjuvanted influenza vaccine in an SOT population and three of them used only one vaccine dose. Those who gave one dose report a seemingly significantly lower seroprotection rate compared with ours; 34%-44%, all in kidney transplanted patients [96-98]. The substantially lower vaccine responses reported in these studies may depend on different immunosuppression and vaccination performed at various times after transplantation. In contrast, our findings are comparable with those in the study by Siegrist *et al.* investigating 202 SOT recipients and 131 controls [99]. After two doses, they found an overall response rate of 70.3%, but, in kidney, liver or pancreas transplanted individuals, the rate was > 80%. Lung transplanted patients ( $n=25$ ) had a significantly lower response (43.6%). Unfortunately, they did not have data on serostatus after only one dose.

The magnitude of anti-H1N1/09 titre rise was significantly higher among the healthy controls; after two doses of the AS03-adjuvanted vaccine, the median titre was 1:640 compared with the SOT recipients' median of 1:80 ( $p < 0.001$ ), (Fig. 13). A comparable difference was noticed by Siegrist *et al.*, who found a threefold lower titre among the SOT recipients [99].

At study start, three SOT recipients and two healthy controls (4.6%) had a protective titre (1:40) against influenza A H1N1/09 (Fig. 12). A Swedish survey published in 2012 conducting a re-analysis of sera from 2007 found HAI titres of  $\geq 1:40$  among 4.5% in the Swedish population, even higher at ages  $\geq 80$  years (9.3%) [100]. An analysis of the H1N1 three-dimensional HA structure from 1918 and 2009 shows that they are alike, older individuals probably have a partly immunological memory of the Spanish flu [101]. However, in the younger cohort, the seropositive reactions are presumably due to cross-reactive antibodies from earlier seasonal H1N1 influenza. The same has been found elsewhere; in a meta-analysis from a number of studies around the world of the pre-pandemic H1N1 antibody status, the overall seroprevalence was in fact 5%, with a lower prevalence among younger persons and a rising incidence with increasing age [102]. Some regional differences were seen and the authors speculated that this was possibly due to assay sensitivity differences. This has been reported by others and there is no standardised method for influenza antibody detection. According to a WHO meeting report evaluating the influenza pandemic in 2009, significant differences have been seen when comparing the HAI assay result between laboratories [94]. Those three recipients with a protective titre before the H1N1/09 had a one-, two- and four-fold titre rise respectively, perhaps a less prompt vaccine response than expected. One peculiar finding reported by some authors is that TIV administered one to two years before the pandemic vaccination might lower the response to the pandemic vaccine [103, 104]. This is the opposite reaction to what could be expected; a booster effect is usually seen after repeated vaccinations. However, in this situation, the antigens used differ between the vaccines. As a result, different TIV vaccination histories might be another factor explaining the discrepancies between the above-mentioned reports.



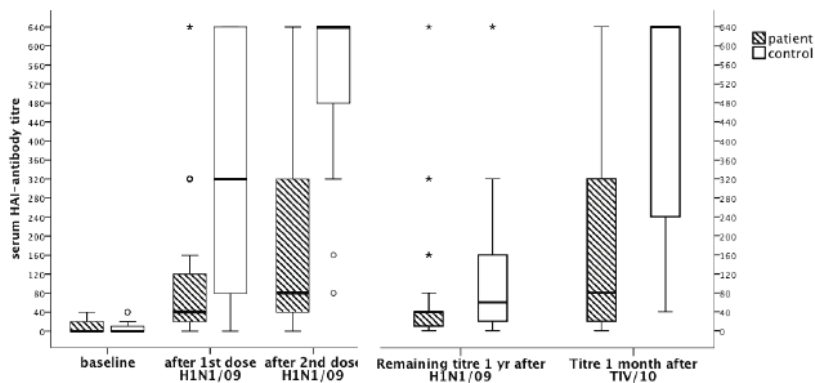


Figure 13. This boxplot shows the distribution of anti-H1N1/09 antibody titres from the start of the pandemic vaccination until one month after the following year's TIV/10 vaccination. The box represents the 25th-75th percentile, the dark line represents the median and the extended bars represent the 10th-90th percentile. Asterisks and rings represents outliers.

#### 4.1.3 Antibody persistence one-year after H1N1/09 pandemic vaccine (*Paper II*)

There was a significant loss of protective titres 10-14 months after H1N1/09 vaccination. Among the SOT recipients 23/49 (47%) had protective titres compared with 80% directly after vaccination ( $p=0.02$ ). The corresponding numbers for the control group were 7/11 (63%), compared with (100%) the year before ( $p=0.008$ ), (Fig. 12). Looking at SOT populations, two studies have been published on the loss of the antibody protection one year after using the H1N1/09 vaccine. Cordero *et al.* [105] reported a decline in protection from 80% to 30% over one year; seemingly a more profound drop than in our study. In that study, only one dose of MF69-adjuvanted monovalent vaccine was administered (Table 2). To some extent, this could account for the lower remaining immunity in their cohort of recipients. Siegrist *et al.* [106] is the only study using two doses of AS03-adjuvanted influenza vaccine, as in our study (Table 2). They found that 67% of the kidney transplant recipients had protective titres one month after immunisation. In contrast to our study, they did not detect a loss of protection, as 65% continued to have HI titres of  $\geq 1:40$  one year after vaccination. There is no obvious explanation of this difference

when compared with our study, as they studied renal recipients with a comparable age, immunosuppression, time after transplantation and renal function. Among healthy individuals one year after two doses of AS03- adjuvanted pandemic vaccine, the seroprotection rate was 79.1% at ages 18-60 years and 54.4% if above 60 years [107]. We did not find an age effect, perhaps due to the small number of subjects. In healthy children and a corresponding vaccine regimen, 98% had remaining seroprotection after one year compared with 51.6 % of the children receiving non-adjuvanted, whole-virion monovalent vaccine illustrating the different immune responses to different pandemic vaccines [108].

#### 4.1.4 TIV/10 booster dose (*Paper II*)

Boosting with TIV/10 enhanced the immune response to H1N1/09 in our SOT recipients to some extent, albeit not significantly. In particular, the frequency of responders increased from 49% to 71% ( $p=0.2$ ) as compared with 100% among the healthy controls ( $p=0.05$ ), (Fig. 12). Three other studies have also shown a booster effect with TIV/10 among organ transplant populations (Table 2). In these studies, the seroprotection rate after TIV/10 vaccination varied between 53% and around 80% [105, 106, 109]. The lower frequency (53%) of responders reported in the study performed by Mulley *et al.* [109] may reflect the low baseline titres after a single dose of non-adjuvanted H1N1/09 vaccine. The distribution of the magnitude of the H1N1/09 antibody titre rise after boosting with TIV/10 is shown in Fig. 13. The median titre increased from 1:20 to 1:80 in the SOT recipients and from 1:80 to 1:640 among the controls. The titre rise was significantly lower among the SOT recipients when compared with the controls ( $p=0.004$ ).

*Table 2. Comparison of percentage seroprotection (titre  $\geq 40$ ) between four studies of TIV/10 booster effect in SOT populations*

	Felldin <i>et al.</i> [110]	Siegrist <i>et al.</i> [106]	Cordero <i>et al.</i> [105]	Mulley <i>et al.</i> [109]
Patients	SOT $n=49$	Kidney tx $n=53$	SOT $n=96$	Kidney tx $n=49$
Vaccine	AS03 2 doses	AS03 2 doses	MF59 1 dose	Non-adjuv 1 dose
Pre H1N1/09	3.6%	13.4%	13.6%	13%
Post H1N1/09	79%	67.3%	83.9%	34.7%
Pre TIV/10	49%	66%	30%	no data
Post TIV/10	71%	72%	79%	53.1%

### 4.1.5 Non-responders (*Papers I and II*)

Some patients did not develop a protective antibody titre after vaccination; in Paper I, 15.8% and, in Paper II, 28.6%. As a result, these patients were “non-responders” to the pandemic vaccine and the H1N1/09 component of TIV/10 respectively. The non-responders did not differ significantly in terms of age when compared with responders in our studies. In the comparable but larger study ( $n=202$ ) after the pandemic vaccine by Siegrist *et al.* [99], an independent risk factors for non-responsiveness was in fact higher age, as also shown by Mulley *et al.* [109]. Since age is usually a dependent factor for seroresponse in vaccine studies, our studies might have been under-powered in this respect.

In both Study I and II, non-responders were more often treated with triple immunosuppressive therapy compared with responders [92% vs 35% and 79% vs 34% respectively]. Moreover, we were also able to show in Paper II that 93% of the non-responders were treated with MPA compared with 48.6% of the responders ( $p=0.0006$ ). This is in line with others who found that MPA treatment reduced the likelihood of achieving seroprotection after H1N1/09 vaccination in a dose-dependent manner [96, 99, 109]. Interestingly, Siegrist *et al.* found that, after the pandemic vaccination, if the MPA trough level was  $\geq 4\mu\text{g/mL}$ , the response was reduced by 80% [99]. In their follow up study after the TIV/10 booster dose, MPA-treatment only tended to have an impact, but this cohort was much smaller (Table 2) [106].

A decreased response was seen in our patients with severe renal impairment. If renal function was better ( $\text{mGFR} \geq 30 \text{ mL/min/1.73m}^2$ ), 87% responded with protective antibody titres after the pandemic vaccination, compared with 61% of patients with a GFR of  $< 30$  ( $p=0.036$ ) and this was also a significant factor for the response after the TIV/10 booster dose ( $p=0.003$ ). The same finding was made by others [109]. In contrast, Dikow *et al.* found a good immune response to H1N1/09-adjuvant vaccine in a haemodialysis population [111] and, in the multivariate analysis of the response to the pandemic vaccine by Siegrist *et al.*, renal function was not an independent risk factor, while age and MPA treatment were [99].

### 4.1.6 Adverse events after vaccination (*Papers I and II*)

**Side-effects** At the time of the administration of the first pandemic vaccine dose and TIV/10, a simple questionnaire was given to all subjects to be

returned two to three months later. We asked about the most common symptoms after vaccination (Table 3) and if there were “other symptoms”. The response rate was 93% and 75% in the SOT cohort in the first year and second year, while it was 96% and 100% respectively in the control group. A tendency was found among the healthy controls towards a stronger experience of muscle pain (Table 2). Another study also comparing with healthy controls reported less frequent local symptoms in the SOT population [99].

*Table 3. Side-effects comparing H1N1/09 and TIV/10 among SOT recipients versus healthy controls*

Reported symptoms <i>n</i> = (%)	Patients			Controls		
	H1N1/09 1 <sup>st</sup> dose	H1N1/09 2 <sup>nd</sup> dose	TIV/10	H1N1/09 1 <sup>st</sup> dose	H1N1/09 2 <sup>nd</sup> dose	TIV/10
Local symptoms <sup>1</sup>	51 (68)	41 (55)	8 (21)	16 (59)	12 (44)	4 (36)
Muscle pain	16 (21)	15 (20)	7 (19)	13 (48) <sup>2</sup>	12 (44) <sup>3</sup>	3 (27)
Fever	10 (13)	13 (17)	2 (5.4)	8 (30)	6 (22)	0
Cough	4 (5)	3 (4)	4 (11)	1 (4)	1 (4)	0
Headache	2 (3)	4 (5)	1 (2.7)	4 (15)	4 (15)	0

<sup>1</sup>Tenderness, redness and pain at the site of injection, <sup>2</sup>*p*=0.01, <sup>3</sup>*p*=0.02

**Adverse neurological events** In our cohort, one patient experienced vertigo and one developed unilateral deafness after the pandemic vaccine. There is a single report of sudden deafness, a healthy teenager after the vaccine [112]. There have been no reports of narcolepsy after the H1N1/09 vaccination among SOT recipients. A meta-analysis performed in 2013 (not mentioning narcolepsy cases) compiled 104 reports of neurological complications in 1,349 persons after the H1N1/09 pandemic influenza infection and 247 after vaccination. The influenza infection group did less well; 97% were children who developed encephalopathy, 4.7% died and 30.1% developed permanent sequelae. The vaccine group were mostly adults (72%) suffering from Guillain-Barré (64%) and they all survived [113].

#### 4.1.7 HLA antibody reaction (*Paper III*)

No SOT recipient became HLA sensitised during the two months after the first dose of AS03 adjuvanted vaccine. Previous studies of *de novo* HLA antibody

development in SOT populations after the same vaccine have reported diverging results. Our data are in agreement with the results presented by Broeders *et al.*, who reported *de novo* HLA alloantibody formation in only one of 111 renal transplant recipients [97]. In contrast to our results, other studies have found a higher production of *de novo* HLA antibodies. Fairhead *et al.* reported that almost 12% of kidney transplant patients developed *de novo* HLA antibodies [98]. This is in line with the findings of Katerinis *et al.*, who reported that 12-17% of renal SOT patients developed HLA antibodies after an influenza vaccination containing AS03 [114]. One possible explanation of the latter finding is that the majority of the patients received not only two doses of adjuvanted H1N1/09 vaccine but also one dose of TIV/09 prior to the H1N1/09 vaccine. It is possible that, the more intense the influenza vaccine regimen, the more frequent *de novo* HLA antibody formation. In our HLA study, only 8/67 patients and 1/20 controls were vaccinated with TIV/09 between the second vaccine dose and the serum sample one month later. Most HLA antibodies in the Katerinis study were detected at low MFI (<2,000) and generally resolved by six months.

At the start of the H1N1/09 vaccination, 26 of 67 (39%) of the SOT recipients had HLA antibodies which were detectable as compared to five of 20 (25%) healthy controls. When comparing demographic data, only the number of years since transplantation tended to be significant for the presence of HLA antibodies ( $p=0.03$ ).

**HLA antibodies with new specificities** were seen in seven (10.4%) SOT recipients, all of whom were HLA immunised before vaccination with antibodies against 16-52 Class I antigens and 0-6 Class II (Fig. 14). They had higher post-vaccination MFI values, in both in Class I and II, albeit not uniformly. Three of the seven recipients also had DSA before vaccination and these MFI values increased post-vaccination. Proinflammatory events were seen in three of our seven patients with new HLA specificities and these can influence the HLA reaction substantially (2.5-fold) as showed by Locke *et al.* in a cohort of immunised renal transplant recipients [90]. One of these three also received a blood transfusion, which also, although nowadays leukocyte-reduced, have been shown to increase the HLA ab strength in patients on the waiting list [115].

**Donor specific antibodies (DSA)** No recipient developed *de novo* DSA after vaccination and no DSA disappeared. Of the 26 sensitised patients, 15

had DSA and 11 non-directed HLA antibodies before the pandemic vaccination. The change in the DSA MFI values after vaccination was not statistically significant when considering the strongest MFI value or the cumulative DSA. However, we are unable to preclude that some of the new specificities of HLA antibodies that were seen were in fact donor specific, as we were not able to re-type the donors. Furthermore, in 13/15 patients, all the DSAs were inferred by genetic linkage to typed loci. The occurrence of *de novo* DSA after the AS03 adjuvant influenza vaccination varies between 0-8.8% in different studies [97, 98, 114]. Apart from clinical differences between these patient cohorts, the single antigen technique is not standardised and differences between centres could therefore also depend on the assay (with a LOT to LOT variation), the execution of the assay and the interpretation of the results including different cut-off values.

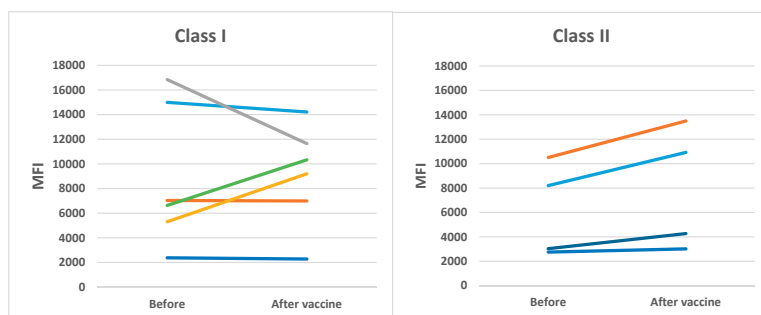


Figure 14. The seven SOT recipients who developed HLA antibodies with new specificities after vaccination. Here represented by their HLA antibody with the highest MFI value in Class I and II. Three patients had both Class I and II HLA-antibodies.

#### 4.1.8 Acute and chronic rejection (*Papers I-III*)

In most studies, no increased risk of allograft dysfunction or clinical rejection has been reported and no acute rejection episode occurred in our study during the first two-year follow-up after the vaccination. Two recipients were diagnosed with chronic rejection but they had a lower or mixed DSA response. In those reports which found new HLA antibodies, single patients with *de novo* DSA have in fact developed acute or chronic AMR [96, 98, 114]. There are, however, possible causes other than the pandemic vaccine coinciding with the development of both DSA and rejection. Non-adherence or earlier

minimisation of the immunosuppression is thought to be the cause of many AMR and subsequent graft losses [116, 117].

#### **4.1.9 Five-year clinical follow-up (Paper III)**

From the time point of the H1N1/09 vaccination, the overall one-, two- and five-year patient survival was 100%, 98.5% and 82%, and the corresponding overall graft survival was 94%, 88% and 80%, including graft loss due to deaths with no impact of HLA antibody status. Five-year graft survival among those seven recipients with HLA antibodies with new specificities was (57%) ( $p=0.8$ ) and, among the 15 recipients with DSA, it was 46.6% ( $p=0.04$ ).

Among renal transplanted patients there was a correlation between the five-year graft survival after vaccination with the presence of both DSA ( $p<0.0001$ ) and low mGFR/09 ( $p<0.0001$ ). In fact, in renal recipients with no DSA, the five-year graft survival was 83% in comparison with 22% in the DSA-positive group. There was a relationship between a rise in the DSA Class II titre at vaccination and graft survival at five years ( $p=0.001$ ), but the impact of this in relation to the pre-existing lower renal function and DSA is questionable. By way of comparison, Lachman *et al.* re-analysed stored serum for HLA antibodies using the single antigen technique in 1,014 renal recipients transplanted in 1984-2004 and calculated the five-year outcome after the test was taken. The graft survival among the HLA negative recipients was 83%, but, in patients who were DSA positive in combination with an estimated GFR of  $< 30$ , the survival was only 12% [118].

#### **4.1.10 Risk and benefit of H1N1/09 vaccination**

To summarise, the risk of both adverse events and HLA immunisation after H1N1/09 vaccination appears to be low and should be compared with the risk of the influenza disease. The pandemic H1N1/09 infection had a mortality rate among unvaccinated hospitalised SOT recipients in 2009 of 4% [56]. A more recent report from Finland highlights the need for continuous TIV vaccination. Due to the reports of negative side-effects after the AS03-adjuvanted vaccination, a lower adherence to seasonal vaccination recommendations developed in Finland. In the spring of 2014, an outbreak of influenza A H1N1 occurred on the ward at the transplant unit in Helsinki. Of 23 patients, six had not been vaccinated with TIV/13, five developed symptomatic influenza disease and three of them died despite maximum ICU support. Those three were all newly transplanted ( $< 1$  month). Among the 17 who were vaccinated,

two tested positive for influenza and only one of them had mild symptoms [119]. This also points to the importance of vaccination ahead of transplantation, despite the knowledge of a lower seroresponse among individuals with end-stage organ failure.

## **4.2 HEV study (*Paper IV*)**

### **4.2.1 The study population**

A total of 196 SOT recipients were followed with regular serum and urine sampling during the first two years after transplantation to establish the prevalence and incidence of hepatitis E virus infection. The prevalence was compared with corresponding data in 500 healthy blood donors [73]. A total of 375 serum and 346 urine samples from the SOT recipients were investigated. The median age among the kidney transplanted patients was 52 years compared with the other SOT recipients, who had a median age of 45.5 years and healthy controls, who were 44 years old. In both the study population and the healthy controls, fewer were women, 34.2% and 36.2% respectively.

Most of the SOT recipients had received a kidney (59%), but they also included liver (20%), lung (12.7%) and heart (7.6%) transplants and one multivisceral graft. Renal recipients had a longer history of transplantation, as 20.5% received their  $\geq$  2nd graft compared with the other SOT recipients (2.5%) ( $p < 0.001$ ). The immunosuppressive treatment was generally a combination of CNI, MPA and corticosteroids, with the exception of 16 liver transplanted patients who only had CNI plus steroids. Acute rejection episodes were seen in a total of 25% of the recipients; this was not always biopsy proven in non-renal organ transplanted patients.

### **4.2.2 HEV infection before transplantation**

In order to find those SOT recipients with previous, recurrent or de novo HEV infection most efficiently, the screening for anti-HEV antibodies was started with the serum samples at 9-15 months post transplantation but for 13 patients the last available sample was drawn at month 6. Further analyses were made in a stepwise fashion to identify when the infection occurred, before or after the transplantation (Fig. 15).



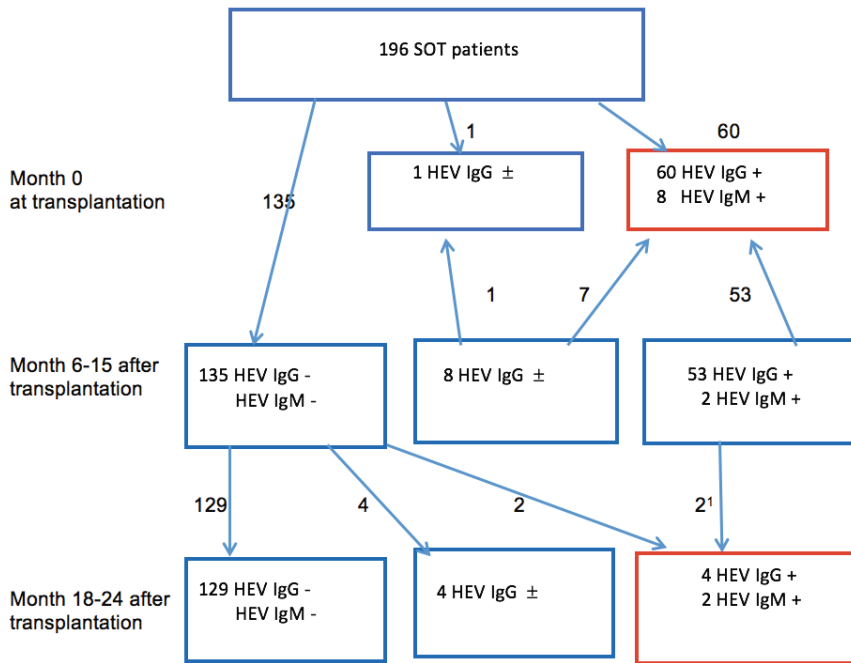


Figure 15. Results from analysing for anti-HEV IgM/IgG in sera from the SOT population starting at month 6-15. <sup>1</sup>Sera drawn at month 18 and 24 from the two patients who were still IgM positive at 15 months. ± = borderline value.

At the time of transplantation, 60/196 (30.6%) of the SOT recipients were HEV IgG positive, significantly more compared with 16.8% of the healthy blood donors ( $p < 0.0001$ ). There were no gender differences, no differences depending on type of organ transplanted or whether the patient received a first graft versus being re-transplanted. However, there was a strong correlation with age; the older both the healthy individuals and SOT-recipients were, the higher was the anti-HEV IgG prevalence ( $p < 0.0001$ ). Among the younger individuals ( $< 50$  years), HEV IgG positivity was somewhat more frequent in the SOT cohort compared with the blood donors ( $p = 0.04$ ), but in fact, it was only 10 SOT recipients  $< 50$  years old who were HEV-IgG positive. Seven of them were renal transplanted (four with unknown renal disease and three with former transplants) and one heart-, lung- and liver transplanted respectively. With increasing age, the difference disappeared and, when both blood donors and SOT recipients were older than 60 years, there was an even and high anti-

HEV prevalence in both groups; 54% and 47%, respectively ( $p=0.7$ ). The development of assays for anti-HEV IgG and IgM detection with both high sensitivity and specificity explains today's higher anti-HEV prevalence figures overall [120]. Our SOT population has a seroprevalence that is almost as high as that found in the high-prevalence area of southern France, where 40% of the SOT population is HEV IgG positive [121, 122]. A recent study from Italy using the same assay as we use found a lower incidence among their blood donors (7%) and individuals with chronic liver disease (9.2%) with median ages almost comparable to our study (45.5 and 52.3 years respectively) [123]. They also had a cohort of patients with chronic renal disease with an anti-HEV prevalence of 30.7%, but they were significantly older (median 74.3 years) and, after adjustment, they found that only age, not kidney disease, was an independent risk factor for HEV IgG. As a result, the anti-HEV prevalence is unexpectedly high in Sweden and SOT recipients appears to be affected by HEV at an earlier age. The reason for this earlier onset is not known but it is probably due to a number of causes; persons with chronic disease run the risk of being infected via blood products [68, 69] or transplanted organs [70, 71]. Single patients with acute HEV infection might have been misdiagnosed [80] and speculatively, single cases of chronic renal disease might be caused by HEV [77]. Our finding with 4/10 younger HEV positive with unknown renal disease needs to be studied further.

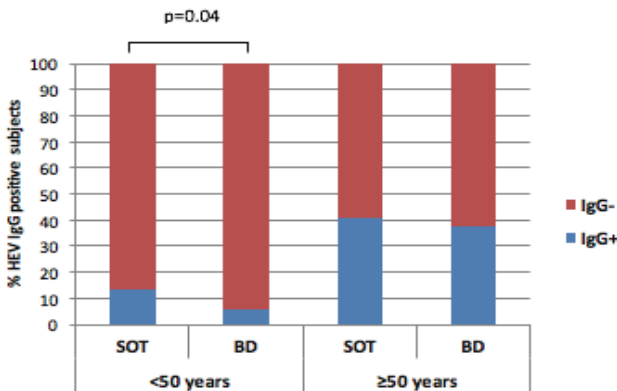


Figure 16. A higher proportion of SOT recipients were HEV-IgG positive compared with healthy blood donors (BD) at ages below 50 years.

### 4.2.3 HEV infection after transplantation

In eight patients we found HEV IgM at the transplantation, indicating a recent infection before transplantation. Two of them continued to have high IgM levels throughout the follow-up (15 and 24 months respectively). All the tested samples were negative for HEV-RNA and the patients did not develop liver affection. Nevertheless, we are unable to rule out the possibility of chronic HEV infection in these two. Another two IgG- and IgM-negative patients seroconverted after transplantation and became HEV IgG positive at the 18- and 24-month follow-ups. No RNA could be detected in serum or urine at conversion or three to nine months before. This might be due to a primary HEV infection occurring during the follow-up after transplantation albeit with an indolent course. Another recipient, HEV IgG positive at the time of transplantation, had detectable HEV-RNA in a single urine sample one month post-transplantation but no signs of infection in other parameters. These five cases of possible new or chronic HEV infections are perhaps too few in an immunocompromised cohort, as 5/500 of the healthy controls were PCR positive in serum, all five with anti-HEV IgG, but only two had a borderline-value of anti HEV IgM [73]. The reason only one sample had detectable HEV RNA might be explained by the handling of the study samples, as the study was not designed for RNA detection but for the detection of DNA viruses. However, the fact that we found HEV RNA in urine is interesting. One other study has reported HEV4 RNA in urine [76]. This indicated that urine could also be used to identify on-going infections. Compared with France, which saw HEV re-infection in 3.3% (3/263) and *de novo* infection during the first year after transplantation in 2.1% (all diagnosed by the detection of HEV RNA) [121], the number of cases we have identified, if correctly interpreted, are what would be expected in the context of our anti-HEV prevalence.

Our patients did well and a decrease in immunosuppression could be enough to resolve HEV infections. In a compilation report of 85 SOT recipients with confirmed HEV infection, 65.9% developed chronic disease [124]. In 32.1% the infection resolved, eight developed liver cirrhosis and two needed a liver re-transplant. There are also case reports of rapidly fibrosing hepatitis due to HEV after liver transplantation [70] and it is therefore important to identify HEV infection at an early stage.

In our view, the unexpected high prevalence of anti-HEV IgG among the Swedish SOT recipients in this study highlights the possibility of hepatitis E

as a new opportunistic infection in the immune compromised host and there is a need of further studies if, and to what extent HEV cause secondary organ involvement.

## 5 CONCLUSIONS

### Paper I

Vaccination with the monovalent AS03 adjuvanted influenza A H1N1/09 vaccine was well tolerated and over all effective in SOT recipients although significantly less compared with healthy controls. A second dose of the same vaccine enhanced the immune response significantly among the immunocompromised patients.

### Paper II

One third of both SOT recipients and controls had lost their protective antibody level one year after vaccination. After a boosting dose of TIV/10, some of the SOT recipients regained seroprotection, while all the healthy controls returned to their former seroprotection levels.

### Paper I + II

Vaccine non-responders had more often lower renal function (mGFR  $<30\text{ml}/\text{min}/1.73\text{m}^2$ ), triple immunosuppression and MPA-treatment. No acute rejection was identified during the first two years after pandemic vaccination.

### Paper III

No SOT recipient became HLA immunised between the start of the pandemic vaccination and two months later. In 10% of patients previously allo-immunised, HLA antibodies with new specificities were seen. No *de novo* donor specific antibodies (DSA) were seen with the limitation of that no new donor tissue typing was possible. Two cases of chronic rejection diagnosed within one year of vaccination had a mixed DSA response and was probably unrelated to vaccination. A subgroup of renal transplant recipients had DSA and low renal function at study start and did very poor at long term follow up.

### Paper IV

Hepatitis E virus have affected 30% of SOT population at the time of transplantation; a significantly higher prevalence compared with healthy, particularly in ages below 50 years. A suspicion of new and chronic HEV in a few patients during two years of follow up could not be verified by HEV-RNA. A single urine sample was HEV-RNA positive in a HEV-IgG positive patient one month post transplantation.

## 6 FUTURE PERSPECTIVES

The balancing act between rejection and infection is a challenge for all transplant physicians and the formula for achieving tolerance appears to be far off. The state of tolerance also appears to be vulnerable and could be challenged by vaccination. This became very clear to me when Dr. Mary Louise Market gave a lecture on thymus transplantation at the 2016 ATC Congress. One of her patients had been tolerant for 10 years but, after a measles vaccination, the tolerant state was lost within 14 days. This exemplifies the need for further research on vaccinology and clinical transplantation, apart from the very fundamental issue of basic transplant immunology.

The rate of seroprotection after the AS03-adjuvanted vaccination was fairly good in our SOT population, but there are a number of non-responders who are in need of better protection. Higher vaccine doses or repeated vaccinations have been suggested [97], but alternative strategies [125], such as the more effective medical treatment of influenza, are needed. Studies testing monoclonal antibodies against conserved surface structures of the virus, such as the M2 ion channel (the antibody TCR-032) or the stalk of the HA molecule (VIS410, CR8020 and others) in particular, are in progress, even if there are obstacles that need to be removed before these drugs can be introduced into the clinic [126]. Our conclusion in Papers I and II was that the use of AS03-adjuvanted vaccines in the SOT population was safe, as was concluded by Cordero *et al.* [105, 127] and Broeders *et al.* [97]. In contrast, those authors who have studied the HLA antibody reaction more closely argue that the vaccine effect needs to be further studied before we use it again, due to the risk of allo-immunisation [96, 98, 114]. None of the current observational studies has fully elucidated the risk since they were not designed for this purpose. To address the question, a controlled study with both a larger and more uniform cohort of patients is needed. The optimal design would include an un-vaccinated (probably not feasible) or non-adjuvanted vaccine SOT control group. Both the serological and cellular immune response need to be studied, from the angle of both protection and immunisation. The diagnosis of rejection still relies on biopsies and, as a result, control biopsies would be needed, together with solid phase data and a long-term follow-up. However, due to the cases of narcolepsy, the AS03-adjuvant will probably only be used again if we are threatened by a particularly dangerous infection, not as an influenza vaccination.

**Hepatitis E** is a common form of hepatitis in Sweden, in particular among SOT recipients, but the infection is still unrecognised by us, the medical professionals. The reports of chronic liver disease, as well as extrahepatic manifestations among SOT recipients, highlight the need for a new awareness of HEV among hepatologists, nephrologist and neurologists in cases with unknown cause of organ disease or rapid decline in organ function.

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