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Erasmus Journal of Medicine: independent scientific journal

Renal failure after liver transplantation

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Colofon

Erasmus Journal of Medicine is a scientific magazine by and for students of Erasmus MC University Medical Center Rotterdam. It was initiated by the MFVR (the students' organization of Erasmus MC).
The journal will appear twice a year. It will be published on paper (3000 copies) and on the EJM website (www.erasmusjournalofmedicine.nl).
The main purpose of the journal is to stimulate Erasmus MC medical students to read and write about medical scientific subjects, early in their career. A secondary purpose is to make the results of excellent student-driven research known to others. The journal will contain papers describing original research (Full articles), systematic reviews (Reviews), summaries of recently conducted studies (Extended abstracts), short descriptions of research projects looking for students to participate (Research News), editorial comments and letters to the editor.
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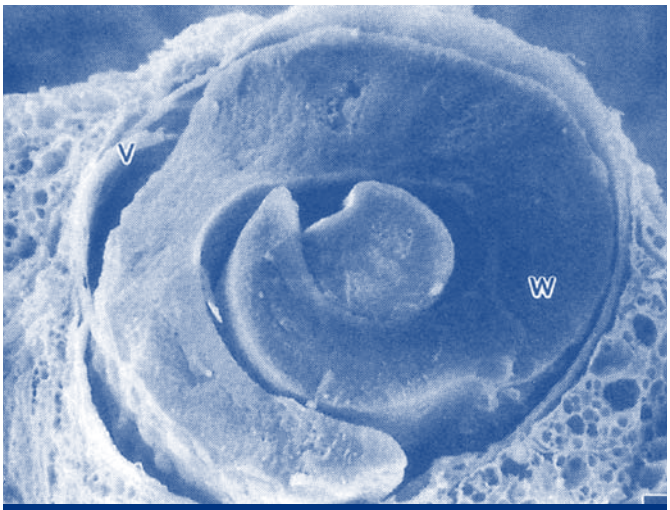

Schistosoma occluding a bloodvessel (pag 52)

Foreword

Here it is! The first issue of Erasmus Journal of Medicine. Written entirely by students and scholars of Erasmus MC. Before you start thumbing through the pages of the journal, probably looking for subjects most relevant to your field of study, I would like to introduce you to this new journal.

A few weeks ago I spoke to the directors of our medical education teaching program on our vision for the future of education. We tried to picture the physician in 2020. Naturally, this did not lead to fixed conclusions. But three images that kept coming back were: Patients, Society and Academia.

At Erasmus MC we speak of 'Patient-centered care' ('De patient prominent'). That goes without saying. Yet it is precisely because we take it for granted, that we are sometimes inclined to forget about the patient. An objective of medical education should be not to make do with the casualness of the human factor but to be constantly aware of the fact that the patient really is to be put at the center.

Social involvement by the medical professionals is a prerequisite in a Society in which patient care no longer takes place in fragmented organizations but increasingly extends beyond the organization's walls. Cooperation is a key word. Cooperating with colleagues and other care professionals, as well as with policy makers and public and private organizations, because healthy people and a healthy society cannot be seen separately.

Academia stands for the scientific astonishment that we would like to get our students acquainted with. This is the best intellectual baggage that we can give them in a world in which medical know-how is being continually renewed. A critical, open attitude and a thorough methodological training are at the basis of finding new knowledge, assessing it and using it, long after having left the lecture rooms of Erasmus MC.

The Erasmus Journal of Medicine offers prospective physicians and researchers enjoyable and exciting reading material. It is always a great joy to quietly get engrossed in the latest issue of your favorite trade journal. As part of our curriculum, EJM offers authors an exquisite opportunity to publish scientific articles on their own research or literature.

Our first issue clearly elaborates on the versatility of our university medical center. You will be amazed. Do not just read the articles closely related to your field of study! Let the enthusiasm of all the authors who have contributed to this memorable first issue of the Erasmus Journal of Medicine get a hold on you.

Enjoy the read.

Prof. Huibert Pols, Dean and vice-chairman of the Board of Directors Erasmus MC

"Concealed talent brings no reputation."
Desiderius Erasmus.

With that statement he inspired us to start this journal. Because, how else than to write and publish will the scientific talents of the youngest generation of medical students and scientists be revealed? That is exactly what the Erasmus Journal of Medicine aims for, to stimulate scientific development and to reveal the scientific talent of investigators and doctors-to-be. Scientific development is an important part of the medical curriculum at the Erasmus MC University Medical Centre. Students are being trained here to become research-orientated doctors, and to broaden their horizon.
Our aim is to publish articles written by students at the Erasmus University Medical Center, to give fellow students and employees an impression of the scientific quality that is present amongst them. The Erasmus MC is known for its scientific expertise, so we have a reputation to safeguard.
We want to stimulate students to think outside the box and extend their knowledge beyond the borders of the medical curriculum and push themselves harder, because that is what makes great doctors.
As student editors of the editorial board we are honoured to present to you the first issue of our Erasmus Journal of Medicine. It has taken us a lot of effort and perseverance, but with strong teamwork and help from many we managed to get the job done.
We hope that by publishing this journal we inspire many others to develop their scientific senses and knowledge and to discover their own or someone else's talent.
Student-editors of the Editorial Board of the Erasmus Journal of Medicine.

Bas Hullegie
Denise van der Linde
Mostafa Mokhles
Maartje van der Schaaf

A thank to you all

All writers of scientific articles remember the mixture of feelings of pride, relief and joy on seeing their own writing in a printed international journal for the first time.

Forgotten are the long hours of struggling with text and tables, the rewriting and reformulating, the frustration when a senior author makes too many changes to the text and the anxiety when a paper is finally submitted to the journal and enters the peer-review process.

The Erasmus Journal of Medicine is a students' journal. The editorial board hosts a majority of student members and all papers are authored by students of Erasmus MC University Medical Center.

The editorial policy of the Erasmus Journal of Medicine and its review process are styled according to procedures that are standard for the large scientific medical journals. In this way, students may develop a taste for writing scientific articles, and they may get acquainted with this process early in their scientific career.

Writing and publishing has changed in the last decades. We are more and more able to define what constitutes a methodologically sound paper. The methodology of systematic reviews has evolved, thanks to the work of, among others, the Cochrane group (www.cochrane.org). Improvements in the conduct and reporting of randomized clinical trials are reflected in the recent updates of the recommendations in the CONSORT statement. Similar recommendations have been published for diagnostic studies (STARD) and studies reporting experimental studies in animals (CAMERADES). It is the policy of this journal to closely adhere to these recommendations.

Apart from getting our students acquainted with up to date writing and peer review, the purpose of the journal is to publish the results of research projects carried out by students of Erasmus MC, first of all because they are worth publishing and worth to be read by you and by other researchers.
In this first issue you will find an abundance of systematic reviews by 2nd year students. We hope that in the next few months the 4th year students, who are currently working on their own research projects, will submit their work in equally large numbers.

This journal is the product of the hard work delivered by many. Each submitted paper has been reviewed and by students and staff members of the editorial board: Maarten Frens, Paul van Daele and our student-editors Maartje van der Schaaf, Mostafa Mokhles, Denise van der Linde did a very good job in this. They reviewed, helped and advised where necessary. Our two student reviewers, Konstantinos Vakalopoulos and Renuka Birbal took up their task with enthusiasm and skill. Bas Hullegie helped in all matters concerning publication. Wu Wei helped starting up the editorial board and made a very nice promotional clip (http://www.erasmusjournalofmedicine.nl) Ed Hull and Charles Frink were great in restructuring the texts and English language editing on a really short notice. Els Springer staffed the editorial bureau before she fell ill. Last but not least we want to thank Annabel te Hennepe who took care of everything we could not do or forgot.

We hope that the journal will stimulate students to write and submit their work to us. I invite you to read the journal's first edition and judge for yourself whether it is worth your while.
Rotterdam, May 2010,

Diederik Dippel

Saccade adaptation and the neurology of saccades

In this issue of the Erasmus Journal of Medicine, van Broekhoven et al report on the role of the cerebellum in the detection of post-saccadic errors.

A saccade is a quick jerky movement of the eye that positions a visual target on the fovea.
Also the quick phases of a nystagmus movement can be considered to be saccades. The term saccade stems form the French word saquer, which refers to the flicking of a sail in a gust of wind. Saccades are highly stereotyped ballistic movements. This means that the amplitude of the movement strictly determines its kinematics (the so-called "main sequence" relationships), and the movements can not be modified during their execution [1].

Saccades are generated by a brain stem circuit in the paramedian zoned of the pontine reticular formation (PPRF). Various areas, such as the midbrain superior colliculus, and the frontal eye fields directly project to the PPRF. Its neural pathways are separate from all other types of eye movements, up until the brainstem oculomotor nuclei that directly control the six extra-ocular eye muscles through the IIIrd, IVth and Vth cranial nerves.

This hierarchy in the organization of saccadic commands can be applied in neuro-ophtalmologic examination [2]. Slow saccades may reflect abnormalities in the motor periphery or the medial longitudinal fasciculus. One can not decide to make a fast or a slow saccade, although factors such as fatigue or drug-intoxication can be of influence. Patients with Parkinson's disease show a characteristic abnormality of saccade initiation. Reflexive saccades are made with appropriate latencies (i.e. around 200 ms) and amplitudes. However, self-initiated saccades are accompanied with large latencies and amplitude decreases. Inappropriate saccades (or "square wave jerks") have been reported in strabismus, Huntington's disease, multiple sclerosis and schizophrenia. Here the patient makes saccades that are uncalled for.

Finally, saccadic dysmetria (i.e. undershooting, but especially overshooting saccades) is the hallmark of cerebellar deficits, but it can also be caused by certain brainstem lesions. The reason behind this is presumably that the saccadic system needs to be continuously calibrated in order to correct for factors such as fatigue, disease or ageing. In healthy subjects, one can actively make saccades hypometric or hypermetric by consistently displacing the target in saccadic midflight [3]. Cerebellar patients do not modify their saccade amplitude in this so-called saccade adaptation paradigm [4]. Most likely it is the visual error after the saccade, i.e. the distance between the target and the fovea that drives this adaptation. Van Broekhoven et al have now shown that a specific area in the cerebellar cortex, lobules VIII and IX are responsible for the detection of such errors [5]. This can further narrow down the relation between saccadic symptoms and the corresponding cerebellar lesion.

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A retrospective study of 23 years of experience with calcineurin inhibitors at a single center

Renal failure incidence and risk factors after orthotopic liver transplantation

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Background: **The 10-year survival rate after orthotopic liver transplantation (OLT) has increased from 18% to 60% in the past two decades. Consequently, more patients are at risk for complications, such as chronic renal failure (CRF), which are related to the long-term use of immunosuppressants. The aim of this study was to assess the incidence of CRF, to identify the risk factors for CRF, and compare the outcome based on the immunosuppressive regimens used in a cohort of long-term OLT recipients.**
Methods: **We retrospectively reviewed the clinical and laboratory data of all patients who received a liver allograft between 1986 and 2008 at the Erasmus Medical Center, Rotterdam.**
Results: **In total 391 patients were analyzed. CRF developed in 168 patients (43%), and the cumulative incidence of CRF was 41.1% at 5 years after OLT. Multivariate analysis indicated that an increased risk of CRF was associated with a number of variables, including sex and age, the use of cyclosporine compared to tacrolimus, and the presence of diabetes mellitus and positive HCV serology. Furthermore, the incidence of CRF was significantly lower in patients transplanted after 1996.**
Conclusion: **Clinicians should be less concerned about acute rejection in OLT recipients, which is the primary reason for immunosuppression, and should focus instead on methods that not only prevent short-term rejection, but also preserve long-term renal function.**

Introduction

In the past 25 years the number of orthotopic liver transplantations (OLT) has increased from 550 to 5400 per year in Europe [1]. During this period, the average one-year survival after OLT increased from 34% before 1985 to the current 90%. Five-year patient survival also increased, from 22% to 70%, and ten-year survival increased from 18% to the current 60% [2]. This improved survival is largely attributable to improved surgical techniques and the introduction of immunosuppressive regimens, including calcineurin inhibitors (CNI) [3, 4]. Most immunosuppressive regiments in solid organ transplantations were initially based on Cyclosporine A; later on, tacrolimus was added. The use of CNIs in the field of clinical transplantation has significantly reduced the risk of acute rejection and improved patient survival [5].
However, one of the most serious complications of CNIs is nephrotoxicity. The incidence of chronic renal failure (CRF), five years after OLT, is reported to be as high as 18% [6]. To try and limit this effect, several new strategies have been introduced in immunosuppressive regimens, including CNI minimization, complete CNI avoidance and CNI withdrawal [7]. The long term effects of these strategies, however, have not been established. If we can determine the incidence of CRF, the risk factors for CRF and the risk of death after CRF, and compare the outcome based on the immunosuppressive regimens used, then we can further optimize the regimens and ideally create a risk profile for the development of CRF after OLT.

In a single-center retrospective study, we addressed the following research questions:

- What is the incidence of CRF after OLT now, compared to previous years?
- What are risk factors for the development of CRF?
- What are the consequences after the development of CRF?
- What is the risk of death after CRF?
- Is there an significant difference in renal function between tacrolimus and cyclosporine treated patients?

Patients and methods

Patients

We retrospectively reviewed the clinical and laboratory data of OLTs performed between October 1986 and April 2008 at the Erasmus Medical Center. Of the 503 liver allograft recipients, there were 98 postoperative deaths during the first year. Of the remaining 405 patients, 391 were followed up primarily for the development of renal failure (dialysis, transplantation or a calculated GFR below 60 mL/min according to Cockcroft-Gault). We analyzed the influence of different immunosuppressive regimens, the cause of liver disease, patient demographics, and pre-existing and de novo conditions for the development of CRF. The primary exclusion criteria were: 1) less than one year survival and 2) having undergone a combined kidney-liver transplantation. A secondary exclusion criterion was the lack of at least one year of follow-up data.
For each patient, follow-up data were obtained at six-month intervals during the first year and annually thereafter until 1 April 2009 or death. Patients were excluded when there was a gap of two or more years in follow-up data or if they needed renal replacement therapy.
If patients had more than one OLT, the starting point of follow-up was the last performed OLT if it occurred within 12 months. If the period between OLTs was more than 12 months, the earlier OLT was used as the starting point.

Table 1 - Baseline characteristics[1]

Characteristic	1986-1990 (N=26)	1991-1995 (N=56)	1996-2000 (N=106)	2000-2005 (N=123)	2006-2009 (N=80)	All patients (N=391)	P value
Male sex, N (%)	16 (61.5)	26 (46.4)	63 (59.4)	74 (60.2)	48 (60.0)	227 (58.1)	0.45
Positive for hepatitis C antibody, N (%)[2]	2 (10.5)	5 (8.9)	15 (14.2)	16 (13.0)	10 (12.5)	48 (12.5)	0.90
Diabetes mellitus before OLT, N (%)	2 (7.7)	5 (8.9)	7 (6.6)	16 (13.0)	22 (27.5)	52 (13.3)	<0.001
Recipient weight (Kg)	71 ± 15	72 ± 15	72 ± 12	76 ± 14	78 ± 17	74 ± 15	0.015
Recipient mean age, yrs (min, max)	41 (20, 60)	49 (22, 66)	46 (19, 68)	44 (16, 66)	51 (16, 67)	47 (16, 68)	<0.001
Primary cause of liver disease, N (%)[3]							
Acute hepatic failure	4 (15.4)	10 (17.9)	25 (23.6)	26 (21.1)	11 (13.8)	76 (19.4)	0.49
Cholestatic disease	8 (30.8)	19 (33.9)	19 (17.9)	31 (25.2)	16 (20.0)	93 (23.8)	0.21
Alcoholic cirrhosis	2 (7.7)	8 (14.3)	11 (10.4)	8 (6.5)	16 (20.0)	45 (11.5)	0.017
Hepatitis B	5 (19.2)	6 (10.7)	12 (11.3)	8 (6.5)	1 (1.3)	32 (8.2)	0.15
Hepatitis C	0	3 (5.4)	9 (8.5)	11 (8.9)	4 (5.0)	27 (6.9)	0.45
Hepatocellular carcinoma	2 (7.7)	3 (5.4)	7 (6.6)	11 (8.9)	11 (13.8)	34 (8.7)	0.018
Metabolic disease	1 (3.8)	1 (1.8)	8 (7.5)	1 (0.8)	2 (2.5)	13 (3.3)	0.069
Polycystic disease	0	0	1 (0.9)	4 (3.3)	2 (2.5)	7 (1.8)	0.52
Other liver diseases	4 (15.4)	6 (10.7)	14 (13.2)	23 (18.7)	17 (21.3)	64 (16.4)	0.64

[1] Plus-minus values are means ± SD. Because of rounding, percentages may not add up to 100.
[2] Data were missing for 6 patients.
[3] Patients may have had a secondary cause of liver disease.

The study population was stratified into 5 groups based on transplantation year (1986-1990; 1991-1995; 1996-2000; 2001-2005; 2006-2009). This was mainly done so the incidence of CRF and differences in our patient population could be analyzed over time.

Renal function

The calculated glomerular filtration rate (cGFR) was indirectly estimated as the creatinine clearance (CCI) according to Cockcroft-Gault (C-G). To this end, we used the creatinine level (µmol/L) and weight, which we gathered from follow-up data.

To allow comparison of results between people of different sizes, we corrected the CCI for the body surface area [12] and expressed it compared to the average sized male (mL/min/1.73 m2) [24].

CRF was categorized in three groups. Patients that had moderate renal failure with a cGFR of 59 mL/min or lower for more than 3 months were categorized as having Stage 3 Chronic Kidney Disease (CKD-III). Patients with severe renal failure with 29 mL/min or lower were categorized as having Stage 4 CKD (CKD-IV). Patients with kidney failure with 14 mL/min or lower who required dialysis or kidney transplantation were categorized as having Stage 5 CKD, better known as End Stage Renal Disease (ESRD). This was in accordance with the Kidney Disease Outcomes Quality Initiative (KDOQI) [11].

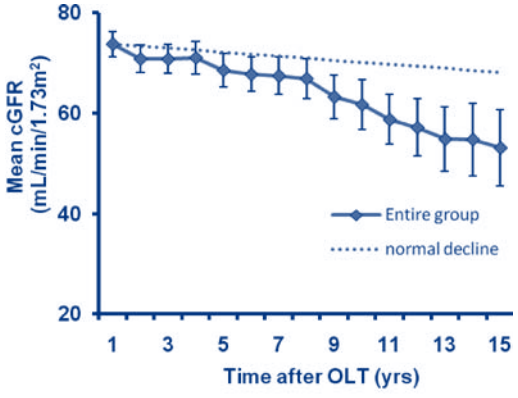

Figure 1
Mean cGFR of the entire group OLT recipients (N=391) shows a decline of 1.6 ml/min/year. In comparison the normal decline, based on the healthy population.

Immunosuppression

We divided the immunosuppressive regimens into three groups based on the treatment patients received: Tacrolimus-based, Cyclosporine-based or CNI-free. As a reference point we used the immunosuppressive regimens each patient received three months after OLT.

Statistical analysis

Survival curves were calculated with the Kaplan-Meier method. Multivariable Cox regression modeling was used to analyze the relation between CRF and covariates. The risk of death after CRF was analyzed by means of a time-dependent Cox regression model. Significance was implied at $p < 0.05$. The SPSS 17 statistical software package was used.

Results

Baseline characteristics

The baseline characteristics of our patients are summarized in Table 1. The median duration of follow-up was 6.4 years (range: 1-21). We stratified our study population into 5 groups based on transplantation year (1986-1990; 1991-1995; 1996-2000; 2001-2005; 2006-2009). We compared the mean age and weight in the 5 groups using a one-way ANOVA. We observed a difference in age and weight between groups: OLT recipients in the last cohort were significantly older (p<0.001) and heavier (p=0.015) compared to the rest. Using the Chi-squared test for trend we saw a linear association between diabetes prevalence and the year of transplantation; the prevalence of diabetes in OLT recipients increased significantly (p<0.001). We used the Chi-squared test to determine whether the prevalence of liver disease cause (primary and secondary) differed in the 5 groups. We observed a significant increase in the prevalence of hepatocellular carcinoma (p=0.018) and alcoholic cirrhosis (p=0.017).

Renal function after OLT: Mean GFR of the groups

The mean cGFR of the entire group (n = 391) was 76 mL/min at 3 months and 53 mL/min at 15 years after OLT, a decline of 30.5%. In the healthy population, a GFR decline of 0.4 mL/min/year [13] after the age of 30 is considered normal. We noticed a decline in mean cGFR of 1.6 mL/min/year in our group (Fig 1).

Incidence of chronic renal failure

During follow-up, chronic renal failure – defined as CKD-III – developed in 168 patients (43%) (Fig 2). Using Kaplan-Meier methodology, the one-year cumulative incidence (CI) of CKD-III was 29.7 ± 2.3%, and the five-year CI was 41.1 ± 2.6% in our entire group (Fig 3). At 0.54 years after OLT, 25% developed CKD-III; 50% developed it 10 years after OLT.

Of the 168 patients diagnosed with CKD-III, 25 improved with a cGFR>60 mL/min for more than one year. Of these 25 patients, 6 suffered a relapse of CKD-III. In total, 19 of the 168 patients (11.4%) had a cGFR above 60 for more than one year, and were not diagnosed with CKD-III by the end of this study.

The log-rank test revealed a statistically significant difference between the group of patients treated primarily with cyclosporine and those treated with tacrolimus (Fig 4).

The log-rank test also showed a statistically significant difference between the 5 groups: patients transplanted after 1996 were found to have a significant lower prevalence of CKD-III ($p<0.026$) compared to those transplanted before 1996 (Fig 5).

Patients who require renal replacement therapy

In our analysis, we used cGFR as the primary end point and renal failure (the need for renal replacement therapy) as the secondary end point. CKD-IV developed in 18 patients (4.6%). The cumulative incidence was 7% ± 2.1% after 10 years and 15.8% ± 4.2% after 13 years.

Of the 18 patients diagnosed with CKD-IV, 11 developed ESDR, requiring either dialysis or kidney transplantation. Of these 11 patients, to date 5 have received kidney transplantation, 3 remained on dialysis and 3 have died. Similarly, using the Kaplan-Meier method, the cumulative incidence of ESDR was 4.3% ± 1.7% after 11 years and 11.1% ± 4.3% after 15 years.

Table 2 - Immunosuppressive regimens, 3 months after OLT. N (%)

	Tacrolimus	Cyclosporine	CNI-free
1986-1990	0	24 (92.3)	2 (7.7)
1991-1995	0	55 (98.2)	1 (1.8)
1996-2000	45 (42.5)	61 (57.5)	0
2001-2005	106 (86.2)	15 (12.2)	2 (1.6)
2006-2009	43 (53.8)	32 (40.0)	5 (6.3)
Total	194 (49.6)	187 (47.8)	10 (2.6)

Immunosuppressant regimens

Three months after OLT, 194 patients (49.6 %) received tacrolimus-based regimens and 187 (47.8%) received cyclosporine-based regimens. A third group, which consisted of 10 (2.6%) patients, did not receive any form of CNIs as part of their immunosuppressive regimens (Table 2). Using the unpaired t-test, we determined that there was no significant difference in age and weight between the cyclosporine and tacrolimus group. Similarly, using the Chi-square test, we found no significant differences in gender, indication of OLT, HCV serology, diabetes and re-transplantation between these two groups.

Risk factors for CRF

Multivariate Cox analysis indicated that an increased risk of CRF was associated with a number of variables, including the patient's sex, age, the use of cyclosporine compared to tacrolimus and the presence of diabetes mellitus and positive HCV serology. For each stage of CRF, a separate analysis was used with the same co-variants (Table 3).

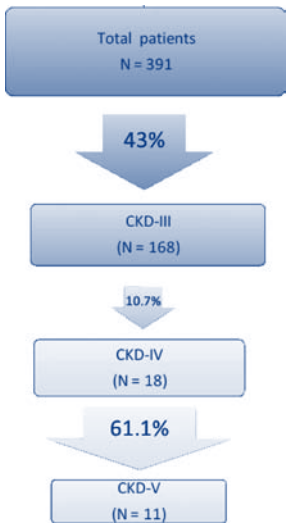

Figure 2
Overview of chronic kidney disease after OLT.

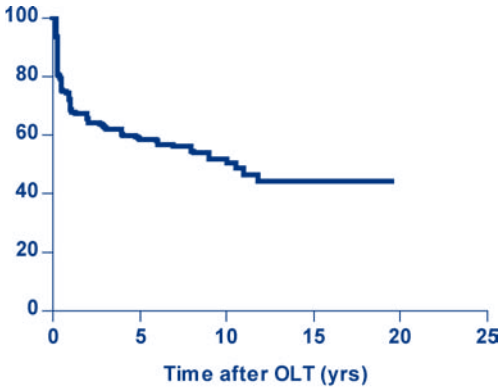

Figure 3
Cumulative incidence of CKD-III in 391 OLT patients between 1986 and 2008.

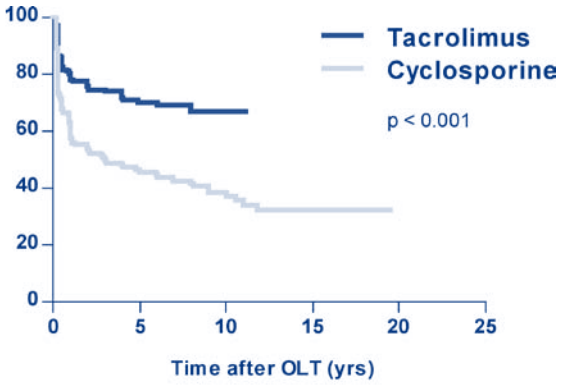

Figure 4
Cumulative incidence of CKD-III in 391 OLT patients between 1986 and 2008, based on immunosuppressive regimens received 3 months after OLT.

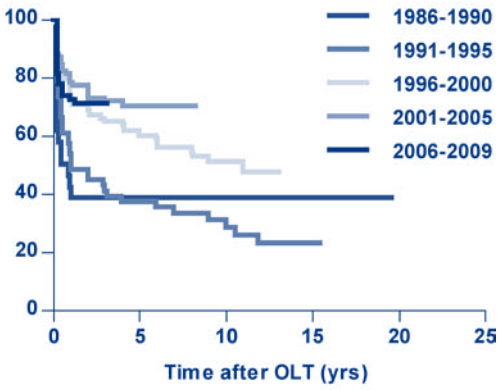

Figure 5
Cumulative incidence of CKD-III in 391 OLT patients between 1986 and 2008, based on transplantation year. Significant difference was seen between the first two and the last 3 cohorts ($p<0.026$).

Table 3 - Significant risk factors associated with Chronic renal failure.

Variable	Hazard Ratio (95% CI)	P Value	CKD stage
Age (10-year increment)	1.57 (1.40-1.74)	<0.001	CKD-III
Treatment based on			
Tacrolimus	1.00 (reference)		
Cyclosporine	2.09 (1.50-2.89)	<0.001	CKD-III
CNI-free	0.95 (0.23-3.93)	0.95	
Male sex	1.56 (1.14-2.14)	0.006	CKD-III
	0.29 (0.09-0.93)	0.038	CKD-IV
Pre-existing diabetes mellitus	4.16 (1.28-13.51)	0.018	CKD-IV
	5.76 (1.26-19.73)	0.024	CKD-V
Positive for hepatitis C antibody	5.04 (1.12-22.45)	0.034	CKD-V

Risk of death after CRF

The risk of death associated with the onset of CRF was evaluated with a time-dependant Cox regression model. Compared to patients that did not develop these disorders, the hazard ratio of death was 0.95 (95% CI: 0.56-1.61; p=0.84) for patients that developed CKD-III, 2.17 (95% CI: 0.87-5.38;p=0.096) for patients that developed CKD-IV and 2.37 (95% CI: 0.71-7.74; p=0.17) for patients that developed ESRD. The hazard of death was corrected for age, sex and diabetes.

Discussion

In this retrospective study, we have shown that chronic renal failure is common in liver transplant recipients, and we have identified potential risk factors other than calcineurin inhibitors.

In our study, renal failure developed in 43% of our population (168/391). The risk of moderate renal failure – defined as Chronic Kidney Disease III – was 25% 6 months after OLT and 50% 10 years after OLT. Most patients who develop renal failure do so within one year after OLT. This study probably underestimated the risk of renal failure because we excluded patients who survived fewer than 12 months from our analysis. Due to our long-term aims, we did not take into account the pre-transplantation renal function. We believe that the renal function before transplantation, if analyzed, could partly explain the rapid onset of renal failure after OLT.

There are many factors that can contribute to renal failure. Wilkinson & Cohen [21] found that the complications arising from the transplantation procedure itself may play a decisive role in the development of kidney failure. Furthermore, disease within organ-specific transplants, such as hepatitis C infection, can be associated with various glomerulonephritides [22]. According to our study, significant risk factors for the development of renal failure after OLT were sex, age, the immunosuppresive regimens, the presence of diabetes mellitus and positive HCV-serology. However, all these risk factors were previously documented in previous studies as possible risk factors for the development of renal failure after OLT [6, 21, 22].

Our study also showed that 11.4% of patients who are diagnosed with moderate renal failure – classified as CKD-III – will retain long-term normal renal function. This is largely due to alterations in therapy that occur after moderate renal failure is diagnosed. Of the 168 patients who developed moderate renal failure, 18 advanced to severe renal failure – classified as CKD-IV – with a cumulative incidence of 5% 10 years after OLT. A high percentage of patients with severe renal failure develop end stage renal failure – defined as CKD-V. Of patients having a cGFR below 29 mL/min, 61% needed permanent renal replacement therapy. In our analysis, the consequence of being diagnosed with moderate renal failure (CKD-III) was mild; only 10.7% of patients diagnosed with moderate renal failure advanced to severe renal failure (CKD-IV). However, the consequence of being diagnosed with severe renal failure was serious: 61% of the patients needed permanent replacement therapy. The risk of death after CKD-IV and CKD-V increased by 2.17 (p=0.096) and 2.37 (p=0.17). Due to the limited number of cases we were unable to determine significance.

In addition, our study showed that the incidence of renal failure was significantly lower in patients that were transplanted after 1996. This drop in incidence can be explained by various changes that occurred around that time. For example, the immunosuppressive regimens changed with the introduction of tacrolimus and triple therapy. Also, because the clinicians were more experienced with using immunosuppressants, they were able to minimize the dosage, limiting the nephrotoxic effect and improving survival at the same time. We believe it is unlikely that the drop in incidence of renal failure can be attributed to a single change. It is more likely that this effect is due to a combination of changes. Furthermore, based on transplantation year, our patient characteristics showed that the average OLT patient was becoming older, more obese and had a higher prevalence of diabetes. Despite the fact that the average OLT patient had more co-morbidities, improved renal function and survival were observed.

Previous studies [6, 8, 14-19] have shown that renal failure is a common problem after OLT. The largest of these studies was performed by Ojo et al. in 2003 [6]. They found a cumulative incidence of 27% 10 years after OLT, while our study found 5%. To explain this disparity, we first compared patient groups. Patient characteristics showed that our patients weighed more, had a higher prevalence of diabetes and had a lower prevalence of positive HCV serology compared to the patients in Ojo el al. This difference, however, was not enough to explain the disparity in the incidence of renal failure. One difference that could explain the disparity in incidence is the CNI levels that clinicians maintain. In our center, clinicians maintain CNI levels that are roughly half the levels maintained in American centers (data not shown). This difference in maintenance levels of CNIs could partly explain the lower incidence of renal failure that we found in our study compared to Ojo et al.

The risk of renal failure in our study was significantly higher for OLT recipients who were primarily treated with cyclosporine compared to those who were treated with tacrolimus (Fig 4). Ojo et al. [6] reported a relative risk of 1.24 between cyclosporine and tacrolimus (p<0.001), while we found a hazard ratio of 2.08 (p<0.001). This disparity can be largely attributed to the fact that we only analyzed the primary immunosuppressive regimens and not the secondary or tertiary ones. Secondary treatments included corticosteroids, azathioprine, mycophenolate mofetil (MMF) and sirolimus, which were introduced at various points during our study period. These secondary and tertiary regimens could possibly play a role in the incidence of renal failure, but were left out of our analysis. Furthermore, higher nephrotoxicity was expected in the early years of OLTs, because the primary focus then was on preventing acute rejection. Due to lack of experience, the only CNI available at that time, cyclosporine, was given at higher dosages.

In previous studies, tacrolimus and cyclosporine have also been compared as primary immunosuppressant regimens after transplantations, but with conflicting results. For example, Martins et al. [26] and Luceya et al. [27] found that tacrolimus was associated with better renal function, while Levy et al.

[28] did not. The debate on tacrolimus versus cyclosporine is far from being settled. In our opinion, tacrolimus is as good as cyclosporine, if not marginally better. However, given the choice between the two drugs, we would prefer tacrolimus above cyclosporine because of patient friendliness. Tacrolimus, compared to cyclosporine, is easier to digest, results in less vomiting, and the blood level is easier to maintain.
Currently, there is a strong focus on limiting the nephrotoxic effects of CNIs. Several new strategies have been introduced in immunosuppressive regimens, including CNI minimization, complete CNI avoidance and CNI withdrawal [7]. Presently, for all solid organ transplants, CNI withdrawal appears to be the best option [29-38]. Neuberger et al. conducted the "Respect" study, where they focused on CNI minimization [39]. They found that reduced dose and delayed tacrolimus regimens led to improved renal function compared to standard-dose tacrolimus regimens. Unfortunately, a high CNI blood level was maintained in their standard-dose group, resulting in high nephrotoxicity as reference. This partly explains the improved renal function they reported.
In conclusion, this study has shown that with the current 90% one-year survival, the challenge of liver transplantation now lies in optimizing the long-term outcome after OLT. One of the limiting factors, CNI-induced nephrotoxicity, has already been reduced thanks to new drugs, treatments and clinical experience. To further improve the long-term outcome after OLT, better and less nephrotoxic immunosuppressive strategies are needed. Clinicians should not be so concerned with acute rejection in OLT recipients, the primary reason for immunosuppression, and should focus instead on methods that not only prevent short-term rejection, but also preserve the long-term renal function.

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Article

The essential amino acid requirement in preterm neonates:

The tracer wash-out study

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Background: **For optimal growth and development, preterm and term neonates need nutrition containing 9 essential amino acids. If these amino acids are not administered in right proportions in the diet, protein synthesis will be reduced, which can have serious consequences. However, the exact requirements for preterm and term neonates is unknown, which means that there are no optimum nutrition formulas. To determine the essential amino acid requirements, stable isotope techniques are used. To prevent accumulation of the tracer, most of the studies wait at least 2 days before starting the next tracer study with the same subject. The time needed before the tracer has left the body is called the tracer wash-out time.**
Objective: **Little is known about the exact tracer wash-out time of the tracers used in the indicator amino acid oxidation method (IAAO method) in preterm infants. In our study, we addressed the following research questions. (1) What is the exact tracer wash-out time? (2) What is the adaptation time to the change of amino acid intake? The latter question will be addressed in a subsequent article.**
Methods: **All the included subjects were preterm infants with a gestational age between 30 - 37 weeks, a birth weight < 2200g and fully enteral feeding. The subjects were adapted to the formula for 5 days, and tracer studies were performed on day 0, 1, 3 and day 5. Directly after the [1-^{13}C]phenylalanine on the tracer study day was stopped, duplicated breath samples for the tracer wash-out study were collected during the next 24 hours by using the nasopharyngeal sampling method. The wash-out time was defined as when the slope of the graph was not significantly different from zero ($P < 0.05$).**
Results: **In total 9 subjects were included in this study; in 4 of these subjects, 2 tracer wash-out studies were performed on different adaptation days. The overall mean time of the slope was 13.1 hours. The maximum tracer wash-out time was 18 hours, which was shorter than the intervals used in previous tracer studies.**
Conclusion: **A tracer wash-out time of 18 hours is sufficient before starting the next measurement on the same subject. This study confirms that it is possible to measure the same subject on two consecutive days. This prevents unnecessary delay in the subsequent measurement, thusly avoiding longer exposure of the subjects to a deficient diet, which was the case in previous studies.**

Introduction

For optimal growth and development of preterm and term neonates, nutrition containing 9 essential amino acids is needed. If these amino acids are not administered in right proportions in the diet, protein synthesis will be reduced, and this will have serious consequences on 1) later cognitive function, 2) blood pressure and 3) later risk of obesity. However, the exact requirements for essential amino acids in term and preterm neonates are unknown. This means that there are no optimum nutrition formulas for preterm and term neonates. By using the indicator amino acid oxidation method (IAAO), it is possible to determine the exact individual requirement for all essential amino acids.
The IAAO method is based on a stable isotope technique. A labelled indicator (labelled with ^{13}C, a safe, non radioactive isotope) with an oxidative pathway different from and unrelated to the test amino acid is used. The main principle is that because there is no storage of free amino acids, a deficiency of one essential amino acid will limit protein synthesis. If the tested amino acid is deficient in the diet, this will limit protein synthesis, and the indicator amino acid labelled with ^{13}C will be oxidized at a high rate. This can be detected from the concentration of $^{13}CO_2$ in expiratory air. If the dietary intake of the test amino acid increases, oxidation of the indicator will decrease until the requirement for the test amino acid is met. When the protein intake meets the requirement, protein synthesis will occur at optimum capacity and the oxidative degradation of all other essential amino acids will reach a plateau. The requirement for the test amino acid will then be identified by this breakpoint (Figure 1) (4). In our study, we used [1-^{13}C] phenylalanine as the indicator, which is the most commonly used tracer (5).
If small amounts of the tracer remain in the body at start of the next measurement with the same tracer, then this affects the outcome, due to increased recovery of labeled $^{13}CO_2$. This is called the carry-over effect. To prevent accumulation of the tracer, most studies wait at least 2 days before starting the next tracer study with the same subject (6-14). The tracer wash-out time is the time needed for the detectable amount of $^{13}CO_2$ to reach the background level in breath, blood and urine.
Many factors can affect background $^{13}CO_2$ enrichment, and one of these is nutrition (15).

Objective

Little is known about the exact tracer wash-out time of the tracers used in the IAAO method in preterm infants. In our study, we addressed the following research questions.

(1) What is the exact tracer wash-out time? If we can determine the tracer wash-out time, and thereby the maximum time needed before starting the next tracer study with the same subject, then it is possible to prevent a carry-over effect. This can also prevent unnecessary delay in the subsequent measurement, and thus avoid longer exposure of the subjects to a deficient diet. Our hypothesis is that the tracer wash-out time is actually shorter than that considered in several previous studies (6-14).

(2) What is the adaptation time to the change of amino acid intake? This is the time needed to adapt to the study formula in preterm neonates by using the IAAO method. The adaptation time to the change of amino acid intake in a preterm neonate is still unknown. If we can determine the exact adaptation time, then the exposure to a deficient diet will be shorter and the study will thus be less invasive. This question will be addressed in a subsequent article.

Methods

Inclusion and exclusion criteria:

The criteria used to compile the study population are listed in Table 1. The subjects also participated in the above-mentioned adaptation study.

Table 1. The inclusion and exclusion criteria of the adaptation and the tracer wash-out study.

Inclusion criteria	Exclusion criteria
• Preterm infants with a gestational age of 30-37 weeks and a birth weight < 2200g	• Congenital anomalies
• weight gain rate > 10 g/kg/d in preceding 5 days	• Sepsis
• On full enteral feeding	• Gastro-intestinal pathology
	• No informed consent from the parents

Study diet

The formula used during the study contained either leucine 166 mg/kg/d or 252 mg/kg/d, which is below the current recommendations (16). We mixed the original neocate with the Infant Formula Minus leucine (both produced by SHS/Numico) to obtain different leucine concentrations. Neocate and the Infant Formula Minus leucine are elemental diets containing free amino acids.

Before starting on the study formula, subjects were fed with Chinese formulas of various brands. The study formula was given during the adaptation study. The subjects were fed according to the feeding schedule of the hospital, i.e. every 2 hours.

During the adaptation study, all subjects received 170 ml/kg/d, 135 kcal/kg/d and a protein intake of 3.7 g/kg/d, according to the current recommendations for the preterm infant (16).

Study design

The tracer wash-out study was performed on the subjects who participated in the adaptation study.

All the subjects who participated in the adaptation study were adapted to the study formula during 5 days and 8 hours on the 6th study day. Tracer studies were performed on adaptation day 0 (A0) directly after starting the study formula, and on adaptation day 1 (A1), adaptation day 3 (A3) and adaptation day 5

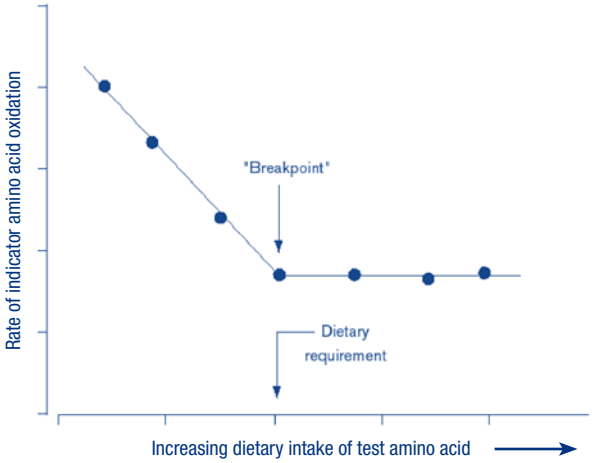

Figure 1
Oxidation rate of the indicator amino acid at different intakes of the test amino acid.

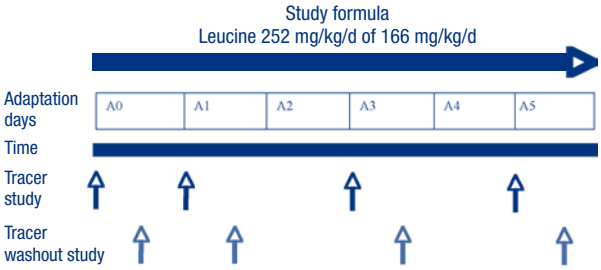

Figure 2
Study design.

(A5). On the tracer study day, the subjects received an enteral, primed (10 µmol/(kg·min) continuous (10 µmol/(kg·h)) [^{13}C] natriumbicarbonate infusion during the first 2.5 hours for quantifying the individual CO_2 production (17), followed by an enteral, primed (30 µmol/(kg·min)) continuous (30 µmol/(kg·h)) [1-^{13}C]phenylalanine infusion for 5 hours.

Directly after the [1-^{13}C]phenylalanine of the tracer study day was stopped, duplicated breath samples were collected at T0 and subsequently every hour during the first 9 hours of our tracer wash-out study. During the following 6 hours we collected samples every 2 hours and then every 3 hours during the next 9 hours until we measured the full 24 hours. For subjects who were not participating in a subsequent tracer study the following day and when logistically possible, samples were taken for longer than 24 hours. To collect the breath samples we used the same nasogastric tube which was used in the adaptation study. This tube was placed into the nasopharynx. From this tube we extracted breath with a 20-mL plastic syringe, injected the air into a 12-mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored it at room temperature until analysis. The nasopharyngeal sampling method used to obtain the breath samples in both this tracer wash-out study and the adaptation study has been validated by van der Schoor et al. (18).

The study design is shown in Figure 2. Our tracer wash-out study was performed on different adaptation days: A0, A1, A3 and A5. On 4 subjects, 2 tracer wash-out studies were performed on different adaptation days.

Table 2. Subject characteristics

Subject No:	1	2	3	4	5	6	7	8	9	Mean
Gender (M/F)	M	F	F	M	M	F	M	M	M	GA*
(in weeks)	33 2/7	32 1/7	30 3/7	30 5/7	29 2/7	29 1/7	32 6/7	31 4/7	31 4/7	31 2/7
Birthweight										
(in grams)	2080	1370	1900	1600	1425	1700	2050	1820	1570	1724
Weight on										
study day										
(in grams)	2105	1420	A0: 2540	1840	2210	2220	A3:2090	A1:1910	A1:1720	2050
			A5: 2720				A5: 2140	A3: 1975	A3: 1765	
Weight gain	10	12,2	17,8	12,2	22,9	22,5	13,5	15	16,6	15,9
rate (g/kg/d)										
GA on	36 1/7	35	A0:35 6/7	34 3/7	35 3/7	35 1/7	A3:34 4/7	A1:33 5/7	A1:33 5/7	34 6/7
study day			A5:36 4/7				A5:34 6/7	A3:34	A3:34	
(in weeks)										

Notes: *GA=gestational age, A=Adaptation day

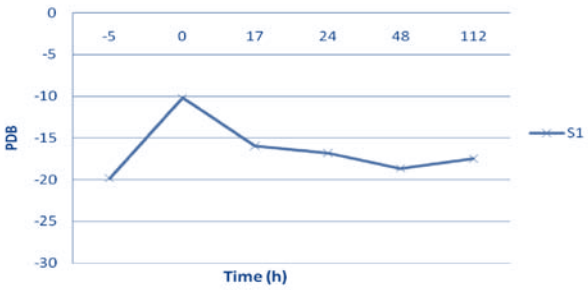

Figure 3
Adaptation day 0 (A0) compared to the background enrichment on A5.

Notes. S= Subject, A= Adaptation day, the point at time -5 presents the background enrichment of A0 before start of the study formula. The point in time 112 represents the background enrichment of A5 (of the study formula).

Analytical method

Breath samples were sent to the laboratory of the Erasmus MC- Sophia Children's Hospital in Rotterdam (the Netherlands) for analysis.

$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as PDB (Pee Dee Belemnite), which is the international standard for the $^{13}C/\ ^{12}C$ proportions in carbon.

The tracer wash-out times were recorded in an Excel file, after which they were plotted on a graph: PDB against time. The wash-out time was defined as when the slope of the graph was not significantly different from zero ($P < 0.05$).

Consent

Informed consent was obtained from at least one of the parents/caregivers. The parents were told that they could refuse to take part or could withdraw from the study at any time for any reason if they wished to do so and without any consequences. The study was approved by both the Institutional Review Boards at the Children's Hospital of Fudan University Shanghai and at the Sophia Children's Hospital, Erasmus Medical Centre Rotterdam.

Results

Nine subjects were included, of which 4 were adapted to leucine 166 mg/kg/day and 5 were adapted to 252 mg/kg/day. In 4 subjects, 2 tracer wash-out studies were performed on different adaptation days. In total 13 tracer wash-out studies were performed: 5 studies on A0, 2 studies on A1, 3 studies on A3 and 3 studies on A5. Subject characteristics are shown in table 2. The mean gestational age was 31 2/7 weeks and the mean birth weight was 1724g.

Subject 1 is described separately, due to the fact that the breath samples were only collected at T0, T17, T24 and T48 (Figure 2) to determine when $^{13}CO_2$ had reached the background enrichment of the study formula. The tracer study on this subject was only performed on A0 and A5. T-5 represents the background enrichment before start of the study formula, and the background enrichment of the study formula measured on day 5 is represented by T112. As shown in Figure 3, after 17 hours the PDB did not significantly decrease compared to T112, with a slope of 0.012 ($p<0.05$). The background enrichment of the study formula appeared to be different than that of the study formula.

The results of the other 8 subjects in our study are shown in Figures 4-6. T-5 represents the background enrichment before starting the study formula (or the background enrichment of the study formula). T0 is the first sample taken after stopping the tracer [1-^{13}C]phenylalanine infusion.

As shown in Figure 4, the slope is not significantly different from zero ($p<0.05$) at 15 hours for subject 2, at 9 hours for subject 3, at 18 hours for subject 4 and at 8 hours for subject 5. In 3 subjects, the background enrichment of the study formula was reached. Subject 5 did not reach the background enrichment of the study formula within 24 hours.

Figure 5 shows that the slope was not significantly different from zero ($p<0.05$) at 11 hours for subject 3, and at 8 hours for subject 6. For subject 7 the slope was not significantly different from zero between T11 and T21, but a further decrease was seen after T21. The background enrichment of the Chinese formula (A0) was not reached within 24 hours in subject 3, while the subjects were again receiving the Chinese formula. In subject 6, the background enrichment of the Chinese formula was reached within 24 hours, and in subject 7 within 48 hours. However as can be seen on the graph the background enrichment in subject 6 changed to a lower background enrichment. On A1 and A3, the subjects received the study formula before, during and after the tracer wash-out study.

As shown in Figure 6, on A1, the slope was not significantly different from zero ($p<0.05$) at 15 hours for subject 8 and at 15 hours for subject 9.

On A3, the slope was not significantly different from zero ($p<0.05$) at 14 hours for subject 7, at 18 hours for subject 8 and at 12 hours for subject 9.

In Table 3, the time of the slopes ($p<0.05$) in hours for each adaptation day are shown separately .

Tabel 3. Timetable (in hours) of the slopes and their size.

Subject No:	1	2	3	4	5	6	7	8	9
Adaptation day 0	By 15h	By 9h	By 18h	By 8h					
	0.044	0.046	0.030	0.039					
Adaptation day 1								By 15h	By 15h
								0.012	0.030
Adaptation day 3							By 14h	By 18h	By 12h
							0.010	0.003	0.033
Adaptation day 5			By 11h			By 8 h	By 12h		
			0.010			0.043	0.010		
Time of the slope (in hours)		12.5 hours			15 hours		14.7 hours		10.3 hours 13.1 hours

Notes: *GA=gestational age, A=Adaptation day

The overall mean time of the slope ($p<0.05$) is 13.1 hours. The maximum tracer wash-out time found was 18 hours, which is shorter than the intervals used in previous tracer studies. (Table 3)

Discussion

Our results suggest that the maximum tracer wash-out time is 18 hours, which is shorter than the times described in previous tracer studies.

A total of 9 subjects were included in our study. As shown in the results, for all the subjects a slope not significantly different from zero ($p<0.05$) was found, with a mean of 13.1 hours and a maximum of 18 hours. This confirms that there was no accumulation of the tracer and therefore no influence from the carry-over effect during all the adaptation and tracer wash-out study days. Background 13C enrichment depends on many factors, such as nutrition. This can be seen in the results, where the background enrichment of the Chinese formula appeared to be different than that of the study formula. Since subjects measured on A1 and A3 received the study formula before and during the tracer wash-out study, these results are more reliable.

To our knowledge, no studies have been done to determine the exact tracer wash-out time of the tracers used in the IAAO studies. In 1990, Zello et al. used a tracer wash-out time of 2 days, which resulted in a latency of at least 3 days before starting the next tracer study in the same subject (6). Kriengsinyos et al. used a latency of 2 days between the measurements to avoid the possibility of the carry-over effect (9). Riazi and Rafii studied their subjects with a 5-day to 7-day interval period between study days. But in their previous studies they assumed that the carry-over effect of the tracer would not affect the background enrichment after 2 days and therefore, the minimum interval between the studies could be 3 days (10). Turner et al. separated their study days by at least one week, and they completed 6 studies in a 2-month period (11). Darling et al. used an interval of 48 hours between the two 24-hour periods of tracer infusions in their 4-day balance study (13). This interval was used in relation to a previous study of the threonine kinetic in preterm neonates (14). All these studies also used this interval to avoid the effect of long-enduring deficient diet on the results of their protein requirement studies.

There are several ways to determine the tracer wash-out time, one of them being the analysis of amino acid enrichment in the urine. Urine collection, the sampling of the free amino acid pool, has been validated in several studies that all used infusions of amino acid tracers for the comparison of the isotopic

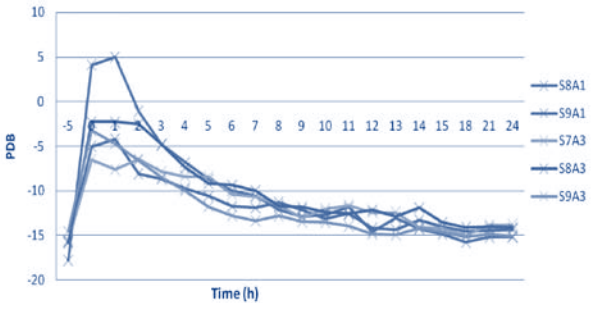

Figure 4
Adaptation day 0 (A0) compared to the background enrichment of A5.

Notes. S= Subject, A= Adaptation day, the point at time -5 presents the background enrichment of A0 before start of the study formula. The single points represents the background enrichment of A5 (of the study formula) for each subject.

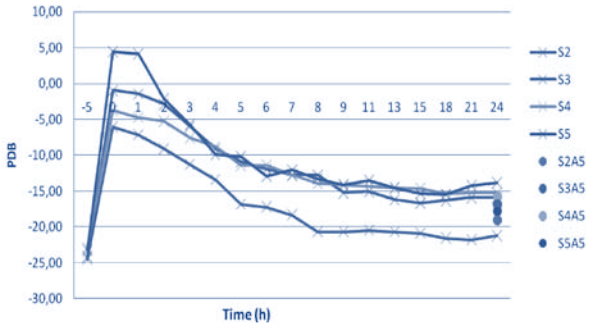

Figure 5
Adaptation day 5 (A5) compared to the background enrichment of A0.

Notes. S= Subject, A= Adaptation day, the points at time -5 presents the background enrichment of A5 (of the study formula). The single points represents the background enrichment of adaptation day 0 before start of the study formula for each subject.

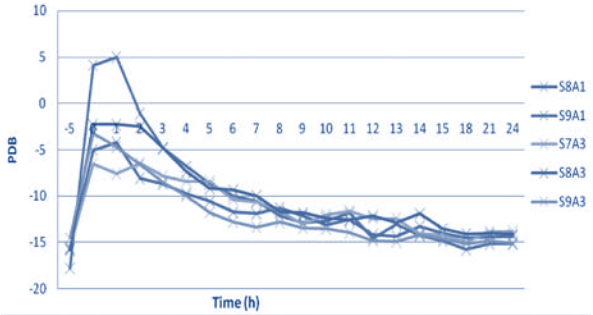

Figure 6
Adaptation day 1 (A1) and A3.

Notes. S=Subject, A= Adaptation day, the points at time -5 represents the background enrichments of A0 and A3 for each subject.

enrichment in the plasma and urine (12,19-21).
In many studies, $^{13}CO_2$ in breath during the infusion of a [^{13}C] bicarbonate-tracer was measured to calculate the CO2 production (22-24). The breath samples are collected by using a closely fitted facemask and an inlet-outlet system with a low dead volume. In addition, the collected air has to pass through a glass spiral condenser filled with sodium hydroxide. After liberating the CO2 pt, the CO_2 can be stored in a septum-capped tube until analysis. However a disadvantage of this technique is that a fraction of the $^{13}CO_2$ can be lost due to the many procedures, resulting in underestimation of substrate oxidation.
In our study, we used the IAAO method in combination with the nasopharyngeal sampling method and [1-^{13}C]phenylalanine as the tracer. The IAAO method had been made minimally invasive by Bross et al. (20); they used a shorter adaptation time and took breath and urine samples instead of plasma. In combination with the nasopharyngeal sampling method, this consequently became the most appropriate method for determining amino acid requirements, tracer wash-out time and adaptation time in vulnerable groups such as preterm and term neonates, children and pregnant women.
A disadvantage of our study is that the subjects were not measured on the same tracer study days. Some were measured on days 0, 1 and 3, when the study formula was continued, and some on day 5, after the study formula was stopped. Therefore, it was difficult to compare these subjects, because their own formula and the study formula had different enrichments. However in our study, subjects measured on day 1 and day 3 had no confounding factors such as the change of formula. For future studies, to make a better comparison between the subjects it will be important to measure subjects on the same adaptation day with the same study formula.

Conclusion

We conclude that in preterm neonates, a tracer wash-out time of maximum 18 hours is sufficient before starting the next measurement. The results of this study confirm that it is possible to measure the same subject on two consecutive days, which prevents unnecessary delays in starting the next study day and thus avoids longer exposure of the subjects to a deficient diet, compared to previous studies.

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Age and Stroke Severity as risk factors

Symptomatic Intra-Cerebral Hemorrhage in Ischemic Stroke Patients Treated with Intra-Arterial Thrombolysis

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Background and purpose: **Intra-arterial thrombolysis is a promising treatment for acute ischemic stroke . The risk of hemorrhage, however, is substantial. This review identifies risk factors for intra-cerebral hemorrhage after intra-arterial thrombolysis for acute stroke patients.**
Methods: **We searched the PubMed database for related articles. All risk factors were summarized and compared. We focused especially on the risk factors age and NIHSS score (National Institutes of Health Stroke Scale).**
Results: **Most large studies found that a high NIHSS score was a strong risk factor.**
Conclusion: **Our study indicates that that age is not a risk factor for symptomatic intra-cerebral hemorrhage (sICH) for acute stroke patients after intra-arterial thrombolysis. This conclusion is important for clinical practice because physicians are reluctant to treat elderly patients with intra-arterial thrombolysis; our results may help to change this clinical practice.**

Keywords
Intra-arterial thrombolysis • Intra-cranial hemorrhage • Risk Factors • Stroke • symptomatic hemorrhage

Introduction
Stroke victims rapidly loose localized brain functions due to ischemia caused by thrombosis or embolism, or due to a hemorrhage. Intra-arterial thrombolysis is often used for acute ischemic stroke therapy, and it can be effective, efficient and safe([1]). Intra-arterial thrombolysis results in rapid recanalization. Whether it improves patient function neurological outcome has not been definitively proven. None of the articles on this topic summarized the major risk factors for sICH after intra-arterial thrombolysis in acute stroke patients. sICH occurs in only some patients (8% - 15%) treated with intra-arterial thrombolysis. Therefore, individual risk factors probably play a role. Treatment decisions depend on the balance between individual risk of hemorrhage and the expected benefit of the treatment. Therefore, it is important to assess the role of risk factors for sICH. There are many potential risk factors regarding intra-cerebral hemorrhage after intra-arterial thrombolysis, see Table 2. These include age, diastolic blood pressure, the use of antiplatelet therapy and the NIHSS score. But so far it is unknown how these factors influence the risk for sICH. We found that that age and NIHSS score were the most common risk factors cited in the articles reviewed, so we analyzed these two risk factors.
The risk factors for sICH have never been summarized. The definitions of sICH differ, but its main characteristics are neurological deterioration and a visible hemorrhage on the CT-scan. If the risk factors for sICH after intra-arterial thrombolysis can be determined, clinicians can screen patients before treatment and thereby decrease the risk of subsequent sICH complications.
In this review, we addressed the following research question.
What are the risk factors leading to sICH in stroke patients after intra-arterial thrombolysis?
To answer this question we performed a systematic review.

Table 1. Search query.

Search Query	Results
stroke	144156
(Thrombolysis OR Thrombolytic	
OR Tissue Plasminogen Activator OR tPA)	10167
(haemorrhage OR hemorrhage)	2181
(intracerebral OR intracranial)	1103
symptomatic	349
(outcome OR outcomes OR risk OR risks)	325
limit: published in the last 10 years	302
limit: English	286

Methods
The National Library of Medicine's PubMed database was searched on June 14th 2009 with multiple search queries. The first search returned 144,156 results. After limiting the search query several times, PubMed returned 286 articles. Many articles were excluded based on their title or abstract. For example, articles considering specific or rare patient groups were immediately excluded. Also, articles about intra-venous therapies were excluded. Finally, after evaluating the content of 11 articles, 6 articles were considered useful for this review. See Table 1 for the detailed search queries and the returned number of results. No MeSH terms were used; see the discussion for further

Table 2 - Risk Factors Overview.

Risk Factor	n	Brekenfeld	Ernst	Lansberg	Shaltoni	Singer	Wechsler	Neumann
Age	6	+		+	+	+	+	+
NIHSS Score	5	+		+	+	+	+	
DWI Volume	2			+		+		
Time to Treatment	1					+		
Medication Kind	2					+		+
Sex	1				+			
Hypertension	1						+	
Cerebrovascular Disease	1						+	
Diabetes	1						+	
Hypodensity CT	1						+	
PWI Lesion Volume	1			+				
Diastolic Blood Pressure	1	+						
Poor Collaterals	1	+						
High Urokinase Dose	1	+						
Signs on CT	1	+						
Early Reperfusion	3	+	+	+				
Leukoaraiosis	1							+
Severe ischemia	1							+
Blood-brain barrier damage	1							+
Cerebral microcirculation damage	1							+
Experience	1						+	
High dose thrombolytic therapy	1					+		

explanation. One additional article was found by checking the reference lists in the 6 selected articles, making a total of 7 articles. All risk factors were collected and placed in a table with more details.

Results

The investigated risk factors for sICH after intra-arterial thrombolysis are summarized in Table 2. Age, NIHSS score and early reperfusion were the only risk factors reported three or more times. Only the articles of Brekenfeld et al, Shaltoni et al, Singer et al, Wechsler et al. and Neuman et al. cited age as a risk factor.

Age as a risk factor

Brekenfield et al. studied 294 patients who were treated with intra-arterial urokinase. sICH occurred in 14 of these 294 patients (4.8%). This study did not conclude that age is a significant risk factor for sICH. See Table 3.

Shaltoni et al. investigated 69 patients. IV-rt-PA was given first, followed by IAT. sICH occurred in 4 of 69 patients (5.8%). This study was unable to conclude that age is a significant risk factor for sICH (P=0.063).

Singer et al. analyzed 645 patients. These patients were treated with either IV or IA thrombolysis. Of this group, 44 patients (6.8%) developed sICH. This study did not show age to be a significant risk factor sICH either. See Table 3.

Neuman et al. studied 449 patients. Of this group, 363 patients were given intravenous tPA treatment and 86 patients were given intra-arterial or combined intravenous/intra-arterial treatment with either tPA or urokinase. Hemorrhage occurred in 25 of the 449 patients. This study reported that the risk of sICH after thrombolysis did not necessarily increase in very elderly patients. They stated only that age is a known risk factor for Leukoariosis, which increases the risk for a sICH.

Lansberg et al. investigated 74 patients who were treated with intravenous tPA. sICH occurred in 7 of 74 patients (9.5%). This study did not find any association between age and sICH. See Table 3.

The study of Wechsler et al. included 180 patients. This study did not mention any relationship between sICH and age as a risk factor. Nevertheless, it showed that age above 68 years is a significant risk factor for good recovery (based on the Modified Ranking Scale Score).

NIHSS score

As shown in Table 2, all articles except Neuman et al. and Ernst et al. indicated NIHSS score as a risk factor. The results related to NIHSS score from these articles are summarized below.

The patients in Brekenfield et al. had a mean NIHSS sore of 16 in the sCIH group and 15 in the no-sICH group. These researchers were unable to determine if the NIHSS score is a significant risk factor for sICH. See Table 3.

Table 3 - Results Risk Factor

Study	Risk Factor									
	Age					NIHSS				
	sICH		No sICH		p-Value	sICH		No sICH		p-value
	N	Mean-age	N	Mean-age		N	Average NIHSS	N	Average NIHSS	
Brekenfield	14	63	280	60	0.338	14	16	280	15	0.507
Singer	44	71	601	69	0.11	44	17	601	13	0.003
Lansberg	7	78	67	70	0.21	7	17	67	12	0.02

The cohort of Lansberg et al. had a NIHSS average of 17 in the sICH group and 12 in the no-sICH group. This study concluded that the NIHSS score is a significant risk factor for sICH. See Table 3.

The population of the study of Shaltoni et al. had a mean NIHSS score of 18. This study did not conclude that the NIHSS score is a significant risk factor for sICH (P=0.789).

The study of Wechsler et al. concluded that the NIHSS score is a significant risk factor for good recovery (based on the Modified Ranking Scale Score).

Finally, in the study of Singer et al. the sICH group had a mean NIHSS score of 17 and the no-sICH group had a mean score of 13. This study concluded that the NIHSS score is a significant risk factor for sICH. See Table 3.

The results of this review are summarized in the tables below, see Tables 4 and 5.

Table 4 - Overview results for age as risk factor

Article	Number of Patients	Predictive value for sICH	p-value
Brekenfield	294	No	0.388
Shaltoni	96	No	0.063
Singer	645	No	0.110
Wechsler	180	Yes (dependent risk factor)	0.001
Neumann	449	No	Unknown
Lansberg	74	No	0.210

Table 5 - Overview results for NIHSS-score as risk factor

Article	Number of Patients	Predictive value for sICH	p-value
Brekenfield	294	No	0.507
Lansberg	74	Yes	0.020
Shaltoni	96	No	0.784
Wechsler	180	Yes	0.001
Singer	645	Yes	0.003

Discussion

From the above studies, we concluded that age cannot be considered a high-risk factor (Tabel 3).

In our review, the NIHSS score had a predictive value for symptomatic hemorrhages in three articles.([4,5,6]). These articles showed good significance and described the results for 899 patients. Brekenfield et al. and Shaltoni et al. mentioned that this score does not have a predictive value for sICH and concluded that the NIHSS score is not a significant risk factor ($p > 0.5$). In total, only 254 patients participated in these latter two studies, so it is still plausible to consider the NIHSS score as having a predictive value for sICH after intra-arterial thrombolysis. It is interesting to note that Wechsler et al. corrected the NIHSS score results for CT hypodensity. The NIHSS score results were therefore not affected by the CT hypodensity severity (dependent vs. independent risk factor).

The risk for sICH after intra-arterial thrombolysis, according to the reviewed articles, is 6.1 %. This percentage has been adjusted to the number of patients in every article (see Table 6).

Age does not seem to be a significant risk factor for symptomatic hemorrhages([1,3,4,5,6,7]). Only Wechsler et al. concluded that age is a possible risk factor. All articles that addressed this risk factor showed poor significance; only Wechsler et al. had a p-value less than 0.05. The number of patients in these studies varied. This does not influence the results, because all articles showed similar results.

Table 6 - Risk for sICH after IA thrombolysis

Article	N	sICH	No sICH	%
Brekenfeld	294	14	280	4.7
Ernst	20	1	19	5.0
Lansberg	74	7	67	9.5
Shaltoni	69	4	65	4.2
Singer	645	44	601	6.8
Neumann	449	25	424	5.6
	1551	95	1456	6.1

Our study has several limitations. First of all, it should be noted that some articles were excluded because of limited access privileges. Most relevant articles did not have any match in their MeSH terms. Instead of MeSH terms only, plain text words were used for the search queries. Despite this limitation, many articles could be used.

The associations between age and NIHSS were assessed on the basis of their p-value. Most articles did not contain enough data to compare odds-ratios and 95% CI. Therefore, our review can only indicate whether a clinical observation should be considered as a risk factor or not. It was not possible to estimate effect sizes.

The definition of intra-cerebral hemorrhage is broad. Our review therefore focused specifically on symptomatic intra-cerebral hemorrhage. There are also many ways to interpret the definition of sICH, which could cause problems such as determining mean frequencies, but this did not greatly affect the results of this review.

It should be noted that only two risk factors were analyzed. Obviously, many others remain to be investigated. In our opinion, these results cannot be applied to all patient groups – children, for example – because they were not discussed in the articles.

In conclusion, this review shows that the NIHSS score is a risk factor which can be useful for clinical practice. Furthermore, doctors are generally reluctant to treat elderly acute ischemic stroke patients with intra-arterial thrombolysis. Our review does not support this reluctance.

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Methods used to induce exon skipping in mdx mice:

A systematic review to determine the most promising technique for Duchenne Muscular Dystrophy

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Introduction. **Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy. In view of the etiology of DMD, genetic therapies could have great potential for treating DMD patients. We systematically reviewed experimental *mdx* mouse studies to determine which of the exon-skipping methods led to the most significant reduction in the progression of muscular dystrophy.** ***Methods.*** **We searched the bibliographic database Medline and article reference lists. We compared eligible articles according to various criteria and sorted the articles by the antisense oligonucleotide used.** ***Results.*** **Fourteen articles were identified. We identified significant differences in efficacy of the different antisense oligonucleotide compounds that were used.** ***Discussion.*** **The data suggest that Vivo-Morpholinos are the most promising treatment modality in reducing the progression of muscular dystrophy; this technique overcomes disadvantages of other antisense oligonucleotide compounds. Further research in this area is needed.**

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that affects 1 in 3,500 live male births. [1] Patients are susceptible to muscle damage because of the lack of functional dystrophin in muscle fibers. This results in an exhaustion of their regenerative capacity. [2] Due to respiratory and/or cardiac failure, DMD leads to wheelchair-dependence and death before the third decade of life [3]. DMD patients are treated with steroids and nocturnal mechanically assisted ventilation, but more effective therapy is required.[4] [5]

Defects in the dystrophin gene result in two forms of muscular dystrophy: DMD and Becker muscular dystrophy (BMD). DMD is characterized by nonsense mutations in the dystrophin gene, resulting in a total absence of dystrophin. [6] BMD, caused by an in-sense mutation, is clinically less severe owing to the presence of partially functional dystrophin. [7]

In view of the etiology of DMD, genetic therapies could have great potential for treating DMD patients. Transcriptional modification has been proposed as personalized gene therapy because the targets are specific mutations in the dystrophin gene. This type of therapy uses small molecules to prevent the incorporation of affected exons in dystrophin mRNA. This technique is called exon skipping; it generates an in-frame mRNA that encodes a shorter, but moderately functional dystrophin protein product, resembling the protein in BMD patients. [3]

Exon skipping could be a solution for most DMD patients. [8] The human dystrophin gene contains 79 exons and has over 2 million base pairs, encoding a 427 kDa protein consisting of 3,685 amino acids. The protein can be divided into four domains. About 75% of the DMD mutations occur within the so-called rod region. Skipping of the mutated exon or exons in the rod domain seems to be noncritical for the function of the dystrophin protein. Consequently, DMD patients with loss of essential coding domains will not be able to benefit from the technique of exon skipping. [9]

To induce an in-frame deletion at transcript level, the main method for skipping out-of-frame deletions or insertions is to use antisense oligonucleotides (AONs). There are several different AONs, which are structurally different at the molecular level. [1] These include synthetic AONs and antisense small nuclear RNAs. AONs are divided into 2'-O-methylated phosphorothioated antisense oligoribo-nucleotides (2OMeAO), phosphorothioate antisense oligodeoxynucleotides and phosphorodiamidate morpholino antisense oligonucleotides (PMO). [1]

Research in this field uses an animal model, the *mdx* mouse. These are mice with a genetic X-linked mutation, resulting in a lack of dystrophin. In our systematic review we addressed the following question. Which of the various compounds used to induce exon skipping in *mdx* mice best slows the progression of muscular dystrophy in the *mdx* mouse?

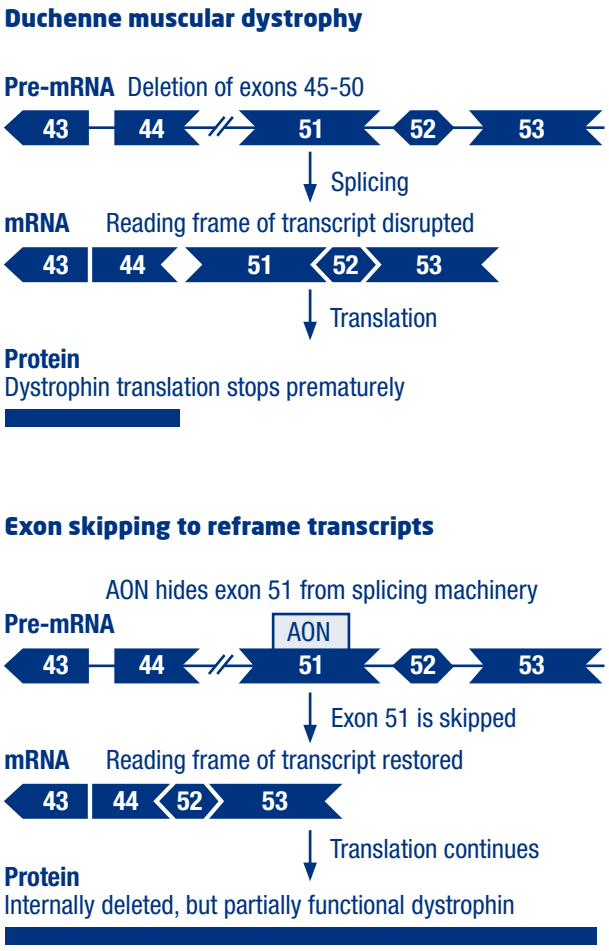

Figure 1. Mechanism of exon skipping
The mutations in the dystrophin gene disrupt the open reading frame of dystrophin. Consequently, protein translation stops prematurely, resulting in a non-functional protein. By using antisense oligonucleotides that target a specific exon in which there is a mutation that truncates the expression of dystrophin, the reading frame can be restored. This enables the production of an internally deleted, but partially functional dystrophin. [20]

Methods

The database Medline was searched on the 10th of June 2009 by using Pubmed. We used a combination search terms, including "Muscular Dystrophy, Duchenne" as a Major Topic Heading, combined with several additional MeSH headings. The reference lists of the included articles were searched by using the Cited Reference Search in the Web of Science.
We examined the articles independently; Our inclusion criteria were: a trial using inbred *mdx* mice and articles written in English. Studies using cultured cells, studies focusing on analytical techniques and reviews, meta-analysis, editorial letters and practical guidelines were excluded. No limits on publishing date were used.
We placed articles that used the same molecular structure of antisense oligonucleotide in the same category. Data on immunohistochemistry, Western blot analysis and RT-PCR analysis were derived from suitable articles. All information was combined in a table.

Results

Using the combination of search terms, we identified 63 articles, of which 50 were excluded. While investigating the reference lists of the included articles, one additional useful article was identified (Wu 2009), bringing the total number of eligible articles to 14. Extracted data from the 14 eligible studies are shown in Table 1.

Immunohistochemistry

In the eligible studies, immunohistochemistry was frequently used to quantify the percentage of muscle fibers as testing positive for the dystrophin protein. The results are expressed as a percentage of dystrophin-positive muscle fibers in *mdx* mice in comparison to wild type mice (Table 1). In five studies, the results of immunohistochemistry were quantified.

Western Blot analysis

Western blot analysis was performed in most of the studies (Table 1). The results were quantified as a percentage of the physiological amount of dystrophin protein normally present in wild type mice. Nearly all studies focused on skeletal muscle, but some included cardiac muscle or diaphragm muscle. In four studies, the results of the Western blot analyses were not quantified.

RT-PCR analysis

The RT-PCR results (Table 1) are expressed as a percentage of dystrophin mRNA where exon 23 was skipped, relative to the total RT-PCR products. The RT-PCR product represents the exon 23 deleted transcript of the mdx mice. The RT-PCR method was used in all but two studies [15, 17]. Two studies [7, 10] also quantified the data of RT-PCR in cardiac muscle; in most trials the data of the RT-PCR were not quantified.

Table 1 - Results of exon skipping in mdx mice by using structurally different compounds

Exon skipping technique	Immunohistochemistry*			Western blot analysis#			RT-PCR analysis°		Ref.
	Skeletal	Heart	Diaphragm	Skeletal	Heart	Diaphragm	Skeletal	Heart	
Phosphorothioate antisense oligodeoxynucleotide (without any carrier)									
	Detected						Detected		[5]
2´-O-methylated phosphorothioated antisense oligoribonucleotide (2OMeA0)									
	<1%	<0.5%	<1%	1-4%	2%	2%	10%	1.5%	[10]
	15-21%**			~20%			Detected		[2]
	>20%			Detected					[15]
	Detected	Detected	Detected	>5%	Undetected	Detected	Detected	Detected	[16]
	23%			2-5%					[17]
Morpholino antisense oligonucleotide									
- Unmodified (PMO)	10-20%	<0.5%	5-10%	10-30%	0.6%	18%	40%	2.5%	[10]
	>80%**	Undetectable	Detected	10-50%	Undetected	<5%	Detected		[18]
	>36%	Detected	Detected	Detected	Undetected	Detected	Detected	Undetect	[13]
- Modified									
Cell penetrating peptide (PPMO)	85%	94%	Detected	91-100%	58%	Detected	80-86%	63%	[7]
	Detected	Undetectable	Detected	8%	Detected	110%	Detected	Detected	[12]
Non-peptide(e.g. Vivo-morpholino)	~95%	>40%	Detected	~50%	>10%	Detected	Detected		[8]
Antisense small nuclear RNA (sRNA) with the use of AAV									
	Detected	Detected	Detected	Detected	Detected	Detected			[3]
	Detected	Detected	Detected	Detected	Detected	Detected		Detected	[11]
	>80%			50-80%			Detected		[19]

* Immunohistochemistry: percentage of muscle fibers positive for dystrophin protein in comparison to wild type mice - **Percentage of cross sectional area (CSA)

\# Western blot analysis: quantification as percentages of wild type dystrophin

° RT-PCR analysis: quantification of exon 23 skipping as level of exon skipping

\+ Detected: data not quantified

Discussion

The results of our review indicate that non-peptide morpholino antisense oligonucleotides (e.g. dendrimeric octaguanidine moiety tagged or Vivo-Morpholino) are the most promising compounds to slow the progression of muscular dystrophy in *mdx* mice.

Vivo-Morpholinos overcome disadvantages of other antisense oligonucleotide compounds. The delivery of Vivo-Morpholinos results in dystrophin protein expression in both skeletal and cardiac muscle (Table 1). The maintenance of dystrophin expression can be obtained by repeated injections, without eliciting an immune response [8].

Morpholino AONs have a better overall dystrophin induction compared to 2OmeAO and oligodeoxynucleotides without carrier (Table 1). Unmodified Morpholino and 2OMeAO are differently charged, which could possibly result in a difference in efficiency between the two methods. Effective delivery of the negatively charged 2OMeAO is inhibited by the like charges of the molecules on the outer cell membrane. Unmodified Morpholinos are able to enter muscle fiber more efficiently, due to their neutral charge. [8]

It is a challenge to realize sustained dystrophin expression in tissues affected by the absence of dystrophin. A disadvantage of the exon skipping treatment with 2OMeAO and Morpholino AONs is the need for periodic re-administration of the compounds to achieve long term expression of dystrophin (data not shown). The use of small nuclear RNAs seems to be able to overcome this constraint. [3] Despite the highly effective and long-term exon skipping induced by snRNAs, further research into this technique is needed to determine the safety of using viral AAV vectors. Immunological problems could arise in humans. [11, 14] Therefore, the most promising strategy is probably to continue the development of Morpholino compounds, especially considering the safe and simple delivery protocol of Morpholinos.

Although unmodified Morpholino AONs have a more efficient distribution compared to 2OmeAO, Wu et al. [7, 8] and Fletcher et al. [12] showed that modified Morpholino compounds further enhance the results (Table 1). Research performed by Wu et al. in 2008 with cell-penetrating peptide phosphorodiamidate morpholino antisense oligonucleotides showed very promising results. However, a major concern with the use of cell-penetrating compounds is the potential adverse effect of an immune response, evoked after repeated injections. [7] The use of dendrimeric octaguanidine moiety tagged morpholino compounds or Vivo-Morpholino has overcome this problem. [8]

Despite the promising results of exon skipping therapy to induce restoration of dystrophin protein in the *mdx* mouse model, there are still concerns about this approach. Successful systemic delivery remains a problem. Most studies compared local to systemic administration. We decided to only display the best results obtained (Table 1). Although local administartion has a less pronounced effect compared to systemic delivery, local administration yields beneficial results as well. For instance, local administration could be used to improve the the motor function of the arm. This also ameliorates the quality of life of a DMD patient.

Currently, researchers are challenged to develop the ideal antisense oligonucleotide: this would be a compound, effective at low doses, which induces prolonged dystrophin expression and has a safe and easy delivery protocol. [12] Vivo-Morpho-

linos met most of these requirements. Further research into both methods is therefore warranted. The translation of these approaches from the mouse model to humans is a future challenge.

When this article went to press, the first Phase I clinical trials in humans had been completed. Trials in Leiden and Londen have demonstrated proof-of-concept after direct injection of antisense oligomers into human dystrophic muscle. Trials focusing on systemic delivery of antisense oligomers have shown promising results, but the clinical benefits of this strategy are still unknown. [21]

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Conscious Sedation in the Intensive Care Unit: the Future?

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Objective: **The objective of this systematic review is to assess the effects of conscious sedation compared to deep sedation in patients who were admitted to Intensive Care Units (ICUs). Furthermore, if conscious sedation appeared to be more beneficial for patients, we analyzed which sedation method should be used.**
Methods: **We conducted a PubMed search using a set of search terms to obtain studies comparing light and deep sedation.**
Results: **The search resulted in 6 articles: 5 randomized controlled trials (RCTs) and 1 prospective cohort. Intervention methods were: daily interruption of sedative treatment, sedative protocols and algorithms, daily awakening of the patient and the use of analgo-sedation.**
Conclusion: **Conscious sedation is associated with a shorter stay in the ICU, a shortened duration of mechanical ventilation and fewer psychological problems.**

Keywords
conscious sedation; intensive care units; critical care; epidemiologic studies

Introduction
Critically ill patients are frequently treated with sedatives and analgesics. This provides comfort for patients in the Intensive Care Unit (ICU). Analgesia is used to prevent and treat pain. Sedation is used to treat anxiety, agitation and stress[1]. It is still a common practice to deeply sedate ICU patients. This results in less oxygen usage by the patient and makes patient care easier, especially for mechanically ventilated patients. However, continuous deep sedation is also associated with a high risk of over-sedation[2]. This can result in prolonged mechanical ventilation (MV), hemodynamic instability, a longer stay in the intensive care unit and mental health problems. The optimal level of sedation for critically ill patients is still debated[3], but recent studies suggest a shift towards "conscious" sedation. Conscious sedation is a method used to induce an altered state of consciousness that minimizes pain and discomfort by using sedatives and analgesics. Daily interruption of sedative treatment, sedative protocols and algorithms, daily awakening of the patient and using analgo-sedation instead of hypnotic sedation have been described as methods to sedate patients more lightly. The Ramsay scale (0-6) is used to measure the sedation level of patients. Conscious sedation can be described as a Ramsay score of 2-3 and deep sedation as a score of 5-6. Light sedation methods have been shown to reduce the duration of mechanical ventilation, shorten the stay in the ICU and the hospital and prevent post-admission psychiatric disorders such as post-traumatic stress disorder (PTSD) and delirium.
The objective of this systematic review is to determine whether conscious sedation is more beneficial for the patient than deep sedation, based on criteria such as mortality, duration of mechanical ventilation, length of hospital stay and occurrence of delirium.

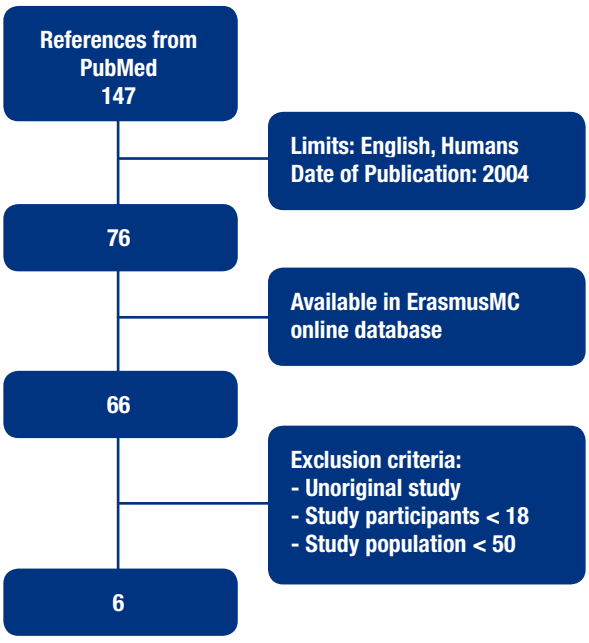

Figure 1 Literature search flow

Methods
On January 14, 2010 we searched the electronic database PubMed with the following Medical Subheadings and free text keywords: "Conscious Sedation"[MAJR] AND ("critical care"[MeSH] OR "intensive care units"[MeSH]) AND ("clinical trial"[ptyp] OR "epidemiologic studies"[MeSH]). We considered length of stay in ICU, mortality, duration of mechanical ventilation and occurrence of delirium as outcome measures. We only searched for articles published after 2004. The articles had to be about humans, had to be written in English and had to be available in the Erasmus MC online database. To further narrow our search, we made a manual selection of the found articles. Both researchers independently read the titles and abstracts of the articles and excluded articles that met the exclusion criteria. Exclusion criteria were: studies containing previously published data comparing conscious and deep sedation, young study participants (younger than 18 years) and a study population

Table 1 - Characteristics of the studies

Study characteristics					
Source	**Publication Date**	**Journal**	**Study Type**	**Country of Study**	**Population**
Treggiari et al.	2009	Critical Care Medicine	Randomized controlled trial	Switzerland	137
Girard et al.	2008	Lancet	Randomized controlled trial	USA	336
Mehta et al.	2008	Critical Care Medicine	Randomized controlled trial	Canada	65
de Wit et al.	2008	Critical Care	Randomized controlled trial	USA	75
Samuelson et al.	2006	Intensive Care Medicine	Prospective cohort	Sweden	313
Breen et al.	2004	Intensive Care Medicine	Randomized controlled trial	10 countries	105

Table 2 - Studied Sedation Method of the Intervention and Control Group

Source	Studied sedation method (intervention vs. control)
Treggiari et al.	light (Ramsey 1-2) vs. deep (Ramsey 3-4)
Girard et al.	daily spontaneous awakening trials (SAT) followed by spontaneous breathing trials (SBT) vs. sedation per usual care + SBT
Mehta et al.	protocolized sedation (PS) vs. protocolized sedation + daily sedative interruption (PS +DI)
de Wit et al.	daily interruption of sedation (DIS) vs. sedation algorithms (SA)
Samuelson et al.	depth of sedation
Breen et al.	analgesia-based sedation (using remifentanil) vs. hypnotic-based sedation

Table 3 - Outcome measures of the studies

Source	Mechanical ventilation	Length of ICU & hospital stay	Adverse effects of sedation methods	Mental health	Mortality
Treggiari et al.	+	+	7+	+	+
Girard et al.	+	+	+	-	+
Mehta et al.	+	+	+	-	+
de Wit et al.	+	+	-	-	+
Samuelson et al.	-	+	-	+	-
Breen et al.	+	+	+	-	-

smaller than 50. Studies about methods to measure sedation levels, studies comparing medicines used for sedation but not specifically pertaining to light or deep sedation, and studies about whether light sedation was achieved were also excluded (Figure 1). After completing our individual searches, we compared our lists of found articles and selected those that were on both lists.

Results

Our PubMed search produced 66 articles. After reading the abstracts and applying inclusion and exclusion criteria, 6 articles remained for our systematic review on whether patients in the Intensive Care Unit should receive deep or light sedation. These articles concerned 6 original studies, of which 5 were randomized controlled trials and 1 was a prospective cohort study. The studies were conducted in the USA (n=2), Switzerland (n=1), Sweden (n=1), Canada (n=1) and one study was conducted in 10 countries [4]. Additional study characteristics of the 6 articles are shown in Table 1. All articles described the effects of conscious sedation compared to deep sedation. However, the methods used for conscious sedation differed in each study (Table 2). Table 3 summarizes the outcome measures evaluated by each of the authors.

Length of ICU & hospital stay

Treggiari[5] compared light versus deep sedation and concluded that a Ramsay score of 1-2 resulted in a shorter stay (1.5 days) in the ICU than patients with a Ramsay score of 3-4. Girard[6] showed that patients who were given a daily spontaneous awakening trial followed by a spontaneous breathing trial protocol were discharged about 4 days earlier from both the intensive care and the hospital than patients who were treated with usual sedation plus a daily spontaneous breathing trial. *Mehta*[7] demonstrated that there was no difference in ICU and hospital length of stay for both protocolized sedation and protocolized sedation + daily interruption. *De Wit*[8] found that both the ICU and the hospital length of stay were longer for patients receiving daily interruption of sedation (DIS). The median hospital length of stay for daily interruption of sedation was 11 days longer than for sedation algorithm. *Samuelson*[9] established that patients with delusional memories had stayed 4.4 days longer in the ICU than those with recall of the ICU without delusional memories. Delusional memories were defined as nightmares, hallucinations and paranoid delusions. *Breen*[4] concluded that there was a trend towards a shorter ICU stay in the remifentanil group by 1 day compared to the standard hypnotic-based sedation with midazolam group, although this difference was not statistically significant.

Mechanical ventilation

Treggiari[5] had mechanical ventilation (MV) as a secondary end point. This study showed that patients receiving lighter sedation required mechanical ventilation for 1 day less than those receiving deep sedation (Figure 2). Patients receiving daily spontaneous awakening trials (SAT) followed by spontaneous

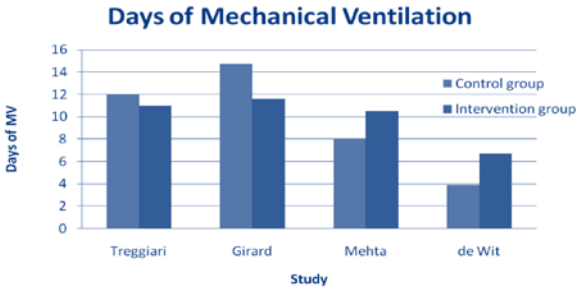

Figure 2 Days of Mechanical Ventilation in Control group vs. Intervention group

breathing trials (SBT) spent 3.1 more days breathing without mechanical ventilation than the control group[6]. There were no significant differences between the protocolized sedation (PS) group and protocolized sedation combined with daily interruption (PS+DI) group[7]. The total duration of MV in the DIS group was 2.8 days longer than in the spontaneous awakening (SA) group[8]. *Breen*[4] described the effect of a newer drug used for analgo-sedation on the number of days spent on MV. The analgesia-based sedation method that used remifentanil reduced the duration of mechanical ventilation by 53.3 hours compared to the hypnotic-based sedation method.

Mortality

Two studies concluded that there was no significant difference in mortality between the intervention group and the control group[5,7]. There was a significantly higher hospital mortality in patients treated by DIS compared to patients treated by SA[8]. However, when looking specifically at mortality in the ICU, there was no significant difference between the two groups (Table 4). *Girard*[6] looked at patient mortality 1 year after enrolment. Analysis of 1-year survival showed that patients in the intervention group were 32% less likely to die than patients in the control group (p=0.01). The number needed to treat to avoid one fatality was 7.

Table 4 - Mortality in Control and Intervention Group

Author	Mortality Control Group	Mortality Intervention Group	p-value
Treggiari et al.	11	12	0.65
Girard et al.	47	58	0.21
Mehta et al.	18	16	0.81
de Wit et al.	13	7	0.04

Adverse effects of sedation methods

All authors found no difference in the incidence of adverse events between the control group and the intervention group. However, the adverse effects of sedation methods were slightly different in each of the studies. *Treggiari*[5] noted adverse events such cardiovascular failure, CNS failure, and coagulation abnormalities, *Girard*[6] recorded tachynpea, hypoxemia and signs of distress and *Breen*[4] noticed hypotension, atrial fibrillation and vomiting.

Mental health

Treggiari[5] conducted a follow-up at 4 weeks to measure the post-traumatic stress disorder (PTSD) score. This score was obtained by using the PTSD Checklist and the Impact of Event Scale-Revised. This study showed that the Ramsay score 3-4 group tended to have higher PTSD scores than patients assigned to the R 1-2 group. In this study, 37% of the R 3-4 group responded with "moderate trouble or worse" to the question "Do you have trouble remembering important parts of the stressful experience?" compared to 13% in the R 1-2 group. Of the deeply sedated patients, 38 % reported "repeated, disturbing memories of the stressful experience" while only 4% of the conscious sedated patients reported this. *Samuelson*[9] conducted a follow-up 5 days after ICU discharge; 82% of the interviewed patients reported memories of the ICU. Patients with no recall had a higher Motor Activity Assessment Scale (MAAS) of 0-2 than those with memories of the ICU. Heavy sedation increased the risk of having no recall, and a longer ICU stay increased the risk of delusional memories.

Methodological quality

All RCTs were properly randomized. However, because each method of sedation involved different practices, the intervention and control treatments could not be blinded.

Discussion / Conclusion

This systematic review, which was based on 6 articles, compared the use conscious sedation and deep sedation in the ICU. Based on the results, we believe that patients should be given lighter sedation. Patients who were consciously sedated spent fewer days being mechanically ventilated, had shorter ICU and hospital stays and experienced fewer psychological problems. However, it is unclear whether the sedation method played a large role in patient outcome. Patients could have a better outcome simply because of the extra attention they received when undergoing the intervention treatment. Moreover, since the study populations and sedation methods compared in each study varied, it is difficult to make a definitive conclusion about which sedation method is best. The only aspect the study populations had in common was that they all concerned non-surgical patients receiving mechanical ventilation. We do not know the effects of different sedation methods on different types of patients, such as those with neurological problems. Each study compared different sedation methods. There was no standard control group and intervention group in all the studies. For example, *de Wit* compared DIS with SA. It was a subjective decision to categorize DIS as the intervention group. This accounts for the wide range in results.

It was difficult to find articles comparing light and deep sedation. Many articles discussed methods on how to measure sedation levels and drugs used for sedation, but few discussed which method of sedation was preferred. More research on sedation methods in the ICU is necessary. We believe that light sedation has many benefits for the patient and should be used in the ICU, even though many hospitals still practice deep sedation. Analgesia and sedation protocols should be optimized to achieve the best possible outcome for critically ill patients.

The data in some studies were incomplete. There was a trend towards a shorter ICU stay in the remifentanil group, but *Breen* only mentioned this in the discussion and not in the results. One study was terminated prematurely. This was not because of findings about increased hospital mortality, although the reason for termination was not mentioned. Adverse effects described were not specific to the sedation method used. We believe that these adverse effects were caused by being in the ICU and receiving mechanical ventilation, not by the different sedation methods. Sedation is essential for critically ill patients. It is clear that sedatives have an impact on the duration of mechanical ventilation, days of hospital stay and the well-being of the patient. An ideal strategy of sedation in the ICU is one where conscious sedation is achieved. The specific method used to obtain lighter sedation does not appear to be as important as ensuring that patients are sedated more lightly and are given more attention.

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Review

What is the effect of influenza vaccination on CD4 lymphocyte count in HIV-infected patients

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Objective: **To systematically review the influence of influenza vaccination on CD4 levels in HIV-infected patients.**
Methods: **We searched The PubMed Database for clinical trials using several MeSH-terms and including criteria.**
Results: **Five articles were included. The different articles show opposite influenza antibody responses to the vaccination. Some studies indicate seroprotection, others don't. Most results didn't point out a significant change in CD4 levels. Also none of the articles demonstrated a significant change in HIV-RNA loads.**
Conclusion: **The influenza vaccination doesn't cause significant changes in CD4 levels, nor in the number of HIV-RNA copies in HIV-patients.**

Keywords
HIV, Influenza Vaccines, CD4 Lymphocyte Count, Clinical Trial

Introduction

Influenza vaccination is recommended for HIV-infected patients, because these patients are at higher risk for infection.[1] HIV attacks CD4-lymfocytes which leads to low levels of CD4 and thereby a failing immune system *(Panel1)*.

Panel 1. Background information on the pathogenesis of HIV
HIV causes AIDS by attacking and destroying CD4+ T-cellen.[2] This is a group of lymphocytes that normally coordinates the immune system during an infection. In this way the virus can not only multiply itself, but also shut down the mechanism whereby the body protects itself to the virus and all other pathogens.[2]
A normal CD4+ level is between the 400 and 1300 cells per milliliter blood.[3] A level of CD4+ between 300 till 400 cells per milliliter blood is an indication to start with highly active antiretroviral therapy (HAART).[3]
By HIV pathogeneses they will also review the number of HIV RNA copies. Below 1000 copies is called a 'sleeping' virus and will progress very slowly.[3] Between 10.000 and 100.000 copies of HIV RNA is a 'normal' virus and progresses normally.[3] Above 100.000 copies it is called an 'active' virus and progresses very quickly.[3]

Infectious disease specialists in the EMC noticed lower CD4 levels in HIV-positive patients during past autumn. Most of these patients were vaccinated for both seasonal influenza (one vaccination) and Mexican Flu (two vaccinations) *(Panel 2)*. Some studies indicate that influenza vaccination may increase HIV replication and thus lower CD4 levels, other studies didn't find a correlation between Influenza vaccination and HIV progression.[4]
We want to know if influenza vaccination causes lower CD4 levels in HIV patients. Because CD4 levels are directly linked with HIV-RNA we will also discuss this subject. We will also glance at the antibody responses and differences between adjuvanted and non-adjuvanted vaccines.

Methods

The PubMed database was searched to the 7th of January 2009, using the following Medical Subject Headings (MeSH) terms: CD4 Lymphocyte count, HIV and Influenza Vaccines. Titles and abstracts were scanned for relevance, to identify papers requiring further consideration.
To be included, studies had to be clinical trials tested on humans aged 19+ years (adults) and the language had to be English.

Results

Our PubMed search produced five publications.

CD4 levels

All five articles describe the changes in CD4 levels. In one study CD4% was measured[5] and in one study both absolute CD4 counts and CD4% were measured.[6] The other three studies performed absolute CD4 counts.[7,8,9] The measuring time-points were variable. Three studies measured CD4 levels before vaccination (or the same day) and compared these data with CD4 levels at different moments after vaccination[7,8,9] *(Table 1)*. Two studies compared CD4 levels of vaccinated patients with a placebo group at different times after vaccination.[5,6]
Most studies showed no significant changes in CD4 levels.[8,9,6]
One study showed a progressive decrease in CD4 counts in patients with CD4 T-lymphocyte counts <200 cells/mm^3 vaccinated with conventional subunit vaccine (p=0,048)[7]
Another study indicated a significant decrease in CD4% after three months.[5] In the vaccinated group CD4 cells dropped an average of 1,6% compared with an average increase of 0,1% in the placebo group (p=0,039).[5] When patients who had added or changed antiretroviral drug therapy during the study period were excluded, the vaccine group had dropped an average of 2,3% compared to a drop of 0,1% in the placebo group (p=0,015)[5]

Panel 2. Background information on influenza vaccines
The annual influenza vaccines contain A/H3N2, A/H1N1 and B.[10] Likewise the used vaccines in the studied researches in this article. A/H3N2 is an antigen against the Hong Kong flu, a type A virus. This virus caused an extreme pandemic in 1968.[11] A/H1N1 is an antigen against the Spanish flu, a type A virus.[12] Since 2009 one of the variances of this virus is better known as the Mexican flu. The H1N1 virus caused also an extreme pandemic in 1918.[12] Understandable B is an antigen against type B virus. Nowadays the influenza vaccines are aligned on the expected dominant strands of the H3N2 and H1N1 virus.[11]

HIV-RNA copies

The level of HIV-RNA copies is discussed in all five articles. The measurements were all different. Only two articles pointed out the lower limit of detection of RNA copies per ml of plasma with reverse transcription-polymerase chain reaction (RT-PCR).[7,8] Two studies measured HIV-RNA up to and including one month after vaccination[5,6] and three studies measured HIV-RNA till about 5 months.[7,8,9] In every article they take the use of highly active anti-retroviral therapy (HAART) into account. The overall results are not different between the two compared groups given the HIV-RNA level *(Table 1)*.
Glesby et al.[6] is one of the studies that measured HIV-RNA until one month after vaccination. They found that both HIV-RNA levels were relatively constant over the 30 days after injection in both the vaccine and placebo group.
Tasker et al.[5] is the other study which measured until one month after vaccination. They encountered that the baseline plasma HIV-RNA copy numbers ranged widely but were similar for both groups, vaccinated or not.[5] At one to two weeks there is no significant difference between the groups.[5] But at one month they found a difference in the vaccinated group compared with the placebo-vaccinated group (P=0.029).[5]
There is a difference between patients who use HAART and those who do not; in vaccinated patients using HAART there is a significant difference in HIV-RNA (P=0.048), in vaccinated patients using no HAART there is no statistically significant difference (P=0.094).[5] Vaccination shows to have the greatest impact on viral load in patients not taking anti-retrovirals by number of HIV-RNA copies that are higher then patients taking anti-retrovirals. But due to the small sample size, this trend was no statistically significant.[5]
Sullivan et al.[9] found a HIV-RNA level decrease with 90 copies/ml per year among all patients. There was no difference in decrease of HIV-RNA copies between those who were vaccinated against influenza and those who were not vaccinated.[9]

Influenza antibody responses

Four out of five articles described the changes in antibody response of the HIV-patients before and after their influenza vaccination. This is important, because there is a discussion about the inadequate immune response induced by the vaccines in severely immunodepressed individuals.[1]
In all four studies[7,8,5,6] the influenza titres were tested with a similar assay. The sera, in general collected at day 0 and day 30, were analysed for antihaemagglutin antibodies to each of the 3 influenza virus strains by the standard haemagluttin inhibition(HI) test. Seroprotection was defined as an antibody titre ≥ 1: 40. That is at least a four-fold increase of the antibody titre.
In the results of the studies there is a dichotomy. In Tasker et al. only 31% of the vaccinated patients had more than twofold increase in influenza antibody titres after 1 month. This was not significantly different from the placebo group, where 25% had a ≥ twofold influenza antibody titre. All placebo patients with an increased influenza antibody titre reported a mild respiratory illness during the study period.[5]
Glesby et al. also concluded that there was a relatively low immunogenicity of the vaccine antigens. Only 29% of the vaccine recipients had more than fourfold rises in influenza antibody titres one month after the vaccination.[6]
The other two articles showed a positive result of the immunogenicity of the vaccines. The study of Iorio et al. reported that for each of the three antigen strains (A/H3N2; A/H1N1;B) and each CD4+ class (≥500 cells/ml; 200-499 cells/ml; <200 cells/ml) the antigen titre increased after vaccination.[8] Thirty days after the vaccination, the vaccine induced a significant increase of the seroprotected individuals.[8]
In Gabutti et al. the seroprotection parameters presented good levels of immunogenicity.[7] This was concluded on the basis of the percentage of individuals who developed seroprotection. Within one month after vaccination ≥70% of the volunteers had a protective antibody titre. This remained high after 180 days.[7]

MF59 Adjuvanted vaccine

Two of these studies use non-adjuvanted influenza vaccines (AGRIPPAL) and MF59-adjuvanted influenza vaccines (FLUAD)[7,8]. MF59 is a substance that can be added to the vaccine to excite an improved immune response.[13]
A previous study showed that MF59-adjuvanted influenza vaccines give a better antibody response in elderly than non-adjuvanted influenza vaccine.[14] Does FLUAD also give better immunogenicity in HIV-infected persons than AGRIPPAL does? And has it a different effect on HIV-RNA load and CD4 levels?
Both studies used the same kind of vaccines *(Table 1)*. Participants were divided in two groups: The first group deceived FLUAD and the other one received AGRIPPAL[7,8].
In Iorio et al. all participants were treated with HAART for at least 7 months8. In Gabutti et al. most participants were on antiretroviral drug therapy (±80%)[7]
The study of Iorio et al. shows that FLUAD induces significant higher antigens comparing to AGRIPPAL, 30 days after vaccination.[8] No significant differences in HIV-RNA loads and CD4 levels were found, comparing FLUAD with AGRIPPAL.[8]
The study of Gabutti et al. doesn't show serious adverse effects of the influenza vaccines.[7] No significant differences were found between AGRIPPAL and FLUAD, with relation to HIV-RNA load, CD4 levels and immunogenicity.[7]

Discussion/Conclusion

This systematic review was designed to investigate if influenza vaccinations causes lower CD4 levels in HIV-infected patients. Hereby we also discussed the HIV-RNA, the antibody response and the differences between adjuvanted and non-adjuvanted vaccines.
With the information of the 5 articles we used, we can conclude that influenza vaccination doesn't cause significant changes in CD4 levels, nor in the number of HIV-RNA copies.

Table 1 - Overview of the results given in the five articles

Article	Comparison	CD4 level	Age	Use HAART	Time measurements	Vaccin	Result CD4	Result HIV-RNA	Result immunogenicity	Result MF59
1. Gabutti et al	Influenza vaccine with MF59 and influenza vaccine without MF59	< 200 200-500 > 500	18-65	Most use HAART, they take account of this in the study	0 days 30 days 180 days	Winter 2002/2003: A/H3N2 A/H1N1 B	<200 = decrease 200-500 = no significant difference >500 = no significant difference	Little change but no significance difference	Seroprotection increase ≥70% in both groups	No serious adverse effect for both vaccines and no significant differences in CD4, HIV-RNA and immunogenicity
2. Iorio et al	Influenza vaccine with MF59 and influenza vaccine without MF59	< 200 200-499 ≥ 500	35-42	All use HAART.	Before vaccination 1 month 3-5 months	Winter 2001/2002: A/H3N2 A/H1N1 B	No significant difference	No significant difference	Significant increase of the influenza antigen titres	MF59 has positive significant influence on immunogenicity, but no significant differences were found in CD4 and HIV-RNA.
3. Sullivan et al	Influenza vaccine and no influenza vaccine	< 200 > 200	≥13	They make a difference between using or not using HAART	Before vaccination 3-12 months	Vaccines given between 1990 and 1999	No significant difference	Little decrease (90 copies/year) but no significant difference	-	
4. Tasker et al	Influenza vaccine and placebo vaccine	< 200 200-500 > 500	25-39	They make a difference between using or not using HAART	1 month 3 months 6 months	Winter 1994/1995: A/H3N2 A/H1N1 B	1 month = no significant difference 3 months = decrease 6 months = no significant difference	1 month = significant difference. Other times no significant difference	No significant increase of the influenza antigen titres	
5. Glesby et al	Influenza vaccine and placebo vaccine	200-500	28-48	Groups were equal concerns use or no use HAART	7 days 10 days 14 days 30 days 6 months	Winter 1994/1995: A/H3N2 A/H1N1 B	No significant difference	No significant difference, level is stable over time	Low immunogenicity of the vaccine antigens	

CD4 levels

Because most results show no significant changes in CD4 levels[8,9,6] and just two studies show a significant decrease in CD4 levels[7,5], there's probably no connection between influenza vaccination and CD4 T-lymphocyte counts. But because the differences between the studies, they were hard to compare. A point of discussion is HAART use. HAART is supposed to suppress HIV replication and improves CD4 levels. So it's possible that antiretroviral drugs cause more stable CD4 levels in HIV infected patients. In some studies all patients were on antiretroviral drug therapy, in other studies some patients. In some studies patients were allowed to change therapy, which could be of great influence *(Table 1)*.
Also the measuring times were different. Measurements 3 months after vaccination cannot be compared with measurements 6 months after vaccination. Most tests were at 1,3 and/or 6 months after vaccination *(Table 1)*.

HIV-RNA copies

Overall the articles contain no evidence for a significant increase in the number of HIV-RNA copies between HIV-patients with and without influenza vaccines. Regardless the fact that the measurements of HIV-RNA differ between most researches, we may assume that all measurements are highly sensitive. So the conclusion that there's no difference in HIV-RNA, is a trustworthy conclusion.

All studies point out that the use of anti-retrovirals affect the HIV-patients. HAART provides a better response on vaccination.[15] Like Sullivan et al.[9] suggested in their article, may the effect of vaccination on HIV-RNA levels depend on whether the HIV-infected person is on a stable anti-retroviral regimen or on baseline HIV-RNA level. Gabutti et al.[7] adds to this point that the higher the number of HIV-RNA copies, the more the patient is sick and the worse he reacts on the vaccine. So HAART can provide this reaction. Tasker et al.[5] is even a little bit detached from the conclusion that influenza vaccine must be recommended to the high-risk groups because of this important point about use of HAART in combination with influenza vaccination. It is important to know that antiretroviral therapy were introduced in 1996, the year that Tasker et al.[5] did research. In the beginning HAART wasn't that good as it is now. In 1996 the therapy consisted of a lot of pills and the effect was meanly lower.[16] Nowadays HAART is possibly one pill a day with very good effect.[16] So it is understandable that Tasker et al.[5] found no hard evidence given the fact that HAART has a big influence on the results of influenza vaccination in HIV-patients.
Sullivan et al.[9] shows that since the first report of increased level of HIV-RNA in the blood of HIV-patients following influenza vaccination there had been concern that influenza vaccination may hasten progression of HIV disease. In the other articles there is no prove for this assumption. On the long term

there is no negative effect of the influenza vaccination on HIV-RNA level and the progression of the disease.[9]
Overall we can conclude that there is no significant difference in the number of HIV-RNA copies between HIV-patients with influenza vaccines and HIV-patients without influenza vaccines. It is clear that anti-retroviral therapy is very important for the level of HIV-RNA in HIV-patients.

Influenza antibody responses

The four different articles showed opposite conclusions about the influenza antibody responses to the vaccines. Two of the studies[7,8] conclude a significant decrease of the seroprotected patients. The other two articles[5,6] which studied the seroprotection don't observe a significant antibody response. Because of this, we can't give a conclusion about the seroprotection of HIV-patients after a influenza vaccination.
All four studies tested the influenza titres with the standard haemagluttin inhibition test. This is advantageous to compare the several studies.
However in the studies we found, influenza antibody response wasn't part of the main question. Therefore not all articles gave extensive or detailed information about their findings around the antibody titres. This makes it hard to criticize the results.

MF59 Adjuvanted vaccine

No significant differences in HIV-RNA loads en CD4 levels are found, comparing MF59-adjuvanted vaccine with non-adjuvanted vaccine. Because of the incompatible results with regard to immunogenicity, we can't draw conclusions from it.

Although this review presents two clarifying conclusions, there are some limitations. There is a lot of development around HIV en HIV-related items. Therefore it is possible that we didn't found the most recent results on PubMed. Our most recent article was published in 2005.
Furthermore, the articles we used were all investigating adults. This means that we don't know if the results are relevant for children with HIV.
Further studies should investigate the influence of HAART on the stability of CD4 levels and HIV-RNA loads in HIV-infected patients. The measurements should be performed with the latest reliable tests.
Overall we can conclude that the influenza vaccination doesn't cause significant changes in CD4 levels, nor in the number of HIV-RNA copies in HIV-patients. Accordingly we can say that the use of annual influenza vaccination is safe for adult HIV-patients.

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Evaluation of results of HIV-1 vaccines tested in humans

Why do HIV-1 vaccines not work?

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Objective: **This review is an overview of prophylactic vaccines that have been tested in placebo controlled, randomized Phase II or III clinical trials on preventing HIV-1 infection. Our research questions were: what are the results of these trials and what are the correlations of protection with immune responses?**
Methods: **We searched the MEDLINE database on PubMed with MeSH terms: "HIV Infections/prevention and control" AND "AIDS Vaccines" NOT "Clinical Trial, Phase I". We only included randomized controlled trials on prophylactic HIV-1 vaccines with the incidence of HIV-1 infection or HIV/AIDS related mortality as primary endpoint.**
Results: **We found four studies that met these criteria. Of these four selected studies, one showed a beneficial effect from vaccination. None of the studies showed an effect on viral load after infection. Correlates of protection remained unclear.**
Conclusions: **Of four clinical phase II/III prophylactic HIV-vaccination trials only one study showed promising results. Despite these initial results further, research is warranted, especially on correlations of protection.**

Keywords
MeSH-terms: HIV-1, Infections/prevention and control, AIDS Vaccines, Systematic Review

Introduction

Worldwide, more than 30 million people have died because of AIDS and each year there are millions of new cases of HIV-1 infection; an estimated 33 million people are living with HIV-1 today (1). An effective prophylactic vaccine would either prevent HIV-1 infection or reduce viral loads after infection. Despite substantial efforts over the last 25 years, no effective HIV-1 vaccine is yet available.
Why is it so difficult to develop such a vaccine? The virus has unique biological mechanisms. First, mistakes in replication lead to mutations in the viral genome. Due to this variability, HIV-1 easily escapes immune responses (2). Second, after infection, virus spread takes place, either by cell-free or cell-associated virus. Therefore, both extracellular and intracellular immune responses will be needed to contain transmission. The virus can also initiate infection either by crossing a mucosal barrier or by directly entering T-lymphocytes or monocytes. Therefore, to prevent infection, both mucosal and systemic immune responses are necessary. Furthermore, infection of T-helper cells leads to cell-death and thereby to a reduction of immune cells needed to fight the virus. The virus also has the capacity to remain latent in the DNA of long-living T-helper cells, including memory T-cells. Consequently, these cells are a potential source of new virus particles for a long period.
The primary aims of a prophylactic HIV-1 vaccine are the following: First, to provide protection against infection by inducing protective immunity. Second, in case of infection, a vaccine could lower the viral load set-point. A vaccine against HIV-1 can stimulate humoral and/or cellular immune responses. Humoral immunity is antibody-mediated; neutralizing antibodies have the capacity to inactivate HIV-1 or prevent it from binding to and infecting cells.
Multiple phase I and II trials of antibody-inducing vaccines have demonstrated their safety and immunogenicity in humans. These studies have shown that it is save and feasible to generate antibody responses in humans with a recombinant glycoprotein 120 (rgp120) vaccine. The rgp120 was developed to induce humoral immune responses against the viral envelope of HIV-1 (3).
This vaccine, consisting of rgp120 protein is called AIDSVAX (4). Cellular mediated immunity refers to the activation of T-lymphocytes, mainly CD8+ T cells that directly kill infected cells. Induction of cellular immunity can be achieved by using certain HIV genes that are carried by viral vectors.
ALVAC is a vaccine consisting of recombinant canarypox viral vector carrying the gp120 gene.
A study on AIDSVAX subtype B/B was canceled because of low CD8+ reactivity.
A combination of ALVAC and AIDSVAX subtype B/E showed no protective effect in a Phase II trial (5) (6). The aim of that study was to induce both humoral response and cellular immune response.
Adenovirus type 5 (Ad5), a common virus in humans, is used as a live attenuated vaccine. An Ad 5 vector-based vaccine carrying HIV genes gag, pol and nef is considered to be the most immunogenic of the cell-mediated immunity vaccines (4). The correlation between host-responses to a vaccine and protection against HIV-1 infection is an important subject in clinical trials that investigate vaccine efficacy (7).
In a systematic review of the literature, we focused on placebo controlled, randomized Phase II or III clinical trials that tested vaccines to prevent HIV-1 infection. We addressed the following questions. What are the results of these trials? Does vaccination significantly correlate with protection and immune responses? In this paper we summarize Phase II/III clinical trials with prophylactic HIV-1 vaccines, discuss their results and make recommendations for future vaccine trials.

Methods

We searched the MEDLINE database of PubMed on January 8, 2010, for publications on the efficacy of prophylactic HIV-1 vaccines. The overall search strategy consisted of the following

Table 1 - Data on study design

Authors	Buchbinder et al.	Rerks-Ngarm et al.	Gilbert et al. / Flynn et al.
Study	STEP Study	RV144 / MOPH-TAVEG	VAX004
Journal	The Lancet	The New England Journal of Medicine	The Journal of Infectious Diseases
Date of publication	November 29, 2008	October 20, 2009	January 27, 2005
Location	North America, the Caribbean, South America and Australia	Thailand	North America and The Netherlands
Vaccine	MRKAd5 HIV-1 gag, pol, nef*	ALVAC-HIV and AIDSVAX subtype B/E**	AIDSVAX subtype B/B (VaxGen)***
Mode of action	Cellular immune responses	Both cellular and humoral immune responses	Both cellular and humoral immune responses
Vaccine schedule regimen	3 injections	4 injections with ALVAC-HIV and 2 booster injections with AIDSVAX B/E	7 injections
Vaccine: placebo ratio	1:1	1:1	2:1
Number of volunteers	2979	16402	5403
Age of volunteers	18-45 years	18-30 years	18-62 years
Percentage of men	62%	61.4%	94%
Risk of HIV-infection of the volunteers	High risk	Community risk	High risk

* Adenovirus type 5 (Ad5) vector based vaccine: mix of 3 separate adenovirus serotype 5 vectors expressing HIV gag, pol or nef gene
** Recombinant canarypox vector vaccine (ALVAC) with recombinant glycoprotein 120 subunit vaccine (AIDSVAX)
*** Recombinant glycoprotein 120 vaccine (AIDSVAX)

key terms: "HIV Infections/prevention and control"[Majr] AND "AIDS Vaccines"[MAJR] NOT Clinical Trial, Phase I[ptyp]. The search was limited to articles with links to full text available for Erasmus MC, articles published in English and randomized controlled trial studies.

Study selection
To be included in our systematic review, articles had to meet three inclusion criteria. First, the outcome measure for vaccine efficacy had to be either incidence of HIV-1 acquisition or mortality related to HIV/AIDS. Second, we looked at whether the participants in the study populations were non-HIV-1 infected healthy adults. We only included articles on prophylactic vaccines. Finally, we excluded studies that were not randomized controlled trials.
By reading the titles and abstracts, we determined whether the studies met the inclusion criteria. We divided the articles that met all criteria into two groups: trials that primarily focused on the efficacy of a certain vaccine and trials that focused on the correlation between an immunologic response and vaccine efficacy.

Results

The literature search in the MEDLINE database described above resulted in 31 potentially relevant articles. Of these 31 articles, 27 did not meet the inclusion criteria: 17 did not investigate vaccine efficacy measured as the incidence of HIV-1 infection or mortality-rate, 8 articles did not meet the criterion of non-HIV-1 infected healthy participants and of the articles left, 2 were not randomized controlled trials. The 4 remaining articles were included in our systematic review. We divided these articles into 2 categories. Three articles focused on vaccine efficacy and therefore fell into the first category. The single article in the second category investigated the correlation between immunology and incidence of HIV-1 infection (8).

Study design
All 4 articles described the results of double-blind, placebo-controlled trials. The STEP study (4), is a Phase II test-of-concept trial, but all the others were Phase III studies.
In Table 1, a summary of study characteristics is shown. The articles of Flynn et al. (9) and Gilbert et al. (8) described different aspects of the same clinical trial, VAX004. Therefore they were combined in one column of the table.
The number of volunteers, age of the volunteers, percentages of men and risk of HIV-1 infection were different between the studies (Table 1). The RV144 study had by far the largest number of volunteers. The VAX004 trial had a placebo to vaccine-ratio of 1 to 2, in contrast to the other trials (which had a placebo to vaccine-ratio of 1 to 1). In this trial the number of injections was also higher. Another important difference is that only the RV144 study used volunteers with "community risk", while the other studies focused on high-risk participants.

Vaccine efficacy
Vaccine efficacy showed wide variability between the trials (Table 2). The RV144 trial reported a modest protective effect from the vaccine (10). Vaccine efficacy in the modified intention-to-treat analysis was 31.2%. The VAX004 study reported a vaccine efficacy of 6%, which is generally considered as no overall protective effect (9).
The STEP Study had to be stopped after the first interim analysis (4). Unexpectedly, rates of HIV-1 infection were the same or slightly higher in vaccine recipients than in placebo recipients. The hazard ratio of HIV-1 infection was higher in Ad5 seropositive men, but was not increased in Ad5 seronegative men (Table 2).

Postinfection viral load
None of the studies reported a significant difference in the mean viral load after HIV-1 infection in the vaccinated group compared to placebo group (Table 2).

Correlation between vaccine efficacy and immune responses
In the VAX004 study, vaccinated participants with the lowest antibody responses to HIV-1 had a higher rate of infection than the placebo recipients (8). In contrast, vaccinated participants with a high antibody response showed a decreased risk of HIV-1 infection.
A Phase III HIV-1 preventive vaccine trial investigated the correlation between HIV-1 infection and the level of 8 different anti-HIV-1 antibodies (9). This study showed no prevention against HIV-1 infection, despite induction of an immune response. The MRKAd5 vaccine tested in the STEP study failed to

Table 2 - Data on study results

Authors	Buchbinder et al.	Rerks-Ngarm et al.	Flynn et al.	Gilbert et al.
Study	STEP Study	MOPH-TAVEG / RV144	VAX004	VAX004
Primary end points and measurements	- HIV-1 acquisition rates: immunoassay and Western blotting - post infection viral load setpoint: HIV-RNA - safety and tolerability	- HIV-1 acquisition rates: immunoassay and Western blotting - post infection viral load setpoint: HIV-RNA	- HIV-1 acquisition rates: immunoassay and Western blotting - post infection viral load setpoint: HIV-RNA and CD4 + lymphocyte counts	- HIV-1 antibody assays of 8 different anti-HIV-1 antibodies - post infection viral load setpoint: HIV-RNA and PCR of the gp120 gene
Vaccine efficacy	Infection-rate: vaccines 4.6 %; placebo 3.1% à overall hazard rate: 1.5 HR in Ad5 seropositive men: 2.3 HR in Ad5 seronegative men: 1.0	Infection-rate: vaccines 51/7960 (0.64%); placebo 74/7988 (0.93%) Intention-to-treat analysis: 26.4% Per-protocol analysis*: 26.2% Modified intention-to-treat analysis**: 31.2%	Infection-rate: vaccines 6.7 %; placebo 7.0% à vaccine efficacy of 6.0%	Peak antibody levels were inversely correlated with HIV-1 incidence. CD4+ blocking was the only significant independent predictor of HIV-1 incidence
Mean viral load values (vaccine compared to placebo)	No significant difference	No significant difference	No significant difference	Not measured in this study

* Subjects who completed all vaccination visits on schedule and were not found to have HIV-1 infection after receiving the full vaccination regimen
** Seven volunteers excluded who were found to be infected on the first test after vaccination

show an immune response (4). In the RV144 trial the correlation between protective effect of the vaccines ALVAC and AIDSVAX and an immunologic response was not determined (10).

Discussion/Conclusions

Vaccine efficacy

Only the RV144 study showed a vaccine efficacy that we considered significant. In that study, HIV-1 infection in the vaccinated group was significantly lower than in the placebo group (10). Both of the other two vaccines (the recombinant glycoprotein 120 vaccine (AIDSVAX) alone and MRKAd5 HIV-1 vaccine) lacked evidence of vaccine efficacy. The latter vaccine, tested in the STEP study, aimed at the induction of cell-mediated immunity, but had to be stopped after the first interim analysis because there were more infections in the vaccine group compared to the placebo group (4). Only the ALVAC and AIDSVAX combination, tested in the RV144 study, seemed promising enough to justify future research, although the results show only a modest benefit (10). We considered these results to be reliable, because the study population was large and because there was a good balance between women and men in this study (Table 1). In contrast to this study, a Phase II trial of AIDSVAX B/E alone showed no efficacy (11). The combination of vaccines therefore seems to be essential, but the immunologic mechanism for this phenomenon has not yet been described.

Potential problems in the RV144 study are that vaccine efficacy may have decreased after the first year after vaccination, and that vaccine efficacy may have been greater in persons at lower risk of infection. This is an interesting finding, because the other studies focused on high-risk groups. The immune mechanisms causing these effects are also still under investigation.

Viral load

None of the studies reported any difference in post-infection viral load. We concluded that none of the tested vaccines influenced viral activity after infection. We, therefore, agree with Rerks-Ngarm et al., that immunologic mechanisms mediating protection against HIV-1 may be different from those mediating early post-infection controls on viral replication (10).

Correlation between vaccine efficacy and immune response

The relative risk of infection was inversely correlated to the neutralization titer against HIV-1 in study VAX004. Two possible explanations are that: the levels of antibodies caused both an increased and decreased risk of HIV-1 infection (depending on height of response), or that they represented a correlation of susceptibility to HIV-1, but had no causal effect on susceptibility. The study did not discriminate between these explanations, but we agree with Gilbert et al. that the latter is more likely.

The vaccine showed no significant efficacy and there was no evidence of antibody-dependent enhancement in Phase I and II trials. Therefore, it is not plausible that low responses to the vaccine would enhance the risk of HIV-1 infection.

Enhancement can occur when a vaccine activates T-cells, thereby making CD4+-cells more vulnerable for HIV-1 infection. When the host has low levels of protective HIV-1 antibodies, the increased numbers of susceptible T-cells can override the protective effect of the antibodies. This could also be an explanation for the finding that vaccination in Ad5-seropositives leads to more HIV-1 infection. The induced immune responses make Ad5 specific CD4+-cells more susceptible to infection when Ad5 immunity (reflected in antibodies and CD4 T cells) is already present (12).

In conclusion, there are numerous issues on vaccine efficacy that deserve greater attention in future studies. Based on the results of this review, we believe that the ALVAC and AIDSVAX combination could be a potentially effective vaccine. Further research on immunological responses and understanding the mechanisms of protection and vulnerability are necessary to improve vaccine efficacy.

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Incidence, treatment and prognosis

Occurrence of HER/2/Neu in esophageal adenocarcinoma

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***Aims*- To determine the incidence of Human Epidermal growth factor Receptor 2 (HER/2/neu) overexpression or amplification in esophageal adenocarcinoma, and to determine its effects on treatment and prognosis.**
***Methods*- PubMed searches were done to identify all studies which investigated the role of HER/2/neu overexpression or amplification in esophageal adenocarcinoma.**
***Results*- Of 55 potentially relevant publications, six were selected and two other studies were added. In esophageal adenocarcinoma, HER/2/neu overexpression or amplification ranges from 11% to 74%. There is no increase in toxicity when the HER/2/neu inhibitor trastuzumab is added to standard therapy, surgery and chemo radiation. Patients with HER/2/neu positive tumors have a significantly worse prognosis and a lower survival rate than patients with a HER/2/neu negative tumor.**
***Conclusion*- HER/2/neu overexpression or amplification appears in esophageal adenocarcinoma, as it also does in breast cancer and in other types of adenocarcinomas. HER/2/neu overexpression or amplification is related to poorer prognosis and survival. Adding trastuzumab to standard chemo radiation did not increase toxicity. Evidently, HER/2/neu is the target for antibody-based treatment of breast cancer, which uses trastuzumab. Adjuvant trastuzumab application was also shown to be dramatically effective in HER/2/neu positive breast cancer patients. We recommend a study that investigates the effect of trastuzumab on prognosis and survival in patients with esophageal adenocarcinoma.**

Keywords
Esophageal neoplasms, HER/2/neu OR her-2/neu.

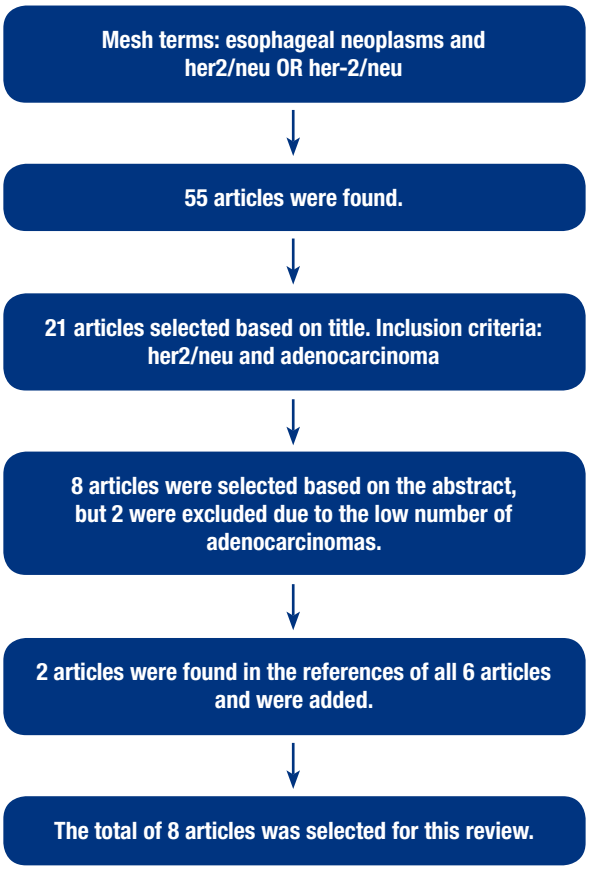

Figure 1
Flowchart of the search for articles.

Introduction

Esophageal cancer is one of the most aggressive tumors. Esophageal adenocarcinoma develops from Barrett's esophagus, and the incidence has increased over the last decade. The prognosis is poor. Because of the late detection of the tumor at the time of diagnoses, the tumor shows local invasive growth and distance metastases (1).

The Human Epidermal growth factor Receptor 2 (HER/2/neu) is an aggressive protein in breast cancers. Its oncogene is located on the short arm of chromosome 17 (2). Overexpression and amplification of HER/2/neu also occurs in various types of human adenocarcinoma. Overexpression or amplification of the gene has not been detected in normal tissue and is very rare in benign tumors. Therefore, the HER/2/neu oncogene seems to be a suitable target for therapy.

In about 20% of breast cancers, HER/2/neu overexpression or amplification occurs (3). The prognosis of these patients is poor (4). The therapy used for these patients is trastuzumab (5). Trastuzumab is a humanized monoclonal antibody which is used against the extracellular domain of the HER/2/neu and is a very effective therapy for patients with HER/2/neu positive breast cancer (6). In esophageal adenocarcinoma, surgery with chemo radiation is the standard therapy. Despite increase in survival, most patients ultimately develop metastases. Trastuzumab appears to have a synergistic effect with when used with cisplatin and an additive or synergistic effect when used with paclitaxel. Radiation has a synergistic effect on the blockage of the epidermal growth factor receptor (7,8).
The aim of this systematic review was to address the following

Table 1 - An overview of all the articles, the number of patients that participated in each study and the aim of the study.

Editors	Number of patients	Aim of the study
Safran, DiPetrillo et al. 2004 (9)	36	To conduct a Phase I study incorporating trastuzumab with paclitaxel, cisplatin and radiation for adenocarcinoma of the esophagus
Safran, DiPetrillo et al. 2007 (6)	19	To determine the overall survival for patients with locally advanced, HER/2 overexpressing, esophageal adenocarcinoma receiving trastuzumab, paclitaxel, cisplatin and radiation in a Phase I-II study
Fléjou, Paraf et al. 1994 (10)	66	To establish the prevalence of c-erbB-2 protein expression in a surgical series of Barrett's adenocarcinomas and to correlate this expression with clinicopathological data and prognosis
Polkowski, van Sandick et al. 1999 (1)	41	To investigate the clinical significance of these factors in adenocarcinoma of the esophagus and/or gastroesophageal junction (GEJ)
Reichelt, Duesedau et al. 2006 (5)	110	Her-2 is the target for antibody based treatment of breast cancer. In order to evaluate the potential role of such a treatment in esophageal cancers, HER-2 amplification and overexpression was investigated in primary and metastatic cancers of the esophagus
Hardwick, Shepherd et al. 1995 (11)	31	To investigate overexpression of the oncoprotein c-erbB-2 in the dysplasia/carcinoma sequence of Barrett's columnar-lined esophagus (CLO)
Brien, Robert et al. 1999 (2)	63	The aim of this study was to evaluate the prevalence and prognostic value of HER-2/neu gene amplification by fluorescence in situ hybridization (FISH) in 63 cases of BEAd
Dahlberg, Blake et al. 2004 (12)	25	The purpose of this study was to (1) determine the frequency of ERBB2 amplification (in comparison to other proto-oncogenes) in tumors from patients with esophageal adenocarcinoma, (2) characterize structural details of an ERBB2 amplicon in the esophageal adenocarcinoma cell line OE19 (contains a 100-fold ERBB2 amplification), and (3) test whether growth of the OE19 cell line is sensitive to the ERBB2 inhibitor trastuzumab

Table 2 - Number of esophageal adenocarcinoma, the amplification or overexpression of HER/2/neu and percentage, and the detection technique.

Numberadenocarcinoma	Overexpression/amplification	HER/2/neu	Percentage	Detection technique
Safran et al. 2004 (9)	36	12	33%	IHC
Safran et al. 2007 (6)	19	14	74%	FISH,IHC
Flejou et al. 1994 (10)	66	7	11%	IHC
Polkowski et al. 1999 (1)	41	10	24%	IHC
Reichelt et al. 2006 (5)	110	16	15%	FISH, IHC
Hardwick et al. 1995 (11)	31	8	26%	IHC
Brien et al 1999 (2)	63	12	19%	FISH
Dahlberg et al 2004 (12)	25	3	12%	Southern blot

questions. 1) Does HER/2/neu overexpression or amplification appear in esophageal adenocarcinoma? 2) What potential effects does overexpression or amplification have on prognosis and treatment?

Methods

We searched with: ("Esophageal Neoplasms"[MESH] AND ("HER/2/neu" OR her-2/neu). To select appropriate articles, based on title and abstract we used "esophageal adenocarcinoma" and "HER/2/neu receptor" or "HER/2/neu receptor" as inclusion criteria. After reading the articles, two articles were excluded because the number of investigated esophageal adenocarcinomas was too low to draw a conclusion. Two articles, Flejou et al. 1994 and Hardwick et al. 1995, did not appear in the search, but were added because all the other articles referred to these articles and often quoted from them. The results of these two articles were therefore important for this systematic review.

Results

Eight articles were selected based on the inclusion criteria. Of these eight articles, two articles addressed of HER/2/neu in relation to treatment and six articles addressed HER/2/neu in relation to prognosis (Table 1).

HER/2/neu overexpression or amplification

HER/2/neu overexpression or amplification ranges from 11% to 74% in esophageal adenocarcinoma (Table 2). HER/2/neu overexpression or amplification does not occur in metaplastic and dysplastic esophagus (Barrett's esophagus) (1,10,11). HER/2/neu overexpression or amplification only occurs in intestinal-type and mixed-type and not in diffuse-type tumors (1,10).

Treatment

There is no increase in toxicity, particularly in cardiac, esophageal, or pulmonary toxicity, when trastuzumab is added to paclitaxel, cisplatin and radiation in patients with HER/2/neu overexpression or amplification (6,9).

Prognosis

Patients with HER/2/neu overexpression or amplification have a significantly worse prognosis and a lower survival than patients without HER/2/neu overexpression or amplification (1,2,10). The presence of HER/2/neu overexpression or amplification is unrelated to TNM-stage, grade, depth of tumor invasion and the presence of lymph node metastases (2,5,10).

Discussion/conclusion

HER/2/neu overexpression or amplification in esophageal adenocarcinoma ranges from 11% to 33%. The most likely explanation for the highly variable frequency in overexpression or amplification is the difference in use of reagents and definitions of positivity (5). Another explanation can be the greater sensitivity of HER/2/neu detection in frozen tissue compared with fixed archival material (11). Safran et al 2007 shows HER/2/neu overexpression or amplification in 74% of the adenocarcinomas (6). This high percentage can be explained by a wider spectrum of inclusion criteria in comparison with the other studies. Overexpression or amplification does not occur in pre-stages of adenocarcinoma. This implies that HER/2/neu overexpression or amplification is a late event in the process of tumorigenesis in esophageal adenocarcinoma (1,10,11). Therefore, HER/2/neu overexpression or amplification cannot be used as a prognostic factor in patients with Barrett's esophagus to predict whether or not these patients will develop esophageal adenocarcinoma.

The standard treatment for esophageal adenocarcinoma is surgery in combination with chemo radiation. Even though this treatment increases the survival, the majority of the patients develop metastases (6,9). It has been shown that in patients with HER/2/neu positive breast cancer, trastuzumab increases the overall survival (median survival, 25.1 vs. 20.3 months; P=0.046) (13). Trastuzumab is synergistic with cisplatin and is additive or synergistic with paclitaxel. Radiation has a synergistic effect with the blockage of the epidermal growth factor receptor (6). When trastuzumab is added to the standard chemo radiation therapy in patients with HER/2/neu positive esophageal adenocarcinoma, there is no increase in toxicity; specifically there is no increase in cardiac, pulmonary and esophageal toxicity (9). Assuming that HER/2/neu in breast cancer is the target for the antibody-based treatment trastuzumab and that trastuzumab does not increase toxicity, perhaps HER/2/neu in esophageal adenocarcinoma can also be a target for this humanized monoclonal antibody treatment. Adding trastuzumab to standard therapy does not increase toxicity, so if trastuzumab provided a few months additional survival, this would be a gain for patients with HER/2/neu positive esophageal adenocarcinoma. Because of its poor prognosis, every additional month is a significant gain. Nevertheless a relation between trastuzumab and overall survival has not yet been demonstrated. Therefore, a study to investigate whether or not adding trastuzumab to chemo radiation increases overall survival in patients with a HER/2/neu positive esophageal adenocarcinoma is necessary.

Patients with a HER/2/neu positive esophageal adenocarcinoma have a significantly worse prognosis and lower survival than patients with a HER/2/neu negative esophageal adenocarcinoma (1,2,10). TNM-stage, grade, depth of tumor invasion and the presence of lymph node metastases are unrelated to HER/2/neu overexpression or amplification (2,5,10). Therefore, HER/2/neu overexpression or amplification is related to a decrease in survival, but is not related to any pathological features. This cannot be explained by a biological mechanism (5), so perhaps HER/2/neu overexpression or amplification can be seen as an independent prognostic factor, which is not dependent on pathological features (10). We recommend a trial which investigates the effect of trastuzumab on the survival of patients with esophageal adenocarcinoma. HER/2/neu in esophageal adenocarcinoma might be also a target for this therapy, and could improve the prognosis for patients with esophageal adenocarcinoma.

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The current status of Lentiviral gene delivery in β-Thalassemia Major

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BACKGROUND. β-Thalassemia major is a monogenic hereditary disease characterized by no or limited production of the β-chain of hemoglobin. Potentially, gene therapy should be curative for such diseases.
OBJECTIVE. To analyze the current status of lentiviral vector gene delivery for treatment of β-Thalassemia major.
METHODS. We conducted a systematic literature search in Pubmed, using certain criteria to obtain a manageable selection of articles. We have compared the currently used lentiviral vectors, the vector design and performance of these vectors in different mouse models. We also briefly looked at the feasibility to correct the endogenous β-globin gene expression instead of adding an exogenous healthy β-globin gene, as will be achieved by lentiviral vector delivery.
RESULTS. We found that a potent vector with a favorable risk profile corrected the phenotype of thalassemic mice with gene transfer.
FUTURE PROSPECTS. A vector similar to the one successful in correcting the thalassemic phenotype in mice will be used in a clinical trial, which will attempt to cure β-Thalassemia major patients.
CONCLUSION. Reversal of symptoms in mice, development of a tailored vector, with a favorable risk profile will bring clinical gene therapy for β-thalassemia within reach.

Keywords
Gene Therapy, Thalassemia/therapy, Lentivirus, Thalassemia major, genetic vectors/genetics

Introduction

β-Thalassemia is a hereditary disease characterized by impaired or no production of the β-chain of hemoglobin (Hb). This is caused by mutations in the two alleles encoding this protein. The absence of the β–chain causes an α/β-globin imbalance. The surplus α-globin precipitates on the membrane leading to ineffective erythropoiesis and haemolysis, causing severe anemia.[1]

The only curative treatment for β-thalassemia currently available is allogeneic bone marrow transplantation (BMT), which involves significant risks such as graft vs. host disease and engraftment failure with considerable morbidity and mortality, and limited by the availability of HLA-compatible donors in less than a quarter of the patients.[2]

The survival for HLA-matched siblings in good condition is >95%, however, in patients in poor condition, with liver dysfunction and iron overload, a high mortality is still a prevailing feature of the treatment.[1]

The current standard treatment consists of regular blood transfusions. A major drawback of this approach is that it will lead inevitably to iron overload, which in turn can cause damage to the liver, pancreas, endocrine glands and the myocardium.1 This effect can be countered with Desferrioxamine, which has to be administered parenterally. It is given as an overnight infusion on 5-7 nights each week. To help iron excretion by the kidneys, ascorbic acid at a dose of 200 mg daily is also given. Therapy compliance is a problem and accumulating doses of Desferrioxamine can cause cataracts, nerve deafness and retinal damage.[1]

In conclusion, the current curative treatment is only available for a small group of patients and may result in significant transplant-related morbidity and mortality. The symptomatic treatment causes damage over time and is intensive. Both these therapies would be unnecessary if the ability to produce the β–chain protein could be restored. In principle, adding a healthy allele encoding for β–globin to hematopoietic stem cells, can cure this disease.

Since erythrocytes are short lived and do not have a nucleus, peripheral cells are not the right target cells for inserting the β–globin gene. They will not only be unable to produce the β–globin, but even if they could, the treatment would have to be repeated often, because the amount of cells that carry the added gene would gradually decrease over time.

In order to achieve a long lasting effect the gene would have to be integrated in hematopoietic stem cells. In order to have the gene present in both daughter cells after cell division, the gene has to be incorporated into the target cell's DNA. This means that specific vectors are needed to deliver the gene. The best choices here are retroviruses and lentiviruses, both viruses integrate into the host's DNA.[3]

However retroviruses proved to be oncogenic in recent trials.[4] Children treated for SCID developed a T-cell acute lymphatic leukemia (T-ALL). It was later discovered that the vector used did not integrate at random sites, but rather near 5' regions within the transcription start sites of active genes. *In vitro* laboratory tests on murine cell lines show that lentiviral vectors are not as prone as the retroviruses to incorporate near the 5' region of proto-oncogenes, thus reducing the risk of oncogenic transformation.[3]

With this promising prospect, we asked ourselves: what is the current status of lentiviral gene delivery in Thalassemia major?

Figure 1
Schematic overview of the vector genome elements

Legend:
LTR Long terminal repeat
LCR Locus control region

Methods

In order to evaluate the current status of lentiviral gene delivery for treatment of β-thalassemia major, we performed a literature search. The main criteria we used to select our articles were: the article describes lentiviral delivery of genes for treatment of β-thalassemia major (Coomb's anemia). Reviews were excluded from our selection.
The search query we ended up using was:

"Gene Therapy"[Majr] AND "Thalassemia/therapy"[Majr] AND ("genetic vectors/genetics"[Mesh Terms] OR "Lentivirus")NOT Review[ptyp]

Two relevant articles, in which a lentiviral vector was used, were not indexed with the correct MeSH term. So we ended up using a free text word search for this subject instead. Other inclusion criteria we used were: the article was available and the article had to be written in English. Before selection there were 10 articles, after applying our criteria this was reduced to 7 articles. The search was conducted on June 10th 2009.

Results

With the criteria described we ended up with 7 relevant articles,[2,5-10] summarized in table 1 for an overview and comparison of the relevant articles.
To ensure high, differentiation stage specific and sustained expression of β-globin, with the lowest risk for insertional mutagenesis, several modifications to the used vector have been investigated. We will address each feature separately.

Vector construction
The viral vector genome is in general build up, from 5'-end to 3'-end, of a 5'long terminal repeat (LTR), transgene, promoter, enhancer, a locus control region (LCR), and a 3'LTR.[5] See figure 1.
The engineered genome to treat β-thalassemia consists of a human β-globin promoter, and uses a human β-globin gene as transgene. Since the promoter is only active in the relatively late stage of proerythroblastic differentiation, the risk for oncogenesis should be reduced. To minimize the risk of vector-encoded enhancer function, insulators or other elements with enhancer blocking capacity may be built into the genome.[2]
May et al. observed that gammaretroviruses did permit erythroid-specific expression of the β-globin gene, however also noted that the expression was low, and subject to position effects.[6]
They hypothesized that implementing large elements spanning DNase I hypersensitive sites (HS) HS2, HS3, and HS4 would further increase expression of the β-globin gene, after concluding from earlier trials that upstream positioning of core elements of HSs had a positive effect on β-globin expression.[6]
Since gammaretroviral vectors can only package a relatively small genome[7], lentiviral vectors were chosen which allow for larger sequences.[6]
May et al. constructed two lentiviral vectors. RNS1 containing the previously tested LCR with core regions of HS2, HS3 and HS4. The TNS9 vector comprised an LCR with large portions of HS2, HS3 and HS4. Both vectors carried the human β-globin gene.[6]
To test tissue and differentiation stage specificity of expression, transduction with both vectors was done on several cell types. The β-globin gene was appropriately expressed with both vectors with respect to differentiation stage and tissue specificity. Also the expression level of both vectors was measured. For RNS1, 1 of 10 tissue clones expressed the human β-globin in measurable quantities, whereas TNS9 yielded measurable levels in 12 of 12 tissue clones (P<0,01). TNS9 also yielded significantly higher levels of human β-globin gene, which was differentiation stage and tissue specific.[6]
In vivo testing of the TNS9 and RNS1 vector showed sustained β-globin expression in the mice treated with TNS9 transduced allogenic HSCs. This was not the case for the RNS1 treated mice. Thus, TNS9 overcame transcriptional silencing of the transgene[6], a prerequisite for sustained efficacy in treating β-Thalassemia.

Hypersensitive sites 1 and 4
The strong influence of HS2 and HS3 on β-globin expression has been well described.[7] Since the effects of HS1 and HS4 were not previously thoroughly investigated, Lisowski et al. went on to find out how HS1 and HS4 influence "the specificity, inducibility, long-term in vivo expression and therapeutic potential of globin lentiviral vectors."[8] The most important rationale was to achieve a minimum vector copy number (VCN) per cell, while maintaining a therapeutic level of β-globin expression. A low VCN is thought to be essential for the safety of stem cell engineering, because it reduces the chance of oncogenesis as discussed earlier.
They used vectors derived from TNS9.[8] 5' upstream addition of HS1 generated no additional effect *in vitro* using two similar vectors. However *in vivo* in mouse models the 5' HS1 addition significantly increased β-globin expression, from 64 g/L and 42 g/L Hb per VCN for vectors T9 and S9, to 95 g/L and 88 g/L Hb per VCN for the vectors T10 and S10. T10 and S10 have the added 5' HS1, T9 and S9 do not. T10 and S10 have a slightly larger promoter than T9 and S9. Lisowski et al. concluded that HS1 significantly improves the LCR function *in vivo*.[8]
In the same study the influence of the flanking region of the HS4 element was examined. Truncating the 5' HS4 significantly decreased β-globin expression, both *in vitro* and *in vivo*, compared to T9.8 It was concluded that the HS flanking regions play an important role in LCR function and are not simply spacers for the core elements of the HS sites. Therefore, HS regions, incorporated in the vector genome, should be as large as possible.[8]
Lisowski et al. conclude that both HS1 and HS4 are important for the expression of β-globin at a low VCN per cell. This will positively affect safety of HSC transduction.[8]

Novel murine models & new vectors
Until the murine model described by Rivella et al.[2] became available, there was no real model to study the effect of vectors on β-thalassemia in vivo. A mouse with the $Hbb^{th1/th1}$ genotype would present with thalassemia intermedia, while a $Hbb^{th3/th3}$ carrying mouse should have a thalassemia major phenotype, but because of the early switch from fetal Hb (HbF) to adult Hb (HbA), the embryo dies in utero.[2]
In the new model, the adult mice would be irradiated and transplanted with fetal liver cells (FLC) with the $Hbb^{th3/th3}$ genotype (n = 31). As a control, two different groups were made.

Table 1 - overview of the articles discussed

	Objective	Methods	Results
Li et al.[7] Year of publication: 1999	To optimize the expression cassette, by determining the sequence requirements of both the Agamma- and β-globin promoters, the importance of flanking and intronic sequences.	The expression was analyzed by RNase protection after stable plasmid transfection of a murine erythroleukemia cell line. With different promoter, intronic sequences and flanking configurations and lengths. To determine how much is needed to create a gene that would fit in a vector, but would also achieve a high enough expression after transfection to be of value.	The Agamma-globin promoter could be deleted to -159 without effecting the expression. While a deletion to -127 increased the expression of the β-globin promoter, which had an overall better expression than the Agamma-globin promoter. The enhancers HS2, HS3, core elements of the LCR and the HS-40 core all increased expression. Although HS-40 was less effective in combination with Agamma-globin.
May et al.[6] Year of publication: 2000	To see if therapeutic levels of heamoglobin could be achieved, after integration of the human β-globin gene together with large sections of the LCR using a lentiviral vector.	To investigate tissue specificity, stage specificity and expression level, RNS1 or TNS9 was transduced into MEL cells, lymphoid Jurkat cells and myeloid HL60 cells. After differentiation, human-globin and mouse-globin transcripts were measured. To investigate the effects in vivo, murine bone marrow cells were transduced and transplanted in lethally irradiated mice. The average vector copy number in peripheral blood cells and Steady state RNA-transcripts were measured for 24 weeks.	In vitro human β-globin levels were 14.2 ±4.7% for RNS1 and 71.3 ±2.3% for TNS9. The expression was stage and tissue specific. In vivo there was a highly efficient gene transfer with both vectors. For RNS1, human β-globin transcripts were in the range of 7.0 ±3.0% after 6 weeks decreasing to 3.4% at 24 weeks. TNS9 maintained transcript levels of 10-20% during the 24 weeks. TNS9 was able to correct Thalassemia intermedia in mice.
Rivella et al.[2] Year of publication: 2003	To see if a lentiviral-mediated human β-globin gene transfer could save an adult mouse model of β-Thalassemia major.	Lethally irradiated mice were engrafted with β-globin-null (Hbb(th3/th3)) fetal liver cells. This made them suffer from the same ineffective eryhropoiesis as found in β-Thalassemia major. These mice were given either TNS9 vector transduced cells or nothing.	Most mice receiving the TNS9 transduced cells survived (11 of 13). While the untreated mice all died within 60 days. Pathological analysis indicated that erytrocyt maturation had been restored in the TNS9 group. The extramedullary hematopoiesis and hepatic iron overload also decreased. However the expression of the transduced gene was too low to fully cure the Thalassemia. It transformed Thalassemia major into a Thalassemia intermedia.
Puthenveetil et al.[9] Year of publication: 2004	To see if a lentiviral vector carrying the human β-globin expression cassette flanked by a chromatin insulator, has a therapeutic effect in transfusion-dependent human Thalassemia major mice.	Gene-corrected human β-Thalassemia progenitor cells were transplanted into immune-deficient mice. β-globin and erythropoiesis were measured during 3-4 months after transplantation.	The transplanted cells expressed normal levels of human β-globin, and displayed normal effective erythropoiesis 3 to 4 months after xenotransplantation. Variability of β-globin expression in erythroid colonies derived in vitro or from xenograft bone marrow was similar to that seen in normal controls.
Xie et al.[10] Year of publication: 2007	To see if RNAi and antisense RNA could restore the α-/β-globin imbalance in a mouse carrying a human splicing deficient β-globin allele (Hbb(th-4)/Hbb(+)).	Next to a control group three groups where created. One receiving only RNAi treatment (down regulating α-globin production), the second receiving antisense RNA (facilitation correct splicing of β-globin) and a third receiving both treatments.	The α-globin levels in the first group decreased 20-35% compared to the control group ($p<0.01$). In the second group there was detectable normal human β-globin, where there was none in the control group. The third group showed signs of better balanced α-/β-globin levels. There was a decrease of poikilocytosis of 30-50% in all three groups. The reduction was sustained for 8 months.
Bank et al.[5] Year of publication: 2005	To announce a stage I/II clinical trial of a lentiviral vector containing a β-globin gene (βA-T87Q). Which produces hemoglobin that can be distinguished from normal hemoglobin.	-	-
Lisowski et al.[8] Year of publication: 2007	To investigate the effect of HS1 and HS4 on the expression of the globin expression.	In vivo tests of different TNS9 vectors. Vector copy number quantification by southern blot and TaqMan analysis. Total RNA was extracted from MEL cells or peripheral blood for β-globin transgene analysis, and hemoglobin levels were measured.	Deletions within HS4 reduced in vivo globin expression. Addition of HS1 to a HS2-3-4 vector increased globin expression by 52%.

One with the Hbb $^{th3/+}$ genotype (n = 21) the other with normal FLC (n = 11).[2]
Analysis of peripheral blood, collected 6 to 8 weeks after engraftment, showed a severe anemia for the first group. (2.8g/dL ± 0.8 Hbb $^{th3/th3}$) (11.1 ± 2.1 Hbb $^{th3/+}$) (13.2 ± 1.0 Hbb$^{+/+}$) The first group also showed a severe splenomegaly and iron deposits which were not found in controls. After 9 weeks all mice in the Hbb$^{th3/th3}$ group died with the β-thalassemia major phenotype.[2]
In an attempt to reverse the β-Thalassemia major phenotype, two groups were made. One group would receive TNS9, with the human β-globin, transduced stem cells (n = 13). The other group would receive mock lentiviral transduced stem cells (n = 20). After 60 days, all mice in the control group had died.
The TNS9 treated survived at least 4 months. Eight weeks after engraftment, 30-65% of the total Hb in the TNS9 group was human Hb (Hbb^{Hu}). From this group, 6 had HbbHu levels of >95%, thus surviving solely on lentiviral β-globin. These mice were selected for long term study since it was sufficiently demonstrated that their original marrow was replaced by the transduced cells.[2]
Hb was on average 6.5 ± 2.9 g/dL during an 8 month period (8.1 g/dL ± 0.3 in healthy control)[2]. This demonstrated that TNS9 has a significant therapeutic benefit. However, the treatment would only convert thalassemia major into a thalassemia intermedia phenotype. For full correction, a vector with higher expression levels was needed.
A further evaluation by Puthenveetil et al. showed us that adding the insulator chicken hypersensitive site 4 (cHS4) increased the expression regardless of where the gene would integrate. After this result, they constructed a lentiviral vector with cHS4, human β-globin and regulatory elements (BGI) to see if this vector would fully correct thalassemia major.[9]
Therefore three groups where made: β-thalassemia bone marrow stem cells (TBM) and TBM transduced with BGI (TBM-BGI) and normal marrow (NBM).
In vitro TBM-BGI differentiation was indistinguishable from NBM. On the other hand, TBM lacked normal erythrocyte differentiation with hemoglobinization. Maturation was assessed by performing differential counts on cytospins of cultures. It was concluded that the TBM arrested at the polychromatophilic normoblast stage, while the TBM-BGI showed differentiation that was indistinguishable from NBM.
In TBM cultures, the proportion of apoptotic cells was 49% ± 12% at day 14. (NBM vs TBM P < 0.004)9 The apoptotic proportion of TBM-BGI was 2% ± 1% the same as seen in NBM cultures (NBM vs TBM-BGI P = 0.1).[9]
After the cultures, the three groups were transplanted in splenectomized, sublethally irradiated, 4- to 6-week-old mice. (TBM n = 11, TBM-BGI n = 13, NBM n = 11)[9]. The data collected from this xenograft showed multilineage engraftment, HbA production in thalassemia bone marrow, a reversal of apoptosis and effective erythropoiesis with circulating HbA+ erythrocytes in the TBM-BGI mice, all of which were indistinguishable from the NBM group, thus demonstrating that the *in vitro* results of successful correction of thalassemia major translated well in the *in vivo* transplant setting.

Future prospects

Clinical trial
The recent successes in correcting the thalassemia major phenotype in mice have led to the design of the first clinical trial with this approach.[5] In this trial a lentiviral vector carrying β$^{A\text{-}T87Q}$, a β-globin gene with a single point mutation, will be used. This will form functional Hb distinguishable from normal Hb. The vector also contains large elements of the LCR and chromatin insulators.[5]
In this trial, 10 patients will be enrolled. Five of them should suffer from β-thalassemia and five from sickle cell disease. Bone marrow will be harvested and CD34+ cells isolated and transduced with the vector. After reinfusion, the patients will be treated as allogenic bone marrow transplant recipients, although no graft vs. host reaction or rejection of the graft is anticipated.[5]

New therapy options
A different approach for curing β-thalassemia would be the use of a lentiviral vector to incorporate a gene in HSCs that will produce interference RNA (RNAi) and antisense RNA. This antisense RNA will alter the way the β-globin mRNA is spliced, fixing for instance a reading-frame shift that occurs with a specific mutation, thus upregulating expression. RNAi will in part suppress the α-globin mRNA to down regulate expression of α-globin. Combining both, the α/β globin balance could be restored, resulting in cell survival. Xie et al. have demonstrated the principle in a test where single cell Hbb$^{th\text{-}4}$/Hbb^{+} embryos had been transduced with the vector.[10]

Conclusion & Discussion

β-Thalassemia major is a monogenic disorder for which allogenic bone marrow transplantation is the only curative treatment.1 Since this treatment is not an option for most patients, we performed a literature search to find out what the current status of curative lentiviral vector gene delivery is.
Reversal of symptoms in mice, development of a suitable vector, and a favorable risk profile brings clinical implementation of gene therapy within reach. A lentiviral vector, consisting of cHS4, human β-globulin and regulatory elements, was able to cure mice with β-thalassemia major phenotype permanently,[9] demonstrating complete phenotype correction *in vivo*. With the addition of HS1 to the vector genome, even higher β-globin levels can be achieved, which leads to a low vector copy number per cell required to achieve therapeutic levels of β-globin.[8]
A low VCN per cell, a lentiviral backbone and the use of insulators in the vector genome, should contribute to an optimal risk profile with respect to insertional oncogenesis compared to the earlier gammaretroviral vectors. Lentiviral vectors are chosen over gammaretroviral vectors mainly for the reason that they can successfully package a larger genome, which can carry a large LCR, insulator elements, and a larger promoter. In addition to these properties, they are less prone to insert their genome at transcription start sites of active genes in the host cell.[3] Production of lentiviral vectors is however more complicated and expensive than that of gammaretroviral vectors.
The development of the murine model by Rivella et al., consisting of adult mice transplanted with FLC's carrying Hbb$^{th3/th3}$, made it possible to study the effect of a vector on the β-Thalassemia major phenotype. However the model used by Puthenveetil et al. to test their vector was a fully hematological humanized and splenectomized mouse. This means that comparing the results was not entirely feasible, although both had similar end-point read-outs.
Concerning alternative gene therapy, effectiveness of treatment with RNAi and antisense RNA was proven in principle, but there are major hurdles to this approach, the most important one being that many different antisense RNAs will have to be developed to correct each specific genetic error. That makes this approach more labor intensive and costly compared to the β-globin gene addition. However the vector used to deliver

these genes can be considerably smaller than the one used for β-globin gene delivery. So production of this vector is relatively easier.
Since β-thalassemia major mice have been cured using gene therapy, a trial to test the effects on humans has been designed and should begin fairly soon.[5] Although nothing can be said about the outcome just yet, this trial marks the possible transition from lab to clinic. Any positive outcomes would be very beneficial for introducing gene therapy as a cure for all β-thalassemia patients. It will also be an important test to see if there are any long-term effects, and to evaluate the occurrence of serious adverse effects, which could jeopardize this approach.

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Review

The effectiveness of lentiviral correction of thalassemia major and the possibility of adverse effects

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Objective: **To review the effectiveness of lentiviral vectors and to reassess the adverse effects of genetic lentiviral therapeutics for thalassemia major.**
Methods: **We carried out a MEDLINE search using predetermined MeSH-terms.**
Results: **Of the 144 search results, 14 met our criteria and were used in this review. We found studies concerning the use of insulators for the prevention of adverse effects as well as several articles discussing the effectiveness of the vectors and various enhancement methods.**
Conclusion: **Lentiviral vectors used in the reviewed articles were able to successfully treat nearly all the symptoms of ß-thalassemia major. The effectiveness of a lentivirus vector can be improved through in vivo and in vitro selection of transduced hematopoietic stem cells. It can also be improved by inserting HS1 in the LCR of the vector. The adverse effects of lentiviral insertion in cells can be reduced by using an insulator.**

Keyword
beta-thalassemia, gene therapy, genetic vectors, globins/genetics

Introduction

The function of hemoglobin in red blood cells is oxygen transport[1]. Adult hemoglobin consists of two β- and two α-globins. Mutations that decrease the rate of synthesis of α- or β-globins can cause thalassemia[2]. The β-globin gene is located on chromosome 16, whereas the two α-globin genes are positioned in a pair on chromosome 11. The β-globin gene mutations related to β-thalassemia fall into two groups. The first group is β^0, in which globin chains are not produced. The second group is β^+, with a reduced β-globin chain production. Nearly 200 β-thalassemia mutations have been discovered.
β-thalassemia major (also termed Cooley's anemia) is the most severe form; it occurs when an individual inherits at least two β^0 alleles or one β^0 and one β^+ allele[1]. A person who inherits one abnormal allele has thalassemia minor. Thalassemia intermedia is a condition between major and minor; affected individuals make small amounts of β –globin, but they still require occasional transfusions.
Mutations that cause β-thalassemia are most frequent in Mediterranean, African, and Asian populations. Among immigrant populations in the Netherlands, the thalassemia major incidence rate is 5 cases per 10,000 per year[2].
When fetal hemoglobin (HbF) production decays 6 months after birth, β-thalassemia major symptoms become visible[1]. Children with thalassemia major develop skeletal deformities, their growth slows down and they develop anemia.
One factor that contributes to the development of anemia in β-thalassemia is insufficient HbA formation. The reduced synthesis of β-globin leads to insufficient HbA formation, causing the mean corpuscular hemoglobin concentration to be low (hypochromic), which in turn causes the cell to become microcytic.

Erythrocyte and erythroblast hemolysis is a result of unbalanced rates of β-globin and α-globin chain synthesis; α-globins that are not paired with β-globins induce cell membrane damage which is sufficiently severe to provoke hemolysis (hemolytic anemia).

The current preferred treatment for β- thalassemia major is allogeneic hematopoietic stem cell (HSC) transplantation[3]. This is a curative treatment, which enables the patient to produce erythrocytes that do have normal β-globin genes. Healthy stem cells with correct genes for β-globin are taken from a donor and injected into the patient. The stem cells then find their way to the patient's bone marrow and take part in the hematopoiesis.

One of the requirements for HSC transplantation is a human leukocyte antigen (HLA) matching donor, which is often not available. If this is the case, then the patient remains transfusion dependent3. The patient receives periodic blood transfusions with normal hemoglobin from a donor to correct the chronic anemia, reduce the skeletal deformities and temper the enormous erythropoiesis. Since an HLA matching donor is available for only one out of four patients, most patients require this therapy. The major disadvantages of this therapy are that *(i)* it does

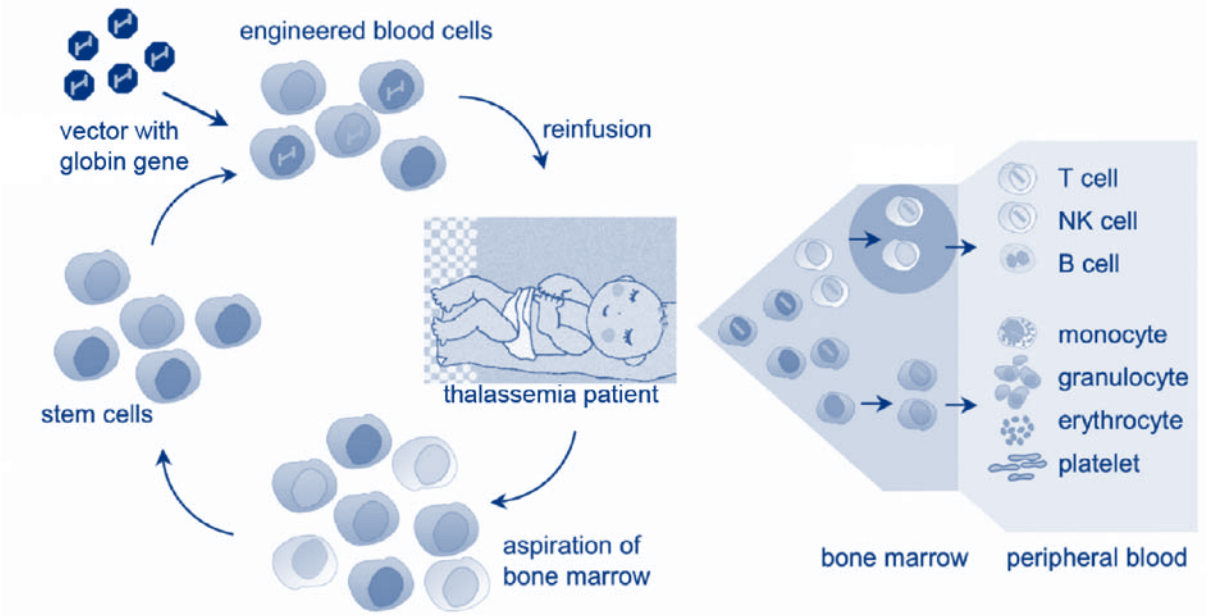


Figure 1
Gene therapy for the treatment of thalassemia

not cure the patient and *(ii)* in the long term it often results in iron overload which, when left untreated, is also lethal.

The drawbacks of both therapies call for an alternative curative therapy, which might be offered by gene therapy.
In gene therapy, a vector is a carrier molecule which is used to transport genetic material (i.e. a gene) into a cell[4]. Because viruses have the ability to efficiently transduce cells, modified viruses are often used as vectors. These virus-derived vectors are known as viral vectors.

A possible way to treat β-thalassemia using gene therapy is as follows. A vector containing either a β- or a γ-globin gene is co-cultured (ex vivo) with hematopoietic stem cells of a thalassemia patient[5]. The vector-particles will transduce these cells, and the globin gene will be integrated into the stem cell DNA. In this way the stem cells now contain a new and functional globin gene. As a result, cells derived from these stem cells will be able to synthesize functional hemoglobin (either fetal Hb, when transduced with the γ-globin gene, or adult Hb, when transduced with the β-globin gene). These transduced hematopoietic stem cells are then injected into the patient's bloodstream where they will find their way back to the bone marrow and start to produce red blood cells (see Figure 1).

Lentiviral vectors derived from HIV-1 have properties that make them superior to other vectors when it comes to the transmission of globin genes[5]. First of all, lentiviruses have the ability to integrate their genetic material (including a globin gene) into the DNA of non-dividing cells. They also have the capacity to transport a sufficient amount of genetic material (as much as 9-10kb). Furthermore, lentiviruses contain the rev response element, which enables a virion to hold unspliced full-length RNA. As stated above, the aim of this article is to review the effectiveness of lentiviral vectors and to assess the potential adverse effects of lentiviral gene therapeutics for the treatment of thalassemia major. Since no results were available from clinical trials, this systematic review mainly focuses on the use of gene therapy in murine models.

Methods

On January 10th 2010 we searched the MEDLINE database for articles concerning gene therapy for the treatment of thalassemia major. The MeSH-terms used for this search were: gene therapy, beta-thalassemia, genetic vectors and globins/genetics. More specifically, the search query was: *("gene therapy"[MeSH] AND "beta-thalassemia"[Major]) OR ("genetic vectors"[MeSH] AND "globins/genetics"[Majr])*. The references of the included articles were also searched for additional relevant articles.

For an article to be included in this review it had to contain original data and it had to be written in English. It also had to meet one or more of the following criteria: discuss the insertion of either a γ- or a β-globin gene with the use of a lentivector, discuss the development or testing of a lentivector suitable for such an intervention, discuss the transcription regulation of one of the previously mentioned globin transgenes, discuss the risks of adverse effects and/or the precautions that can be taken to prevent these effects.

To determine which of the search results matched the above criteria, all results were reviewed by title, abstract and ultimately by reading the entire text.

THAL or Hbb$^{th1/th1}$ is a murine model based on a naturally occurring mutation of the beta-major globin gene, causing homozygote mice to have a thalassemia intermedia phenotype[19].
Hbbth3/+ is a mouse with a manmade heterozygote deletion of both the β1- en β2-globin gene[20]. Homozygotes die prenatally. Heterozygotes have a severe thalassemia intermedia phenotype.
β2mnull NOD-SCID is immunodeficient mouse model that can be used to investigate xenografts[21] (in the case of Puthenveetil et al.[9] induced human stem cells)
C57Bl/6J is a healthy mouse model[22].

Panel 1
Background information on used murine models

Panel 2 Function of insulators

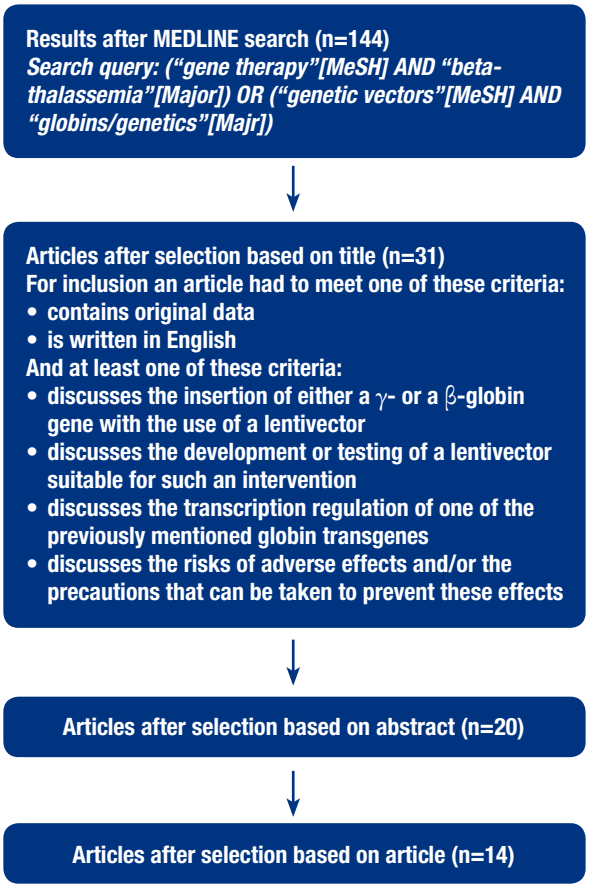
When inserting a gene in an existing strand of DNA, an enhancer is often inserted as well to increase transcription of the transgene. However, this can lead to adverse effects[23]; depending on the integration site and the used enhancer, the inserted enhancer can activate the promoter of a nearby host gene. In other words, inserting an enhancer into the host DNA can lead to deregulation of gene expression of normal genes. This can, for example, lead to overexpression of a oncogene, which in turn can lead to a higher risk of cancer.

An enhancer blocking insulator is a DNA element that can be used to form a shield between the inserted enhancer and a promoter, thus making it less likely for a promoter to become inappropriately activated[24].

Barrier insulators are elements that can protect a transgene from heterochromatin-mediated silencing, thereby increasing the chance of expression of that gene[24].

The cHS4-insulator possesses both of the above properties[24]. Using cHS4 can therefore prevent inappropriate activation of genes outside of the inserted domain and simultaneously protect the inserted gene against heterochromatic silencing.

Figure 2 Literature selection



Because different mouse models and genetic insulators play an important role in the development of the vectors, we also added background information on these subjects (Panels 1 and 2).

Results

Our literature search provided us with 144 results, of which 14 met our selection criteria and were included in this systematic review (Figure 2).

The different vectors

As mentioned previously this review focuses on the use of lentiviral vectors (other vector types will not be discussed). An overview of these vectors is shown in Table 1.

TNS9 is a lentiviral vector created by May et al.[6], wich contains the human β-globin gene and a locus control region (LCR) containing large fragments of the hypersensitive site (HS) HS2, HS3 and HS4. Testing this vector in a murine model ($Hbb^{th3/+}$) resulted in elevated plasma Hb levels. More specifically: 7.4 mmol/L with an average vector copy number (VCN) of 0.8 in peripheral blood cells, compared to untreated mice with a 5.0 mmol/L plasma Hb level. If TNS9 was used, the spleen size and iron depositions in the liver of the mice seemed to decrease. Based on these results, and taking into account that these results did not change over a period of 40 weeks, the authors assumed that TNS9 could be used for the sustained correction of anemia caused by β-thalassemia[7].

Lisowski et al.[8] carried out different modifications to TNS9; they ultimately came up with a vector with significantly increased expression. One of these modifications was the addition of HS1 to the LCR. Tested in $Hbb^{th3/+}$ mice, this modification alone caused the β-globin expression to rise by approximately 50%, up to 5.9 mmol/L Hb per VCN.

Imren et al.[9] reported the sustained correction (which lasted for more than 7 months) of β-thalassemia in mice using a lentiviral vector for the transfer of the β-globin gene based on a vector they previously used to successfully treat sickle cell anemia in mice. THAL/Hbb^{th1} mice were used to test this vector. The results were positive. There was an increase in Hb plasma levels (5.0 in untreated to 7.4 mmol/L in treated mice), but to achieve this result a high VCN of 3 was needed.

A different vector, the so called GLOBE vector[10], with a LCR-β-globin transcription element including a 2.7-kb section surrounded by LCR elements HS2 and HS3, was created by Miccio et al. This arrangement was connected to a fully working small β-globin gene with 265 bp of 5' and 300 bp of 3' flanking sequences.

In thalassemic th3/+ mice, the GLOBE vector caused an increase of the Ter119+ erythroid section (60.0 ± 2.9% of total bone marrow cells compared with 37.1 ± 1.8% in normal control mice)[10]. In mice transplanted with GLOBE-transduced cells the Ter119+ erythroid cells were significantly reduced (43.5 ± 2.9%, $P = 0.003$ in mock-transduced vs. transduced th3/+ group; $P > 0.05$ in wild type vs. GLOBE transduced th3/+ group).
The effect of the GLOBE vector was also examined in a murine model of thalassemia major th3/th3 fetal liver cells (FLCs). All of the mice transplanted with the GLOBE vector infected th3/th3 FLCs displayed long-term survival ($P < 0.0001$ th3/th3 GLOBE vs. th3/th3). Hematocrit (Hct), erythrocyte count and hemoglobin (Hb) level were enhanced compared with those of th3/th3 controls (Hematocrit: 39.49 ± 3.04% vs. 12.64 ±2.22%;

Table 1 - Overview of discussed vectors

Reference	Globin gene (and other genes)	LCR-elements	Mouse model	Serum Hb increase	VCN
May et al.[6,7]	β-globin	HS2, HS3, HS4	$Hbb^{th3/+}$	2.4 mmol/L	0.8
Lisowski et al.[8]	β-globin	HS1, HS2, HS3, HS4	Hbbth3/+	2.5 mmol/L	0.4
Imren et al.[9]	β-globin	HS2, HS3, HS4	$THAL/Hbb^{th1}$	2.4 mmol/L	3
Miccio et al.[10]	β-globin	HS2, HS3	th3/th3 FLCs	4.1 mmol/L	4
Puthenveetil et al.[11]	β-globin	Chicken HS4	$\beta 2m^{null}$ NOD-SCID	N/A	N/A
Persons et al.[12]	γ-globin	-globin HS40	C57Bl/6J mice	1.6 mmol/L	2.4
Zhao et al.[13]	- γ-globin, (MGMT and Mp)			- from 11% to 47%	0.7
	- γ-globin, (MGMT and EF1α)	HS2, HS3	Hbbth3/+	- from 24% to 86%	

(Puthenveetil et al. did not measure the serum Hb increase or VCN, because this was not possible with the mouse model they used.[11])
EF1 α = elongation factor 1 alpha, Hb = hemoglobin, HS = hypersensitive site, LCR= locus control region, MGMT = methylguanine methyltransferase, Mp = murine stem cell vir-us enhancer/promoter, N/A = not available, VCN = vector copy number

erythrocyte: 6.40 ±0.48 x106/ µl vs. 2.02 ±0.22 x106/ µl; Hb: 6.330 ± 0.670 mmol/L vs. 2.18 ± 0.31 mmol/L). Bone marrow cells harbored 4 vector copies per cell.

Puthenveetil et al.[11] created a lentiviral vector with the β-globin gene and the chicken hypersensitive site 4 (cHS4). The authors used transduced human CD34+ cells from thalassemia major patients and transplanted these into the $\beta 2m^{null}$ NOD-SCID mouse model. Puthenveetil et al. claimed to have fully corrected the β-thalassemia major phenotype using this method, but were unable to measure the increase in Hb, because this was not possible in the mouse model used in this study.

All the vectors discussed above contained the human β-globin gene. However, some of the articles in our results discussed vectors with the γ-globin gene. One of these vectors was created by Person et al.[12] In this study, normal murine bone marrow cells were infected with the d432 β-^{A}γ -3' RE vector[12] and transplanted into lethally irradiated normal C57Bl/6J mice (thalassemia intermedia). The polyadenylation site of HS4 element (decreasing its size from 756 bp to 445 bp) in the vector plasmid (yielding d432 β-^{A}γ) was deleted. Thirty-two weeks after transplantation, the pattern and level of γ -globin production were stable.

Using this method, human γ-globin mRNA expression was raised from 9% to 19% (mean, 11.8% # 2.4%) of the level of total mouse γ-globin mRNA[12]. This mRNA expression led to a level of 12% to 25% (mean, 15.7% # 3.2%) per vector copy per copy of γ-globin; 2.4 vector copies per cell were needed to obtain these results.

Zhao et al.[13] created and tested two different γ-globin/MGMT self-inactivating lentiviral vectors for correcting β-thalassemia in murine models. The first lentiviral vector employs an internal murine stem cell virus (MSCV) enhancer/promoter (V5-Mp-MGMT) to drive MGMT expression, while the other vector makes use of an internal cellular elongation factor 1 α (EF1 α) promoter with γ-globin expression cassette. γ-globin expression cassettes in both vectors were the same. These two cassettes are controlled by 3.1 kb of β -globin locus control region elements and a 130-bp β-globin promoter. The function of MGMT (methylguanine methyltransferase = alkyltransferase) is to restore cellular DNA damage at the O position of guanine. BCNU (1, 3-bis-chloroethyl-1-nitrosourea) has cytotoxic effects on alkylating agents. Such an effect can be prohibited with sufficient expression of MGMT. Mice transplanted with cells infected with the two vectors were randomly selected for injection with BCNU and O^6-benzylguanine, or with saline. F cells (erythrocytes that make γ-globin) in mice treated with BCNU that responded in the V5-Mp-MGMT group increased from 11% to 47% (P < .001), whereas untreated mice showed a drop from 5% to 2%. Moreover, the F cells in the responding V5-EF1-MGMT group expressed a strong increase after in vivo selection with BCNU (24% increasing to 86% F cells, P < .001). Bone marrow cells in the mice of the drug-treated group retained an average of 0.67 vectors per cell.

Adverse effects

One of the challenges in the development of a vector for the treatment of a genetic disease is to minimize the possibility of adverse effects that could be caused by the viral integration and the transgene product.

In 2008, Hargrove et al.[14] published a study on the possible genotoxic effect of globin gene transduction using a lentiviral vector. CFU-S cells, which are hematopoietic stem cells found in mice that give rise to erythroblasts, were transduced and analyzed. A total of 18 transduced cell clones were analyzed and compared to 15 untransduced clones. The transcription rates of the genes in a 300kb window on either side of the integrated gene were determined for all the transduced cells and compared to the transcription rates of those genes in untransduced cells. Six genotoxic events were found. Extensive in silico simulation suggested these were caused by the integration of the globin gene and were not merely due to chance. Hargrove et al. speculated that these events might be prevented by using an insulator.

To minimize the frequency of these genotoxic events, Hanawa et al.[15] developed a lentiviral vector containing an optimized cHS4 insulator. This vector, however, was tested for its effectiveness and not for its potential to reduce abnormal gene regulation.

Arumugam et al.[16] evaluated the possibility of oncogenesis caused by a lentiviral vector used for transferring γ-globin; they also looked at the effect of cHS4 on this process. They found that cHS4 could indeed significantly reduce the chance of interfering with other genes when using lentivirus vectors with the inclusion of the LCR.

Discussion

Gene therapy for β-thalassemia major is at the experimental phase. All the vectors we reviewed have only been tested in β-thalassemia intermedia and major mice. Most of the vectors have successfully treated nearly all the symptoms of β-thalassemia. After vector treatment hemoglobin concentration increased significantly[6-9, 12-13], while spleen size[7], Ter119$^+$ erythroid compartment[10] and iron depositions in the liver decreased[7] significantly. Hematocrit (Hct) and erythrocyte count levels were also enhanced[10].

The efficacy of a lentivirus vector can be improved through improved in vivo selection and in vitro selection of transduced hematopoietic stem cells (for example selection by MGMT/BCNU therapy[10]). Gene expression can be regulated through modification of the locus control region. Both HS1 and HS4 improved expression of globin at the mRNA and protein level. Therefore, HS1 and HS48 are important elements for obtaining therapeutic expression from globin vectors.

Insulators[11] can inhibit the action of a distal enhancer on a promoter. They also can act as an obstruction that shields the gene from the silencing effect of heterochromatin. This can improve gene expression and prevent the inserted promoter from interfering with other genes, consequently avoiding adverse effects.

We are aware that the number of articles used in this review is fairly low in comparison with the number of initial search results. This is due to two factors: *(i)* the selection criteria used, which focused on lentiviral integration methodology, and *(ii)* the fact that the field is too young to be covered entirely by one combination of MeSH terms. Because we did not want to exclude valid information useful for this review, we had to carry out two search actions. Our first one focused directly on gene therapy for beta-thalassemia (using "gene therapy"[MeSH] AND "beta-thalassemia"[Major]). We noticed that this resulted in insufficient information on the development of vectors and prevention of adverse effects, so we decided to carry out a second search action. This one focused more precisely on the vectors suitable for globin gene transfer ("genetic vectors"[MeSH] AND "globins/genetics"[Majr]) The latter term resulted in a large number of irrelevant articles, but also in several relevant articles that we were unable to find using other MeSH-term combinations.

In our opinion, future research on the treatment of thalassemia major using gene therapy should focus on the possible undesired side effects of the integration of a globin gene. There has been insufficient research that specifically focuses on the adverse effects of transferring a globin gene using the vectors discussed in this review. In view of the trial in Paris that began in 2004 on the treatment of X-SCID patients[17] we conclude that it is too early to start a clinical study at this stage. Nevertheless, a clinical trial has recently been carried out in Paris[18], of which we have not yet found any results. Although there is insufficient knowledge to warrant further clinical trials, we are looking forward to the results of the above trial.

Acknowledgments

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A review on the prevention of thrombus formation by S. Mansoni in its direct micro-environment

SCHISTOSOMA MANSONI: HIDING IN PLAIN SIGHT

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Although schistosomiasis mansoni constitutes a major health problem with over 200 million people infected worldwide, there is also much that we can learn from this blood dwelling parasite. One particular mechanism of interest is their ability to prevent thrombus formation in their direct micro-environment. This would be expected due to the fact that schistosomes expose a foreign surface to the bloodstream and cause partial occlusion of the blood vessels. Four mechanisms have been demonstrated and/or hypothesized so far that contribute to this remarkable achievement: The inhibition of factor XII, the expression of antigen Sm22.6 that inhibits thrombin, the expression of ATP-Dehydrolases that inhibit ADP-induced platelet activation and the possible expression of an echicetin-α ortholog that prevents platelet activation by blocking the platelet Ib-receptor. Discussion of the currently available information leads to the conclusion that although much is known about how S. mansoni can locally prevent thrombus formation, we are still some way off mapping out the complete mechanism through which this is achieved. Future research should therefore consolidate first and focus on the interaction between known mechanisms before new possibilities are being investigated.

Keywords
Schistosoma mansoni, coagulation, platelet activation, gene expression, proteome

Introduction

Schistosomiasis mansoni is a variant of the tropical infectious disease schistosomiasis, also known as bilharzia. The cause of this disease is a small helminth parasite. An infection with *S. mansoni* can occur through contact with stationary water. It is mainly seen in Africa, the Arabian Peninsula and South America (1). The parasite makes use of a certain type of freshwater snail that lives in these waters as an intermediate host.

The schistosomes leave the snail in the form of cercaria (larvae) which are able to penetrate the human skin. Once inside the human body, they migrate through the blood via the lungs to the liver. Here they mature into adult schistosomes. These are worms with a length of 7-22 mm. When full-grown, the schistosomes migrate to the portal vein where male-female pairs are formed. These couples move to nearby veins where they reside permanently.
The average lifespan of a *S. mansoni* parasite is 3 to 5 years, but there are also extreme cases known with schistosomes surviving up to 30 years in the human body (1). This is fascinating for two reasons: First of all the outer tegument of *S. mansoni* is a foreign surface that has a constant interaction with human blood, yet it does not provoke an adequate immune response. Secondly the schistosome-pairs can cause large occlusion of the vein in which they reside due to their relatively large size (fig.1), but do not cause thrombus formation as has been observed in other occlusive processes (e.g. atherosclerosis).

It has been known for a long time that *S. mansoni* has an overall negative influence on coagulation (2). However, an important distinction has to be made between its systemic and local effects. Systemic effects arise not because of the presence of adult schistosomes in the veins, but due to their eggs. These are produced at astonishing rates by the schistosome-pairs and can become trapped in body tissue. This can happen during migration of the eggs to the intestines prior to excretion with the feces or after embolisation, which occurs mostly in the liver and spleen. The eggs secrete proteolytic enzymes that provoke typical eosinophilic inflammatory and granulo-matous reactions, which are progressively replaced by fibrotic deposits. Chronic infection eventually leads to liver cirrhosis, causing impaired liver function. This leads to a diminished production of haemostatic proteins, thereby creating a misbalance in haemostasis (1).

The mechanism that is responsible for the *local* avoidance of thrombus formation by *S. mansoni* is fundamentally different from its systemic effect and far more subtle.
However, it is also more complicated and still not completely understood. The aim of this review is to create a comprehensive overview of all that is currently known about the local anti-thrombogenic effects of *S. mansoni*.
The complete understanding on how *S. mansoni* prevents thrombus formation in its micro-environment might facilitate the development of a new generation of anti-thrombogenic medicines. These could work strictly on a local level without systemic side-effects and could be applied as a coating on stents for example.

Methods

There are four known mechanisms through which *S. mansoni* exerts its antithrombogenic effect. To make sure that all relevant articles were found we have conducted a separate search for each mechanism.

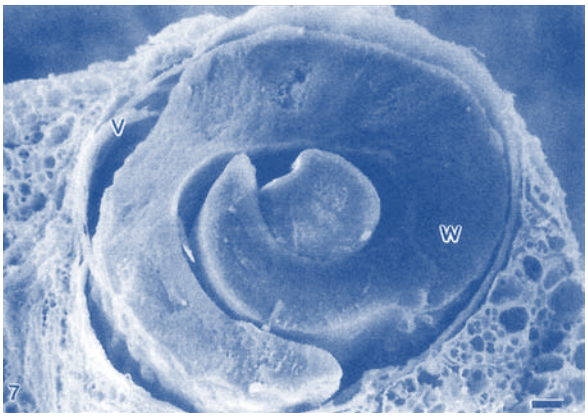

Figure 1
Schistosoma occluding a bloodvessel (file, S, 1995, J. Parasitol.81, 234-8) V=vessel, W= adult schistosome

1. The Hageman factor
On the 17th of March we searched in the PUBMED/MEDLINE database with the limits 'English' and 'human'. The following combination of MeSH terms was used: 'Schistosoma mansoni' AND 'Factor XII' OR 'Liver Failure/parasitology'. The articles were selected with the use of the following inclusion criteria: 1 The article concerns adult schistosomes and not a different life-cycle stage (e.g. eggs). 2 The article concerns coagulation effects and not immune responses. 3 The article concerns anticoagulation in the micro-environment of the parasite.

2. Sm22.6
On the 20th of March we searched in the PUBMED/MEDLINE database with the limit 'English'. The following combination of MeSH terms was used: ´Schistosoma mansoni´ AND ´Proteome´ OR `Platelet adhesiveness` OR ´Glutathione/ metabolism´ OR ´Enzyme Inhibitors/ metabolism´ OR ´Thrombin/ antagonists and inhibitors´ OR gamma-Glutamyltransferase/ metabolism´ AND ´Glutathione Transferase/metabolism´ AND ´Isomerism'. Inclusion criteria were the same as for Hageman factor.

3. ATP-Dehydrolases
On the 17th of March we searched in the PUBMED/MEDLINE database with the limit 'English'. The following combination of MeSH terms was used: 'Schistosoma mansoni' AND 'Apyrase' OR 'platelet aggregation inhibitors/immunology' OR 'chemokines, CC/agonists'. Inclusion criteria were the same as for Hageman factor with the addition that the article only concerned ADP release from platelets and not from other cells.

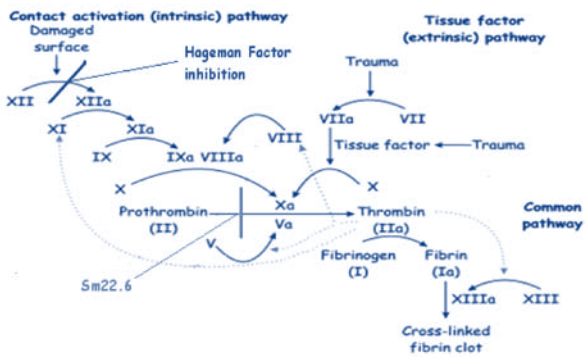

Figure 2
Schematic overview of blood coagulation

Echicetin-α ortholog
On the 21th of March we searched in the PUBMED/MEDLINE database with the limit 'English'. The following combination of MeSH terms was used: 'Chromosome Mapping' AND 'Schistosoma mansoni/ genetics' AND 'Genes, Helminth'. Inclusion criteria were the same as for Hageman factor.

Results and discussion

1. The Hageman factor
The Hageman factor (factor XII) is the first component of the contact activation pathway of blood coagulation. It is synthesized in the liver in an inactive form. Subsequently it circulates the bloodstream and is activated when it comes into contact with a negatively charged substance or material (3). The activation of factor XII is the starting point of a cascade reaction that activates the common coagulation pathway. Ultimately this results in the formation of fibrin (fig 2). Fibrin forms the network in a thrombus that holds the platelets together.

Three articles were found suggesting that S. mansoni is able to intervene in this process by irreversibly inhibiting factor XII (2,4,5). Although a systemic effect would be expected, since the inhibition is irreversible, empirical evidence suggests it to be a mechanism that exerts its effect only on a local level (2). The inhibition is achieved through secretory products and tegument bound proteins of schistosomes (2). The exact structure of these proteins remains to be determined (4). The exerted effect has been demonstrated through in vitro tests with extracts of S. mansoni tegument and whole worm homogenate (2,4,5). The tegument is the outer surface of the S. mansoni (fig.3). These tests showed a prolonged bleeding time in the presence of the tegument extract. It has also been demonstrated that once factor XII is activated it cannot be inhibited by S. mansoni anymore and coagulation will occur (2,4). The mechanism of factor XII inhibition is not unique to schistosomes. It has also been described in other helminth parasites such as Brugia malayi (4).

There are a few points of concern regarding this inhibition mechanism that was first described in 1977 (2).
Although it has been observed that tegument extracts prolong bleeding time and that it inhibits factor XII (2,4,5), it has not yet been clarified if these two effects are caused by the same component(s) of the tegument of S. mansoni.
A second point is that the Hageman factor itself only has a limited effect on thrombin activation. As demonstrated in fig. 2 thrombin is also activated by various other mechanisms (3). For this reason it remains to be determined to which degree inhibition of factor XII contributes to the prevention of thrombus formation observed in S. mansoni. A large role should probably not be expected since persons that suffer from inherited Factor XII deficiencies generally do not display any notable symptoms (6). Finally the results described in the relevant articles (2,4,5), are results from in vitro tests. It is not known if the results will be the same in vivo.

2. Sm22.6 antigen
Prothrombin plays a central role in blood coagulation. It is part of the common final coagulation pathway. Through activation by factor Xa and factor V it is converted into thrombin. The functions of thrombin are to convert fibrinogen into fibrin and to activate factor XIII. This factor forms covalent bonds that crosslink fibrin polymers, which ultimately results in the formation of a fibrin clot (fig.2).

Two articles were found that state that S. mansoni can intervene in this process (7,8). At the tegument surface of *S. mansoni* an antigen is expressed which intervenes with thrombin formation (7,8). This antigen is called sm22.6. Recombinant forms of this protein have been prepared artificially as a fusion protein called glutathione S-transferase. From this protein Sm22.6 can be released (9).
Sm22.6 consists out of 190 amino acids and has a molecular mass of 22.6 kDa (7). It can intervene in the formation of thrombin through direct and irreversible inhibition of α-thrombin and by reversible inhibition of γ-thrombin. This last process has a lower affinity than the first (7). These results have been demonstrated in Western Blot analyses (7). In vitro experiments also show that sm22.6 concentrations of 400-800 ng/ml result in a 3-4-fold delayed coagulation time (7).

In short, the Sm22.6 protein is fully characterized and its anticoagulant properties on human blood quiet well understood. This makes the expression of Sm22.6 the most established mechanism through which S. mansoni is able to prevent thrombus formation on a local level.

3. ATP-Dehydrolases
A thrombus consists out of platelets that are held together by a fibrin network. Sm22.6 and Factor XII inhibition have an effect on the coagulation cascade. Their aim is to prevent the formation of fibrin.
ATP-Dehydrolases aim at the second component that is required to form a thrombus: platelet activation and aggregation.

ADP is one of the most important agents in platelet aggregation (3). When a platelet becomes activated, it releases ADP out of small storage vesicles. By doing so, the platelet activates other platelets and also sets the coagulation cascade in motion (3). This ultimately leads to thrombus formation. ADP also potentiates the ability of other substances, such as chemokines, to stimulate platelet aggregation (10). Removal of ADP out of the extracellular environment eliminates platelet recruitment and can also result in the return of platelets to their resting state (11).
Extracellular ADP removal can be achieved through apyrases called ATP-Dehydrolases (ATPDases). These are enzymes that hydrolyze the tri- and diphospate nucleosides ATP and ADP into AMP. In humans these enzymes are part of the CD39-like gene family. This family consists out of five different isoforms (CD39 to CD39-L4) (12). Regarding prevention of coagulation ATPDases are mostly expressed on the surfaces of vascular endothelial cells, erythrocytes, leukocytes and platelets themselves (13). It is important to realize that ATPDases are not only involved in coagulation. They serve a purpose in many other processes as well, including immune responses (14,15). This is also relevant considering S. mansoni. However, the parasite's immunogenic properties are a topic on its own and beyond the scope of this review.

Six articles were found that suggest that *S. mansoni* has ATPDases as well (11,12,13,14,15,16). In 1992 the first ATPDase was characterized and localized on the external surface of the tegument of S. mansoni (13). It was called smATPDase1. Immediately it was hypothesized that this protein was part of the mechanism by which the parasite could evade a clotting reaction. This view has been supported by others as well (12,14,15). Further research revealed that smATPDase1 concerned a 66 kDa protein with two active isoforms which both had different catalytic efficiencies (14,15). It has also been found that smATPDase1 has a very high similarity with the human ATPDase CD39 (14).
This is in favour of S. mansoni, because it decreases the chance of an immune response. Molecular mimicry is an efficient way for a parasite to merge into the background of its host environment (14).
In 2006 the 55kDa smATPDase2 was discovered (12). This protein has a lot in common with the human ATPDase isoform CD39/L2 (12,16) and has some differences compared to smATPDase1. First the location within the parasite is different (12). SmATPDase2 is contained in an internal cellular structure of the tegument syncytium (12). The syncytium is the detergent insoluble fraction of the tegument between the apical membranes and the basal membrane.
The second difference is that smATPDase2 can be secreted while smATPDase1 is bound to the outer tegument surface (12). The current hypothesis is that smATPDase2 is being produced by the subtegumental cells, then transported across the tegument and finally secreted into the exterior environment (fig. 3). By doing so it complements the effect of smATPDase1 (12).

There are also some points of discussion concerning the ATPDases. So far a solid theoretical basis has been provided and it has been demonstrated that *S. mansoni* tegument extracts are able to inhibit ADP-induced platelet aggregation in vitro (13). However, the problem is that it has not yet been proven that the ATPDases are indeed responsible for this inhibition and if they are; to what degree. This is problematic, because it is know that there is also another possible way through which *S. mansoni* can prevent platelet aggregation (next section).

Another point of concern is that the ATPDases probably play a role in the evasion of a cytotoxic immune response as well (14,15). This suggests that the ATPDases could also be expressed for other reasons than to prevent platelet aggregation. This possibility is reinforced by the observation that the smATPDase proteins have been detected in many life stages of S. mansoni including those outside the human host (14).
A final point of concern is that although the ATPDases bear

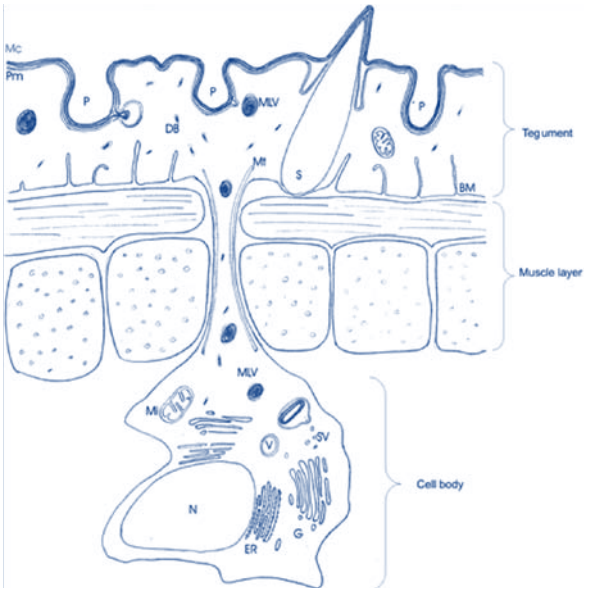

Figure 2
Diagram of the schistosoma mansoni tegument and an associated cell body (not to scale). BM: basal membrane; DB: discoid body; ER: endoplasmic reticulum; G: golgi apparatus; MC: membranocalyx; MI: mitochondria; MLV: multilaminate vesicle; MT: microtubule; N: nucleus; P: pits; PM: plasma membrane; S: spine; SV: shuttle vesicles; V: vacuole (21)

many similarities with human ATPDases they are not the same. SmATPDase1 contains some unique motifs in its sequence that may have an influence on its kinetics (14).

4. Echicetin-α ortholog

One article was found that suggested that *S. mansoni* might also be able to prevent platelet activation through a last, different mechanism (17). Von Willebrand Factor (vWF) is an important regulatory protein in platelet activation (3). Under normal conditions vWF circulates in the bloodstream without interacting with platelets. When it comes into contact with sub endothelial matrices like collagen vWF binds to it and becomes active (3). This can happen in case of vascular endothelial damage for example. Also when vWF is forced towards the endothelial cells in situations of high shear stress, like rapid blood flow in a narrowed vessel, it sticks to the endothelial cells and becomes active (18).

Activation of von Willebrand Factor is most likely caused by conformational changes of the vWF molecule (19). When vWF becomes active it expresses a high affinity for the glycoprotein Ib receptor. This receptor is present on the platelet surfaces. By binding both the vascular endothelia and the platelet surface vWF creates a situation that favours further platelet aggregation. This is complemented by the fact that activation of the Ib receptors in turn also causes platelet activation (3).

Schistosomes can cause large occlusions in the vessels (fig.1), thereby creating turbulence in the blood flow. For this reason they would benefit from a substance that could interfere in the mechanism described above. Indeed, analysis of the transcriptome of *S. mansoni* revealed transcripts for an ortholog of the echicetin-α subunit (17). Echicetin is a snake venom protein that inhibits binding of von Willebrand Factor to the platelet glycoprotein Ib receptor (20). By doing so it prevents platelet activation and thus has an anti-thrombogenic effect. This is supported by the observation that echicetin prolonged bleeding time in both in vitro and in vivo mouse models (20).

A logical theoretical basis has been hypothesized and there is evidence that *S. mansoni* has the potential of expressing an echicetin-α ortholog. This makes it assumable that this protein is also a part of the mechanism by which schistosomes prevent platelet activation and aggregation. However, the main concern is that although the possibility exists, it has not been determined yet if *S. mansoni* expresses the gene, where the protein is located and to which degree it proves relevant in the prevention of thrombus formation.

Conclusion and future recommendations

Four mechanisms have currently been identified by which *S. mansoni* avoids local thrombus formation. Out of these mechanisms, only the inhibition of factor XII through an unknown compound and the inhibition of thrombin through Sm22.6 have empirically proven to effectively lengthen the coagulation time of human blood (2,4,5,7). The demonstrated presence of enzymes in the outer regions of *S. mansoni* that are highly similar to human ATPDases strongly suggests a role of these in the prevention of thrombus formation as well (11,12,13,14,15,16). Finally, analysis of the *S. mansoni* transcriptome suggests the possibility of inhibition of the platelet Ib receptor through an echicetin-α ortholog (19).

The problem is that a large part of this evidence is based on genome, transcriptome and proteome studies. These studies make it possible to identify a mechanism, but give very little information about its actual effect and interaction with other mechanisms in vivo.

For this reason it can be said that although much is known about the possible ways in which *S. mansoni* can locally prevent thrombus formation, we are still some way off mapping out the complete mechanism through which this is achieved.

Fortunately, studies aimed towards revealing the genome, transcriptome and proteome of *S. mansoni* also have a very positive side. They provide directions for future research regarding the identification of possible new proteins involved (19,21). An important example of this is the fact that 55% of all genes specific to *S. mansoni* have no known assignable function yet and have no homology with any known gene (21). However, only 27% of all proteins characterized by proteomic studies are specific for S. mansoni (21). This means that between that 27% and 55% there are still possibilities of discovering new proteins, unique to S. mansoni, which could also play a role in the prevention of thrombus formation.

Then again, it could also be argued that since those genes do not show any similarity with any known mammalian gene, it is also very unlikely that they will encode for a protein of which it is known that it can be involved in anticoagulation in mammals (e.g. the echicetin-α ortholog). This would suggest that all anti-thrombogenic mechanisms relevant for *S. mansoni* have been identified. We tend to support this view more than the first. Therefore we believe that future research should consolidate first and focus on the interaction between known mechanisms before new possibilities are being investigated.

Acknoledgement

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a systematic review

The prevalence of the aryl hydrocarbon receptor-interacting protein gene in Familial Isolated Pituitary Adenomas

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Objective: **The primary objective of this review was to assess the prevalence of AIP gene mutations in families with Familial Isolated Pituitary Adenomas (FIPA). Secondly, we wanted to determine the association of tumor types and clinical characteristics with AIP gene mutations in FIPA.**
Methods: **We performed a computer-based systematic literature search. Three reviewers independently examined the inclusion and exclusion criteria.**
Results: **The prevalence of AIP gene mutations in Familial Isolated Pituitary Adenomas is 15%. Nearly all tumors associated with AIP gene mutations are somatotropinomas, prolactinomas, or mixed prolactin/GH-secreting tumors. Mean age at diagnosis is significantly lower in subjects with AIP mutations compared to subjects without AIP mutations. Also, the mean maximum tumor diameter is significantly larger in the group with AIP mutations than those without AIP mutations.**
Conclusions: **Screening for AIP gene mutations in FIPA is useful as a tool for genetic counseling and clinical surveillance. The clinical characteristics and tumor types associated with AIP gene mutations can help clinicians to recognize them.**

Keywords
Pituitary Neoplasms, Aryl hydrocarbon receptor-interacting protein, Adenoma, Germ-Line Mutation

Introduction

Pituitary adenomas are usually benign. However, patients with pituitary adenomas may have severe hormonal disturbances due to hormonal excess and/or visual disturbances due to the mass effect of the tumor. Clinically diagnosed pituitary adenomas occur in about one case per thousand of the population (1,2). However, autopsy and radiological studies show a prevalence of 16.7% (1). About 5% of the pituitary adenomas are familial (2,3). Familial Isolated Pituitary Adenomas (FIPA) compromise pituitary adenoma of all phenotypes occurring in a family (5). Germline mutations in families with FIPA have been identified in a gene encoding the aryl hydrocarbon receptor-interacting protein (AIP) (7). Box 1 shows known information about the AIP gene. In this review, we examined the prevalence of AIP gene mutations in families with FIPA. If there is evidence of a substantial prevalence of AIP gene mutations in FIPA, then this means that screening for AIP gene mutations could be a useful tool for genetic counseling and clinical surveillance of families with FIPA (5). Furthermore, we determined the association of tumor types and clinical characteristics with AIP gene mutations. These associations help clinicians to recognize AIP gene mutations.

In this review we addressed the following research questions.
1. What is the prevalence of AIP gene mutations?
2. Which tumor types are associated with AIP gene mutations in FIPA?
3. What are the clinical characteristics of AIP gene mutations in FIPA compared to FIPA without AIP gene mutations?

Methods

We completed a computer-based systematic literature search using the Medline electronic database on January 12, 2010. For our review we included English articles that were available in free full text for Erasmus MC. We used the Medical Subject heading 'Pituitary Neoplasms' in combination with the substance name 'aryl hydrocarbon receptor-interacting protein' using the term AND. Other reviews were excluded.

The inclusion criteria were:
1. Articles had to be original research studies.
2. The main subject of the article was the relationship between the AIP gene and Familial Pituitary Adenomas (FIPA).
3. The study contained data related to the prevalence of the AIP gene in FIPA or the clinical features of the AIP gene in FIPA.

Articles were excluded if they concerned only Sporadic Adenomas.

Three reviewers independently examined the abstracts regarding inclusion and exclusion criteria.

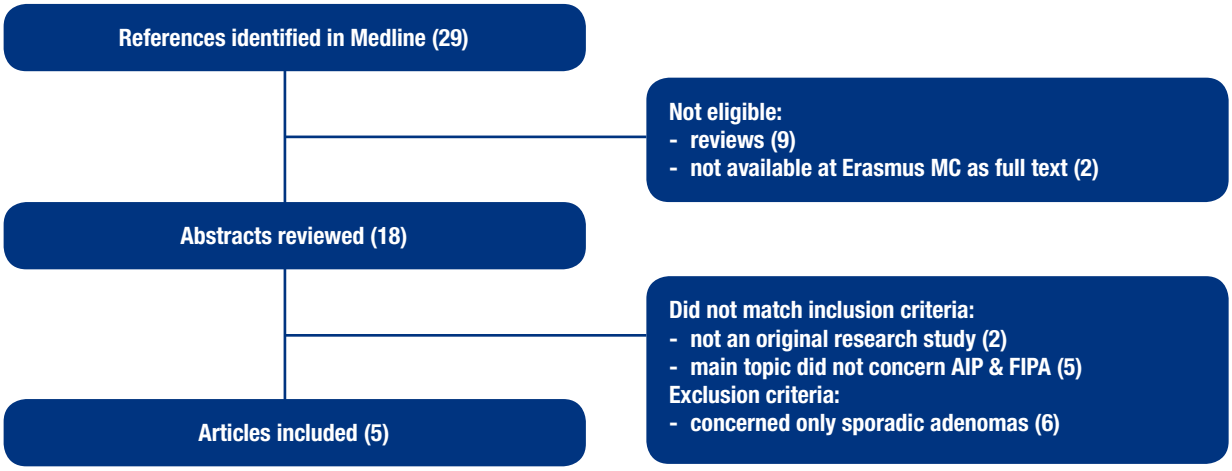

Figure 1
Flowchart of study selection.

Results

We identified 18 potentially eligible references. We excluded 13 of these references (Figure 1). The five remaining references provided data that satisfied the inclusion criteria.

Study Characteristics

The baseline characteristics of the included trials are shown in Table 1. Three of the included articles were case reports and two of the included articles were cohort studies. All of the three case reports described one family diagnosed with Familial Isolated Pituitary Adenomas (FIPA). Two case reports were performed in Brazil (8,9). One case study was performed in Australia/New Zealand (10). One cohort study investigated 73 families diagnosed with FIPA in several industrialized and developing countries (11). The other cohort study included 26 families in several industrialized countries (12).

Prevalence of AIP gene in Familial Isolated Pituitary Adenomas

The prevalence of the AIP gene in FIPA families is shown in Table 2. Both cohort studies investigated families with FIPA for the AIP gene mutations (11,12). The largest cohort study involved 73 families (156 patients) diagnosed with FIPA (11). Of this group, 22 families (26 patients) had mutations in the AIP gene. The other cohort study involved 26 families (67 patients) diagnosed with FIPA (12). Of this group, 9 families (31 patients) had mutations in the AIP gene. All three case reports described a different family diagnosed FIPA and carrying an AIP gene mutation (8-10).

Tumor types associated with the AIP gene

Different tumor types are associated with AIP mutations in Familial Isolated Pituitary Adenomas. The two cohort studies showed a majority of somatotropinomas, prolactinomas or mixed GH/prolactin-secreting tumors in FIPA families with AIP mutations (11,12). Also, one patient with a non-secreting tumor was found (11). One case report described three related patients with AIP gene mutations who had incipient gigantism, somatotropinomas, and a prolactinoma (10). Two other case reports concerned Brazilian families (8,9). One study described AIP gene mutations in three patients diagnosed with acromegaly (9). The other described three patients diagnosed with a somatotropinoma, a prolactinoma, and a mixed GH/prolactin secreting tumor, respectively (8). Adrenal carcinomas and lipomas were also observed in two different families with AIP mutations, although no relationship has been proven as yet (12).

Clinical characteristics of the AIP gene

Two studies report significant differences between FIPA families with AIP mutations compared to FIPA families without AIP mutations (10,12). Both showed that the mean age at diagnosis was significantly lower in subjects with AIP mutations. The mean maximum tumor diameter was also significantly larger in the group with AIP mutations. However, the proportion of patients with macro adenomas in the AIP mutation-positive group was not significantly larger compared with the proportion macro adenomas in the AIP mutation-negative group (11). It appears that the proportion of women with FIPA is larger than the proportion of men with FIPA (12). Three case reports described the clinical presentation of three families with a R271W mutation, an Y268X mutation or an E174fs mutation in the AIP gene. The families with the R271 or E174fs mutation showed variability in tumor phenotype, while the family with the Y268X mutation only had somatotropinomas (8,9,11). Surprisingly, two patients with acromegaly and the E174fs mutation had poor responses to Octreotide (8). In one cohort study, poor responses to somatostatin analogs were also observed in 7 of the 13 treated families (12).

Table 1 - Study Characteristics

Author	No. FIPA families	Study	Country families
Daly et al.	73	Cohort	Belgium, France, Italy, USA, Spain, Brazil, Argentina, the Netherlands, Czech Republic
Jennings et al.	1	Case study	Australia/New Zealand
Toledo et al.	1	Case study	Brazil
Leontiou et al.	26	Cohort	United Kingdom, Sweden, Australia, USA.
Naves et al.	1	Case study	Brazil

Panel 1. Background information about the function of the AIP gene."

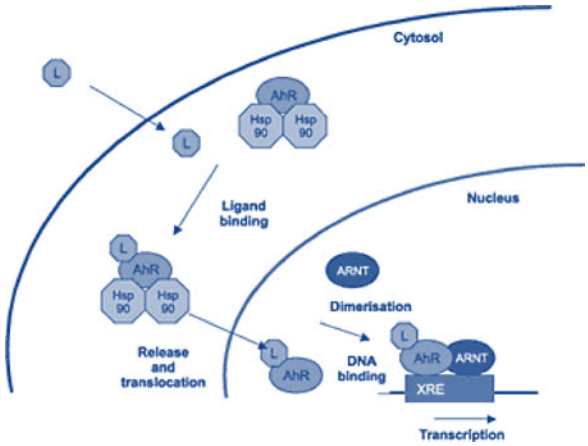

Figure 2
The role of the AhR and Hsp 90 molecules in relation to gene transcription (13)

The AIP gene, also known as XAP2 or AHRA 9, is located on chromosome 11q13 and encodes for 330 amino acids (8-12). The amino acids create a protein known as the Aryl hydrocarbon receptor interacting protein (AIP) (8-12). AIP contains three tetratricopeptide repeat domains (TPR) and an FK506 binding protein-type peptidyl-propyl cis-trans isomerase (FKP-PI) (11). The third TPR domain is necessary for interacting with the heat shock protein 90 (hsp90) and the aryl hydrocarbon receptor (AhR) (8,10-12). The protein, when bound, forms a complex with the AhR and two hsp90 molecules (8,10-12). When mutations occur in the last of the three TPRs and the protein loses the ability to interact with the hsp90, the AhR binding decreases by up to 80% (10). When a ligand activates the AhR, which is also called the dioxin receptor, the complex moves to the nucleus and stimulates gene transcription (10). The effect of the AhR is noted as being anti-carcinogenic and anti-toxic and plays a significant role in the cell cycle and programmed cell death (9,10).

Discussion/Conclusions

In this review we primarily searched for the prevalence of AIP gene mutations in Familial Isolated Pituitary Adenomas. Two studies showed data on the prevalence of the AIP gene in FIPA (11,12). One study found that 15% of the total FIPA families had AIP gene mutations (11). The other study showed a prevalence of AIP gene mutations in the FIPA families of 35 % (12). The difference might be explained by the fact that latter study was performed with patients who were not chosen randomly. Taking this into account, 15% is likely to be a better approximation of the actual prevalence of AIP gene mutations in FIPA. Further investigation on prevalence is necessary to confirm this. However, both studies show that screening for AIP gene mutations is useful. The prevalence of AIP gene mutations in FIPA is substantial, which shows the relevance of screening for the AIP gene mutations. Screening is useful as a tool for genetic counseling and clinical surveillance of families with FIPA.

Secondly, we determined the tumor types that are associated with AIP gene mutations in Familial Isolated Pituitary Adenomas. All references showed patients with somatotropinomas, prolactin-secreting tumors or combinations of these types (8-12). Adrenal carcinomas and lipomas were also observed in two families with AIP mutations (12). This suggests that more tumors could be associated with AIP mutations. Therefore, clinicians should be aware that adrenal carcinomas and lipomas could also be associated with AIP gene mutations in FIPA.

Finally, we showed the clinical characteristics of AIP gene mutations in FIPA compared to FIPA without AIP gene mutations. Mean age at diagnosis was significantly lower in patients with AIP gene mutations compared to patients without AIP gene mutations (11). The mean maximum tumor diameter was significantly larger in the group with AIP mutations relative to the group without AIP mutations (11). Both findings suggest that tumors become more aggressive when they have AIP mutations. Some patients with AIP gene mutations had poor responses to Somatostatin analogs (8,12). Further research is needed to determine the relationship between AIP mutations and poor responses to Somatostatin.

In conclusion, we showed that the AIP gene is mutated in 15% of the families with FIPA. Patients with AIP gene mutations in families with FIPA are diagnosed at a significantly younger age and have larger mean maximum tumor diameters. The expression of adenomas associated with AIP gene mutations is strongly related to somatotropinomas, prolactinomas and mixed GH/prolactin secreting tumors.

Table 2 - Prevalence AIP gene mutations in FIPA

Authors	No. FIPA families	Prevalence AIP gene in FIPA families	No. patients with FIPA phenotype	No. patients AIP mutation	Mutations found
Daly et al.	73	11 (15%)	156	26 (17%)	Q241X,Q217X,Q239X, R304X,R16H,R271W, K241E,G47_R54del, E174 frameshift, Q285 frameshift.
Jennings et al.	1	1(100%)	3	3(100%)	R271W
Toledo et al.	1	1(100%)	3	3(100%)	Y268X
Leontiou et al.	26	9 (35%)	67	31(46%)	E24X,R81X,R304X,C238Y,R304Q, c.749_823du, F269F, a double promotor.
Naves et al.	1	1(100%)	3	3(100%)	E174 frameshift

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a systematic review

Interfering with Huntington

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Objective: **In this review we will examine the current state of development in siRNA therapy for Huntington disease.**
Methods of Design: **We performed a search on pubmed on the 19th of January 2010. Using the following syntax: ("Huntingtin"[All Fields]) AND ("RNA Interference"[Mesh] OR "RNA, Small Interfering"[Mesh] OR "MicroRNAs"[Mesh]) AND English[lang] NOT Review[ptyp].**
Results: **This search yielded 58 results of which 43 were discarded using the inclusion criteria The majority of articles provided evidence of reduction of mutant Htt protein in the used models.**
Conclusions: **SiRNA therapy has been shown to have a positive effect on pathological processes connected to Huntington's Disease, and might prove to be a viable therapy in the future.**

Keywords
gene silencing, Huntington's disease, gene therapy, small interfering RNA

Introduction

Pathophysiology. Symptoms such as chorae and psychological changes have led to Huntington's disease's reputation as one of the most frightening neurodegenerative diseases. Its pathophysiology is not fully understood. Huntington's disease is thought to be an autosomal dominant disease caused by a mutation in the Huntingtin (HTT) gene. Individuals unaffected by Huntington's disease have alleles with a normal number of CAG repeats (with a range of 35 or lower). Individuals with 36-40 repeats may or may not develop Huntington's disease and more than 40 repeats means full penetrance [1]. More repeats tend to lead to an earlier onset [2].
Current research has resulted in the hypothesis that the mutant form of Huntingtin does not cause loss of function but rather causes toxicity (toxic "gain of function") [1]. This has been supported by the fact that heterozygous patients have similar clinical onsets as homozygous patients [3]. Knockout mice without the rodent form of Huntingtin die at an early stage in the embryonic gestation, proving that Huntingtin is essential for the development of the nerves and that a total loss of Huntingtin function is fatal [4].
The function of wild type Huntingtin and mutant Huntingtin is poorly understood but the current hypotheses point to involvement in various intracellular pathways, disruption of which may lead to cell death. Though Huntingtin is found in many parts of the body, the main pathological change in Huntington's disease patients is in the nervous system. One of these changes is severe atrophy of certain areas of the brain, the neostriatum. Another change is aggregation of Huntingtin fragments in the neurons [5].
The diagnosis of Huntington's disease is based on several disturbing symptoms. These symptoms are sudden uncontrolled movements and sounds, and a decreased sense of stability. Chorea, involuntary irregular movements, is the most striking symptom and is characterized by a specific way of walking. The typical HD patient also experiences psychiatric symptoms characterized by mood changes, rigidity, aggression and an overall change in behavior. The diagnosis is based on these symptoms and family history. Genetic testing, which is very sensitive and specific for HD, can confirm the diagnosis [6].
The gene defect can be found by means of a specific PCR test looking for a repeat in the CAG fragment of chromosome 4 [7]. When the CAG fragment is repeated more than 40 times, the test result is considered positive. Prenatal testing is a valid but rarely used diagnostic tool [1].

Current therapy

The lack of a curative treatment for Huntington's Disease has created an arsenal of symptom orientated medicines which are often based on clinical experience with Huntington or other neurological diseases with similar presentations [8]. As a result of this approach, the efficacy of the various treatments has in many cases not been properly assessed in clinical trials [8]. Placebo controlled trials show limited clinical potential because of low therapeutic effects or adverse events [8].
Chorea in a mild form is one of the least limiting aspects of Huntington and because the detrimental effects of the available treatments can be severe, it is only managed with drugs when the involuntary movements get more violent [9]. Therapeutic options then consist of dopamine blocking or depleting agents, glutamate antagonists, GABA and fatty acids [9]. The rationale for using GABA is that it is reduced in the brain of HD patients compared to the brains of controls [9]. Unsaturated fatty acids have been shown to inhibit dyskinetic movements in animals, possibly because of their positive effect on the quality of the membrane and have therefore been employed in trials [10-11]. Glutamate antagonists are being used because of the excitotoxin theory, which hypothesizes that Huntington symptoms are caused by an abundance of excitatory neurotransmitters such as glutamate [9]. Tetrabenazine, a dopamine depleting agent, shows the best anti-choreic results but its use is hampered by adverse events such as drowsiness, parkinsonism, depression, insomnia, nervousness and anxiety [8, 12]. Alternative drugs do not show convincing results or have not been properly tested in clinical trials [8]. Other Huntington-related involuntary movement disorders such as rigidity, akinesia

and dystonia have been largely ignored in placebo randomized controlled trials [13]. Problems with voluntary movements, such as speech impairment, swallowing problems and gait disorders, can be managed non-therapeutically, respectively, with speech therapy, dysphagia therapy and physiotherapy but neither this nor a therapeutic approach is evidence-based [9]. The treatment of psychiatric symptoms shows the same trend. No controlled trials have been undertaken to evaluate the best treatment for dementia, frontal lobe problems, behavioural and sexual dysfunction, psychotic symptoms or depression [13]. There are leads, though: depression has many treatment options and small, non-blinded studies have shown beneficial results in the other areas [13].
Attempts to modify disease progression have also been made with agents like Baclofen, coenzyme Q10+Remacemide, creatine and ethyl-eicosapentanoic acid [14]. None of those showed significant decreases in deterioration in randomized controlled trials but the study of coenzyme Q10, which is essential for the mitochondrial electron transport and has free radical scavenging activity, suggested a reduction in the decline of cognitive, behavioral and functional capacities [14-15]. A new trial has been started to confirm this beneficial result [14].

SiRNA
The last few years RNA interference (summarized in figure 1 [16]) has been a hot topic. It is a process in which small interfering RNAs (siRNA) bind to a cell's innate RNA (RNA which complements the siRNA base order) and thereby knock down the activity of the gene involved [17]. The realization that, in theory, with RNA interference any gene could be knocked out has led to its proposed use in battling many single gene disorders. Micro RNAs are based on the same principle, and also silence mRNA of the host cell by producing a RISC complex. In the human cell miRNAs are an important regulation mechanism [17]. Huntington's disease is a prime candidate for experimenting with siRNA. Specific mutant Htt silencing siRNA, or their uncleaved form (short hairpin RNA), are transduced into the brain cells using a vector. Both viral vectors (based on a genetically modified virus) and non-viral vectors have been used.
Two forms of siRNA can be discerned in HD related research. Non allele-specific silencing makes use of siRNAs that do not distinguish between the wild type and mutant form of the Htt gene, simply because both consist of CAG repeats. Therefore both forms of the Huntingtin protein are down regulated. Loss of wild type Huntingtin may lead to toxicity and contribute to HD pathogenesis [18]. This has led to the development of allele-specific silencing, which can distinguish between the two types and only depletes the mutant form.
These approaches are subjected here to a systematic review of the various HD siRNA related research projects.

Methods

A Pubmed search using the following syntax was conducted on the 19th of January 2010: ("Huntingtin" [All Fields]) AND ("RNA Interference" [Mesh] OR "RNA, Small Interfering" [Mesh] OR "MicroRNAs" [Mesh]) AND English[lang] NOT Review[ptyp]. All studies which performed an experiment with anti-Huntingtin siRNA's on animals or cell lines were included. From the articles we extracted the data on the type of mouse or cell, the beneficial and adverse events and how they were obtained, the virus and study design, and the follow-up time.

Results

The Pubmed search yielded 58 results of which 43 were discarded when applying the inclusion criteria. The flowchart of this process is depicted in Figure 2. Table 1 summarizes the study characteristics of the retrieved articles, Table 2 the toxicity and efficacy.

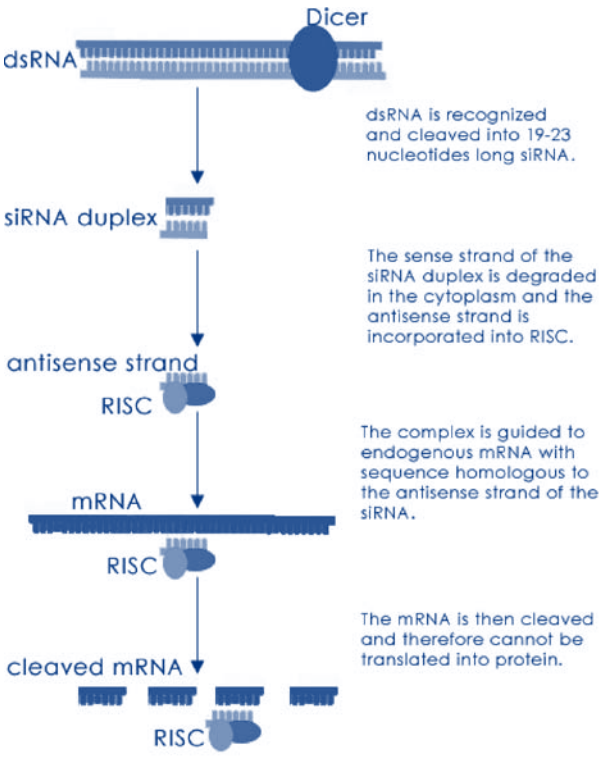

Figure 1
Mechanism of small interfering RNA gene regulation.

Study Design
The majority of the studies used viral vectors to transduce either mice or cell lines with the siRNA genetic material. Huntington Disease model mice and healthy mice that were transduced with mutant Huntingtin material were studied. The same goes for the cell lines: both Huntington fibroblasts and infected cells were used. Two articles described the use of miRNA for the same purpose [19-20]. From the 15 studies, 12 targeted a sequence that is located on all Huntingtin (mouse, man or both) proteins. Three studies [21-23] designed a mutant specific siRNA by either targeting a D2642 deletion of one of the four tandem GAG repeats located on exon 58 (38% of mutant HD alleles and 7% of wild type HD) [21, 23] or by directing the siRNA to the Single Nucleotide Polymorphism rs363125 [22]. The deletion and the polymorphism are not the cause of the disease but are linked to the poly CAG tail [21-23].

Toxicity
The articles in this systematic review all used a number of techniques to show toxicity in histological samples, such as immunohistochemistry. One technique was proving the loss of DARPP-32, using staining, a protein which is seen as a sign of neural degeneration. Htt Aggregates were also analyzed by staining. Northern blots and histological analyses show a disparity between the toxicity stemming from miRNA treatment and shRNA treatment. Microglial activity was also analysed with RT-PCR, showing a significant reduction of toxicity by miRNA (relative compared CD11b mRNA, compared with formulation

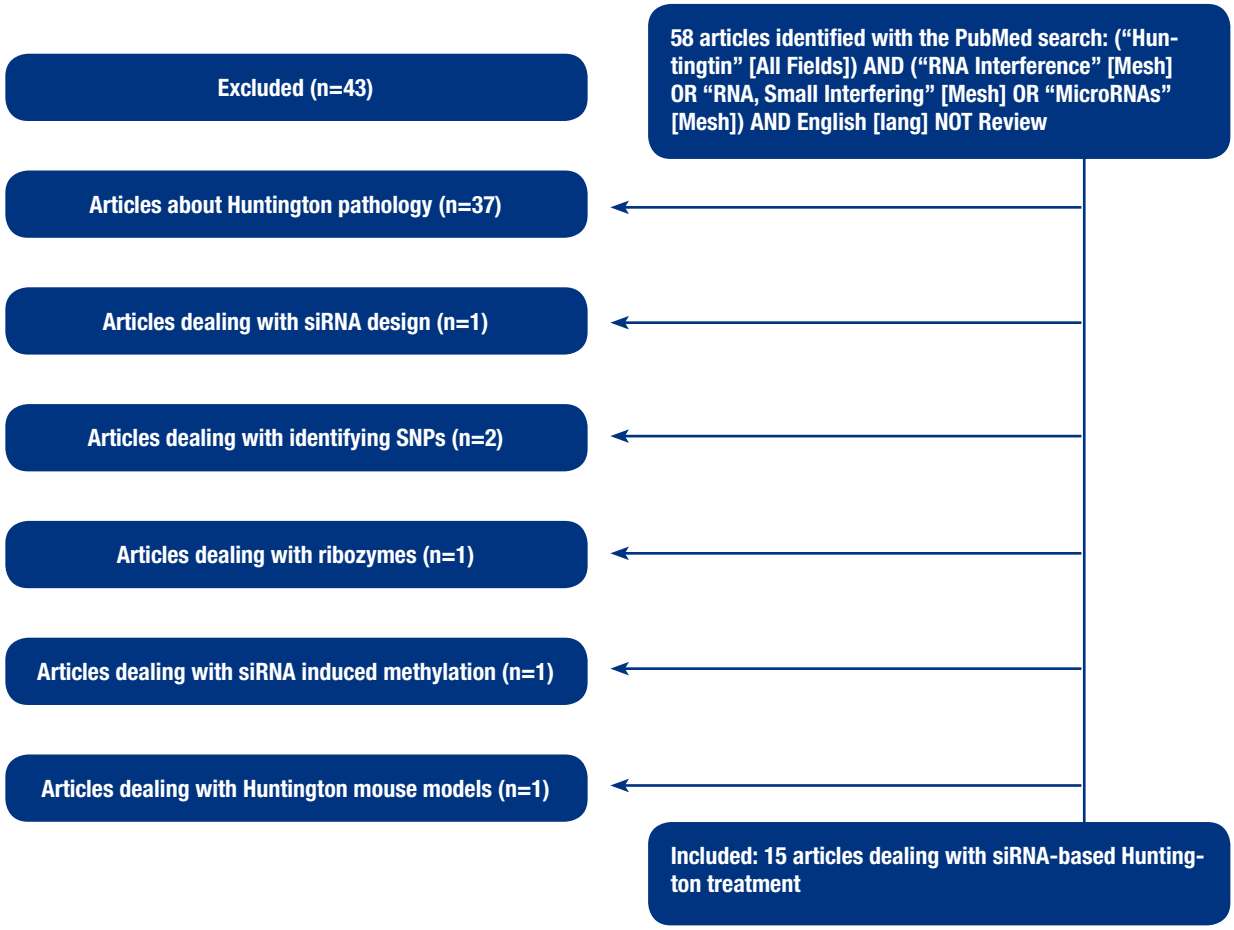

Figure 2
Flowchart of the PubMed search

buffer: miRNA ≈ 2,5 and shRNA ≈5,0, P<0.001) [19].
Two studies [19, 24] performed a microarray analysis to map the changes in molecular pathways as a result of non-specific RNA interference by comparing the transduced cells to control cells. One of them [19] found 107 downregulated and 366 upregulated genes. The genes that changed more than two times were involved in lipid metabolism or were destined for the extracellular space (downregulated) or intracellular processes such as microtubule or cytoskeleton formation and synaptic transmission (upregulated). The genes that are corepressed by REST, which is inhibited by Huntingtin, increased 1,76 times but this was a non-global increase. A comparison between this data and other arrays showed that some proteins changed in the same direction while others changed in the opposite way. In both of the studies the Huntingtin pathway related genes were the ones that changed the most [19, 24].
The mutant Huntingtin specific studies showed no significant decrease in wildtype Huntingtin and did not report any siRNA (allele specific) related toxicity [21-23].

Efficacy
Efficacy of gene silencing was measured in different ways. Much used methods were RT-PCR and protein Western blots. Practically all of them showed significant improvements. Histological changes were also noted, such as the lowering of pathological aggregates in brain cells [24-25].
Both non-allele specific and allele specific [21-23] gene silencing showed improvements in downregulating mutant Htt, same as siRNA and miRNA [19-20].

Some of the articles also looked at the motor skills of the rodents (e.g. by putting rodents on a rotarod or looking at clasping, retracting of the limbs done by neurologically impaired rodents). They showed significant improvement [19, 26-28].

Discussion/Conclusions

Search term. We originally constructed a Pubmed search term that contained the "Huntington Disease" Mesh-term. However, the results of this search did not contain a relevant article about allele-specific silencing which we identified in the process. Even though the article is called "Allele-specific silencing of mutant Huntington's disease gene" the term "Huntington Disease" was missing from the list of Mesh keywords. We therefore replaced "Huntington Disease"[Mesh] with "Huntingtin"[All Fields] because Huntingtin is the disease causing protein in Huntington Disease and we figured it would be mentioned in all relevant articles. This new search yielded the same relevant articles as the first search and in addition it rendered additional articles. A drawback of the new search strategy was the increase in the number of irrelevant articles that were related to Huntington pathology.

Efficacy
Many of the research articles listed in the results table have shown that gene silencing with the use of siRNA is an effective method of down regulating the mutant Htt protein. The positive results are not restricted to only protein or gene expression, but also include the results of clinical motor skills tests. The rodents treated with siRNA have shown significant improvement; the

Table 1 - Overview of the study characteristics (disease model, vector, RNAi construct and timeframe analysed) of included articles.

Article	Model	Vector	siRNA	Timeframe
Allele specific silencing Huang et al				
Hu et al (2009) [21]	Human HD fibroblasts (GM09197) containing the D2642 deletion, Htt:151 CAG repeats; Wt: 21	Cationic lipids	SiRNA4 spanning the D2642 deletion	Unknown
Zhang et al (2009) [23]	Human HD fibroblasts (amongst others GM09197), HeLa cells, SH-SY5Y	HiPerfect transfection reagent	SiRNA4, I-Htt, RI-Htt (Reverse, zero-function I-Htt)	72 hours
van Bilsen et al (2008) [22]	Human HD fibroblasts (GM04022): heterozygous on position rs363125 (adenosine or cytosine)	Lipofectamine 2000	Contains the SNP on position 16	48 hours
Non-allele specific silencing				
Boudreau et al (2009) [19]	B6C3F1/J HD-N171-82Q mice; C2C12 HD-N171-82Q Htt cells	AAV1	SiRNA2.4; SiRNA 8.3; miRNA2.4	5 months
McBride et al (2008) [20]	CAG140 heterozygous knockin mice	Recombinant AAV serotype 2/1	3 ShRNA: sh2.4, sh8.2 and sh30.1 (target sequences in exon 2, 8 and 30 of HD mRNAs.) Mi2.4	15 weeks
Franich et al (2008) [29]	Rapid-onset HD rat model using AAV vector-mediated gene transfer of a mutant Htt construct into the brain	AAV expression constructs encoding shRNAs targeting HD70	ShRNAs targeting HD70 and ShRNA targeting EGFP (control)	2 weeks
Huang et al (2007) [25]	R6/2 HD mouse model Viral transgenic HD mouse model	HC-Adenoviral vector	ShRNA targeted to exon 1 of the Htt gene	4 weeks
Harper et al (2005) [27]	N171-82Q mouse model, HEK 293 cells	AAV serotype 2	SiRNA2.1 (Human Htt)	4 Months
Rodriguez-Lebron et al (2005) [30]	R6/2 HD mouse model	Recombinant AAV-5	SiHUNT-1 (nucleotides 262–281, 5'UTR). SiHUNT-2 (342–363, upstream of the CAG repeat domain)	4 Months
DiFiglia et al (2007) [26]	Rapid-onset viral transgenic mouse model of HD	Cholesterol-conjugated small interfering RNA duplexes	cc-siRNA-Htt	2 weeks
Wang et al (2005) [28]	R6/2 HD mouse model, httQ72-d1EGFP cotransfected mice, Cos-7 cells	ExGen 500	siRNA-HDExon1	4-17 Weeks
Drouet et al (2009) [24]	Wister rats infected with Htt171-82Q via a lentivirus, C57/BL6 mice	Lentivirus	SiRNA1.1 (human specific exon 2); 3 (human and mouse exon 2); 6 (human and mouse exon 3/ 4); 13 (universal exon 8/ 9)	9 months
Machida et al (2006) [31]	HD190G mouse model	Lentiviral vector-mediated delivery	EGFP attached to the HD190G	12 Weeks
Chen et al (2005) [32]	HEK293, DAOY, cerebellar medulloblastoma glioblastoma	psiRNA-hH1-zeo vector + Sleeping Beauty Transposon	SiRNA against exon 1 or 4	5 Months
Gary et al (2007) [33]	HEK 293 cells Mouse L-cells	-	ShRNA	-

AAV: Adeno-associated Virus; cc: cholestrol conjugated; DAOY: cerebellar meduloblastoma cells; EGFP: Enhanced green fluorescent protein; ExGen 500: polyethylenimine solution; HEK293: Human Embryonic Kidney 293; HD: Huntington Disease; Htt: Huntingtin; miRNA: micro RNA; RNA: Ribo Nucleic Acid; SiRNA: Small interfering RNA; SNP: single nucleotide polymorphism

rodents had longer endurance on the rotarod and showed less neurological impairments like clasping. Positive results are extended to every sort of gene silencing used in the articles, non allele and allele specific, shRNA and miRNA.

Toxicity

Several articles highlighted the toxicity caused by shRNA independent of HDh RNA silencing. Research was done to test whether using miRNA instead of shRNA would yield better results. McBride et al found that moving the HD sequences into a miRNA form significantly reduced toxicity without loss of gene-silencing efficacy [20]. A follow up research article found new evidence to support the use of miRNA instead of shRNA [19]. It's worth noting that another shRNA experiment has not shown the same level of toxicity as McBride and Boudreau; Drouet et al show normalized DARPP-32 and increase GABAergic neuron survival [24]. The toxicity may have been caused by the silencing of off-target genes after antisense RNA build-up. Three of the articles were about allele specific gene silencing with shRNAs. These articles show no toxicity, whereas a few of the articles with non allele specificity do show toxicity, showing that the allele specific method might be a good choice.

Table 2 - Toxicity and efficacy of included articles.

Article	Toxicity	Efficacy
Allele specific silencing		
Hu et al (2009) [21]	100 nM, combination of siRNA and lipids. No decrease in wildtype Htt with s4.	60% reduction of the mutant Huntingtin compared to a mismatched siRNA (Western blot)
Zhang et al (2009) [23]	Increase in caspase-like activity with I-Htt, not with s4. Higher caspase response to H2O2 in I-Htt treated cells, not in s4 cells. Measured in a fluorometric protease assay after 72 hours, both compared to wt.	41% reduction of the mutant Huntingtin protein (Western blot). 51% reduction of the mutant RNA (RT-PCR). Both s4 compared to RI-Htt. 51% (HeLa) and 63% (Sh-SY5Y) reduction in the luciferase signal. 40% reduction in abnormal nuclear morphology compared to RI-Htt. All after 72 hours.
van Bilsen et al (2008) [22]	Nearly all Huntingtin lost with the non-specific siRNA. Wildtype Huntingtin not lower than controls with the specific siRNA (PCR with molecular beacons). Both after 48 hours.	80% reduction in mutant RNA compared to the controls ($p < 0.01$; PCR with molecular beacons) after 48 hours.
Non-allele specific silencing		
Boudreau et al (2009) [19]	After four months: More loss of DARPP-32 (immunohistochemistry) with siRNA2.4 than with mi2.4. Fivefold increase in CD11b mRNA (a readout for microglial activation) with SiRNA2.4, threefold increase with mi2.4 (QPCR). More HD2.4 antisense RNA with siRNA than miRNA, build-up of shRNA precursors (Northern blot). Microarray after 4 weeks, comparing siRNA gene expression to control. 107 genes downregulated, 366 upregulated in siRNA/miRNA Group >2,0 fold ($P < 0.01$). 1,76 fold rise in REST target genes.	60%, $P < 0.01$ reduction in endogenous and mutant Htt in mice with miRNA2.4. 75% reduction in endogenous and mutant Htt after 20 weeks with miRNA2.4 compared to HD-N171-82Q mice. The mi2.4 treated mice showed improved rotarod performance at 14 ($p=0.02$) and 18 weeks ($p=0.02$). Failure to normalize weight compared to control mice. Trend to improved survival of the mi2.4 treated mice compared to HD-N171-82Q treated mice (75%; 50%; p=0,1)
McBride et al (2008) [20]	Loss of DARPP-32 and an increase in microglial activity. Striatal toxicity in mice injected with sh2.4 and sh3.0	PCR shows significant reduction of HDh mRNA expression (≈60%) compared to control mice [$F(3,11)=32.3$, $P<0.001$]. Western blot shows significantly reduced Htt protein levels [$t(8)=3.9$, $P<0.01$]. Results were the same for siRNA and miRNA.
Franich et al (2008) [29]	Not determined	RT-PCR (showing signficant reduction of mRNA [85%, P=0.012]) and Western blot (reduction, P=0.064)
Huang et al (2007) [25]	Not determined	Significantly less aggregates in the brain compared to controls (10% aggregates compared to 80%)
Harper et al (2005) [27]	Decline in rotarod performance in injected mice at 10 week, resolved at 18 weeks.	Dose dependent reduction of Htt mRNA (20% -10ng; 50% -100ng; 60% -100ng shRNA plasmid) in HEK 293 cells (QPCR). Reduction in HD-N171-82Q mRNA with 51–55% (QPCR) and Htt inclusions and polymers (fluorescence) in the mouse brain. No normalization of the weight, improvement of stride length ($P< 0.0001$) and rotarod performance.
Rodriguez-Lebron et (2005) [30]	No abnormal changes in cellular morphology or astrocyte activation (cresyl violet and GFAP staining) ten weeks after infection. Reduction of ppEnk (16%) and DARPP-32 (30%) with si-HUNT-2 (ISH)	75% ($p<0.001$) reduction in mHtt mRNA (Northern blot), 60% reduction in Htt expression (Western blot) compared to GFP controls in HEK293 cells. 80% ($p=0.01$) reduction in Htt mRNA compared to the uninjected site (QPCR), 25% ($p=0.01$) reduction in Htt protein (Western blot) after 10 weeks. 31% ($P=0.04$) reduction in NIls compared to contralateral side after 10 weeks (ImageJ). All siHUNT-1 results, siHUNT 2 comparable. Efficient transduction of the vector throughout the striatum but not the white matter tracts (ISH). Increase of ppEnk (24%) and DARPP-32 (16%) with siHUNT-1 (ISH). No weight or rotarod performance improvement. Increased ability to stay on the rotarod [$F(1,22) = 16.7$, $p = 0.0005$]. Less clamping of the hind leg (20%; 80% not treated) after 16 weeks
DiFiglia et al (2007) [26]	No specific immunogenic response	Significantly less clasping (33% clasping untreated versus 18% treated in group 1, $P\leq0.01$; 75,6% versus 51,9% in group 2, $P\leq0.02$) and decreased Htt protein expression (56%, P=0.03)
Wang et al (2005) [28]	Not determined	>80% decrease of Htt (Western blot) compared to siRNA control in cells. Decrease in Htt in the cotransfected mice (Western blot). Decrease in weight loss ($p < 0.01$ by ANOVA), increase in longevity ($p < 0.0001$ by log-rank), increase in rotarod performance ($n = 11$; $p < 0.05$ by t-test), reduction in Htt aggregates in treated R6/2 mice compared to the untreated.
Drouet et al (2009) [24]	SiRNA 6 and 13 mediated down regulation of molecular pathways linked to Htt functions. Alterations of HD pathway genes in mice (micro-array on dissected brains). Wildtype Htt knocked down rats showed no difference in GABA neuron survival or Htt inclusion load. Same levels of LacZ-positive cells after nine months with siHtt1.1 and siHtt6 in mice.	SiRNA 1.1; 3 and 6 prevented loss of DARPP-32 and NeuN expression and induced partial recovery of the glucose metabolism in the rat. Decrease in loss of DARPP-32 expression and ubiquitin positive inclusions. Significant reduction in HD pathology (DARPP-32, ubiquitin inclusions) after 9 months.
Machida et al (2006) [31]	Cytoplasmatoic and nuclear aggregates	60% reduction in Htt (fluorescence). 73,0% reduction of protein, 80% ($p < 0.0001$) reduction in the number of aggregates in the striatum in the model mouse (filter trap assays)
Chen et al (2005) [32]	Not determined	80% reduction in mRNA (QPCR) in HEK293 cells with siRNA 4 (exon 1). Transduction by the vector. 90% ($P < 0.001$) reduction in DAOY cell mRNA content, 93% ($P < 0.01$) reduction in protein levels (Western blot) 3 – 4 months after transduction with SB.
Gary et al (2007) [33]	Not determined	No difference in Htt expression

AAV: Adeno-associated Virus; cc: cholestrol conjugated; DAOY: cerebellar meduloblastoma cells; EGFP: Enhanced green fluorescent protein; ExGen 500: polyethylenimine solution; HEK293: Human Embryonic Kidney 293; HD: Huntington Disease; Htt: Huntingtin; miRNA: micro RNA; RNA: Ribo Nucleic Acid; SiRNA: Small interfering RNA; SNP: single nucleotide polymorphism

Vectors

AAV vectors are often used in the articles we analyzed. The AAV vector has several advantages such as transduction into post mitotic cells, a low immunogenicity [34]. Its disadvantage of a limited insertion capacity (~4,7 kb [34]) is not a problem in the case of siRNA treatment. One article describes how the vector spreads throughout the CNS [30]. It efficiently spreads throughout the striatum but not through the white matter, showing that especially neuronal cell bodies are targeted after injection into the brains. It remains to be seen how the vector distributes in the human brain, which is several orders of magnitude larger than that of the mouse.

Mouse models

Different types of mouse models were used. Many of the articles use the R6/2 mouse model, though its true merit has been questioned. Other HD medicines shown to be effective in animal models were not as successful in humans [35]. Another, newer, model has also been used, a virally transfected rodent with a rapid-onset. Researchers using this model believe that this model is a better approximation of the HD pathofysiology in humans.

Conclusion

SiRNA therapy has been shown to have a positive effect on pathological processes connected to Huntington's Disease, and might prove to be a viable therapy in the future.

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Review

The association between vitamin D and cancer risk

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Vitamin D is known to be important for calcium homeostasis and optimal skeletal growth, but in recent years more and more non-musculoskeletal effects of vitamin D have been discovered. For example, epidemiological studies have shown a link between low vitamin D levels and cognitive disorders, auto-immune diseases, cardiovascular diseases as well as an increased risk for certain types of cancers. In this article we discuss the anti-cancer activity of vitamin D.

Introduction

Vitamin D metabolism

In the human body sunlight promotes the conversion of 7-dehydrocholesterol into cholecalciferol. In the liver cholecalciferol is hydroxylated into 25-hydroxycholecalciferol, 25-(OH)D3. Subsequently a second hydroxylation step takes place in the kidneys, resulting in formation of the active form of vitamin D, 1,25-(OH)2D3. This promotes intestinal absorption of calcium and phosphorus, parathormone-independent reabsorption of calcium and provides calcium mobilization from the bones.[3]

Before 1985 it was generally accepted that the kidneys were the only organs of the body responsible for 1,25-$(OH)_2D_3$ production. However, in 1985 Schwartz et al. reported that cultured cancer cells from the prostate were able to convert 25$(OH)D_3$ into 1,25-(OH)2D3. Since then it has been reported that many normal tissues and various cancer cells are capable of making 1,25-$(OH)_2D_3$.[4-6]

Besides its key role in calcium homeostasis and healthy bones, vitamin D has an essential physiological function in almost every organ of human body.

In this overview we will address the following question: are vitamin D levels associated with cancer risk?

Vitamin D and sunlight

The relationship between UV light exposure, UV damage and skin cancer is well known. This knowledge can result in a lifestyle where UV light exposure is avoided, especially for the elderly. This is often because they have a history of skin cancer or are afraid of developing this disease. However, because sunlight is the most important source of this vitamin, they also risk vitamin D deficiency. Exposure to sunlight produces about 90% of the daily vitamin D requirement. The other 10% is obtained from dietary sources like fatty fish (herring, mackerel etc.), eggs and fortified foods.1

The following groups are at risk for vitamin D deficiency: [7-9]

- the elderly
- institutionalized or housebound people
- dark-skinned individuals
- refugees, especially veiled women
- people with inflammatory bowel disease, e.g. Crohn's disease
- obese persons
- individuals with cholestatic liver disease
- individuals with Systemic lupus erythematosus who avoid sunlight exposure

In children, Vitamin D deficiency results in rickets and in adults in osteoporosis and osteomalacia. Vitamin D deficiency has many symptoms; patients may have muscular pain, skeletal pain and there may be increased incidence of falling. As a result, the elderly are at risk for fractures and muscle weakness.[10]

On the other hand, ultraviolet radiation (UVR) is responsible for the majority of skin malignancies. In 1936 Peller reported that people with skin cancer had a lower incidence of non-skin cancers. He concluded that skin cancer could be protective against internal cancers.[2] Since then, epidemiological, clinical and experimental studies have indicated that vitamin D may reduce the risk of certain solid organ cancers. The incidence of several types of cancer is high in the elderly; therefore it is important for them to have sufficient exposure to sunlight so they obtain adequate vitamin D. This could protect them against several diseases, possibly including various cancers.

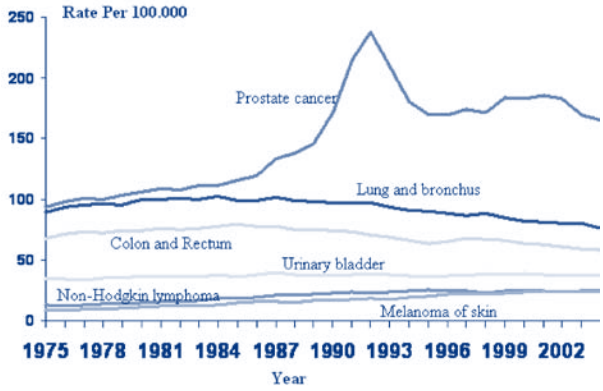
Table 1 - Adequate intake and acceptable upper limit of vitamin D in micrograms per day[33]

Age	Light skin and sufficient sun exposure*	Dark skin, insufficient sun exposure	Upper limit
0 – 11 months	5	10	25
1 – 3 years	5	10	50
4 – 50 years	2.5	5	50
51 – 60 years	5	10	50
61 – 70 years	7.5	10	50
71 and older	12.5	15	50
Pregnant women	7.5	10	50

*Sufficient sun exposure means at least 15 minutes of exposure with at least the hands and face uncovered.

CANCER

In 2008 more than 1.4 million new cases of cancer were diagnosed in the USA. Prostate cancer in men and breast cancer in women were the most frequently diagnosed cancers in that year, followed by lung and colorectal cancer.
After heart disease, cancer was the most frequent cause of death in the USA.[11]



The above figure illustrates the incidence of several cancers in men during the period 1975 – 2004.[11]
The sharp decrease in the incidence of prostate cancer after an initial increase is probably due to the introduction of prostate cancer screening with PSA.

Vitamin D and cancer

In 1915 Hoffman was the first to describe the association of cancer mortality with sun exposure and latitude.[12] In 1936 Peller reported that persons who had skin cancer had a lower incidence of non-skin cancers. He concluded that skin cancer could be protective against internal cancers.[2] More than 65 years ago, Apperly suggested a link between solar radiation and lower cancer mortality in North America.[13] Gorham et al. and Garland et al. also showed a negative correlation between vitamin D deficiency, sun exposure, latitude and risk for developing various types of cancers.[14,15] In a recent study Grant and Holick reported that between 50,000 and 63,000 Americans and 19,000 and 25,000 adults from the UK die prematurely every year from cancer due to vitamin D insufficiency.[16]

Approximately 200 of human genes have vitamin D response elements. Many of these genes encode for proteins which are important for regulating cell proliferation, differentiation and cell apoptosis.[17]

There is significant evidence for the relationship between vitamin D and cancers of the colon, breast and prostate. Many studies have reported conflicting data for the link between vitamin D and other cancers (e.g. esophagus, stomach, lung, pancreas, bladder, kidney, uterus, ovarian, multiple myoma and non-Hodgkin lymphoma).

Results

Colon carcinoma

In 1989 Garland et al. studied the association of serum 25(OH) D3 and cancer risk for the first time. They found a relationship between high levels of serum 25(OH)D3 and reduced colon cancer incidence.[18] In a meta-analysis Gorham et al. found that an increase of 84 nmol/L in serum 25(OH)D3 level led to a 50% reduction in the incidence of colorectal cancer.[19]

In the higher latitudes of the USA, such as New York and New Hampshire, the solar radiation is less than in the lower latitudes. Notably, the incidence of colon cancer is also higher in the northern part than the southern part of the USA. For example, the incidence of colon carcinoma was 17.3 per 100,000 in New York, and only 10.1 per 100,000 in Arizona during the same period. This was probably due to more sunlight exposure in Arizona.[20] This inverse correlation between sunlight and colon cancer could mean that adequate serum vitamin D levels prevent colon carcinoma.

In a National Health and Nutrition Examination Survey III (NHANES III) cohort an association was also found between 25(OH)D3 and colorectal cancer mortality. Persons with a 25(OH)D3 level higher than 80 nmol¬/L had a 75% reduction in mortality due to colon cancer compared to persons with lower levels of vitamin D. A serum 25(OH)D_3 level >95 nmol/L correlated with 55% reduction in colorectal risk, compared to those whose serum 25(OH)D_3 level was < 40 nmol/L.[14,21]

Breast cancer

In 2008 was breast cancer the second leading cause of cancer-related death among women in the USA. In a NHANES III cohort, Freedman also reported that women whose serum 25(OH)D3 was more than 62 nmol/L had a 75% decrease in mortality due to breast cancer.[21] In two other studies the authors concluded that there was a 58% lower risk of breast cancer in women with vitamin D levels more than 95 nmol/l compared to women with a 25(OH)D_3 level lower than 37.5 nmol/L.[22,23]

In a dose-response meta-analysis Garland and colleagues reported that women with the highest levels of 25(OH)D_3 in their blood had a reduced risk of breast cancer. A total of 1760 women were stratified in 5 groups from the lowest 25(OH)D_3 to the highest levels.[24] There was an evident dose-response association. The highest breast cancer rates were found in the group with the lowest 25(OH)D_3 levels (less than 32 nmol/L). The cancer rates were clearly lower in women with serum 25(OH) D3 above 130 nmol/L. The higher levels of serum 25(OH)D_3 levels led to a 35% risk reduction for breast cancer.[24]

Prostate cancer

Prostate cancer is an increasingly common disease in men. In 2008 it was the second leading cancer-related cause of death in the USA. In that year, 25% of all new cancer cases in US were prostate cancer.[11]

There is conflicting data on the association between 25(OH) D3 levels and prostate cancer risk. The incidence of aggressive prostate cancer in men with serum 25(OH)D_3 lower than 70 nmol/l and 1,25$(OH)_2D_3$ less than 77 pmol/L was twice as high than in men whose serum levels were higher.[25] However, this association was not confirmed by Jiyoung Ahn et al. in a large prospective study. In fact, they reported an increased risk of aggressive prostate cancer associated with higher levels of 25(OH) D_3.[26]

Ovarian cancer

There is a strong inverse correlation between serum vitamin D concentration and ovarian cancer mortality. Geographical latitude is also a determining factor. In northern latitudes, the

mortality rates were higher, probably because of insufficient vitamin D levels due to less solar radiation.[27,28] However, in a recent case-control study Toriola et al. did not observe a significant correlation between serum 25(OH)D_3 and ovarian carcinoma risk.[29]

Table 2 - Overview of types of cancer and risk reduction due to sufficient vitamin D levels.

Cancer type	Vitamin D levels risk reduction for cancer
Colon cancer	Serum 25(OH)D_3 >84 nmol/l : 50 – 55%
	risk reduction [14,19,21]
Breast cancer	Serum 25(OH)D_3 >95 nmol/l : 35 – 58%
	risk reduction22-24
Prostate cancer	Conflicting data [25,26]
Ovarian cancer	Conflicting data [27-29,32]
Other types cancer	Conflicting data

Discussion

In recent years our knowledge about the role of vitamin D in preventing different types of cancer has progressively increased. Many studies have indicated that sunlight exposure has beneficial effects on the outcome of many cancers. At the same time it is also known that UV irradiation is one of the most powerful suppressors of immune responses which cause erythema, edema and initiation of skin neoplasms. [2,14,16,18,19,21,31]

There are indications of the significant anti-cancer activity of vitamin D, but no hard evidence to confirm this activity. Only for colon cancer and breast cancer have there been more studies showing a positive correlation between higher levels of vitamin D with lower cancer risk. There is mixed data for other types of cancer. For example, some studies found a positive association between prostate cancer and higher vitamin D levels, but this has not been confirmed in every study.

Nevertheless, Holick advised people who are at risk for vitamin D deficiency to expose their face, hands and arms for 5 or 10 minutes to sunlight at least two or three times a week . This is especially important in the months April to October, since this is the period with the most hours of sunlight during the day. This exposure leads to adequate vitamin D levels.30 These recommended brief daily exposures are safe with regard to the risk for developing skin cancer.

The serum level of vitamin D is clearly not the only factor involved in the etiology, pathology and prognosis of cancer. Furthermore, it still unclear if adequate levels of vitamin D and sufficient sun exposure could prevent or treat any type of cancer. More research is needed to demonstrate the exact role of vitamin D in the types of cancer discussed in this review.

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33. *Gezondheidsraad: naar een toereikende inname van vitamine D 2008*

Abstract

Recurrences, vaccinations and residual long-term symptoms in GBS and CIDP

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Supervising staff member: Prof. dr. P.A. van Doorn

Introduction

Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyradiculo-neuropathy (CIDP) are immune-mediated polyneuropathies associated with a variable clinical course and recovery. After the recovery phase, however, most patients are able to walk again. GBS is usually preceded by an infection and is characterized by acute and rapidly progressive muscle weakness in all extremities. CIDP patients have a slower onset of weakness. A molecular mimicry reaction of the immune system, mostly after an infection, is a possible cause of GBS/CIDP. Some case reports have been published that have linked vaccination to GBS/CIDP. Although hypothesized regularly as a cause of GBS/CIDP due to the effect of a vaccination on the immune system, little is known about the consequences and risks of having vaccinations – like the yearly flu vaccination – after an immune-mediated polyneuropathy. These risks might include deterioration or a full relapse of the disease. If we can determine the risk of recurrence or deterioration of GBS/CIDP and the association with flu vaccinations, the necessity of vaccinations after an immune mediated-polyneuropathy could be reconsidered. In addition, little is known in general about the development of relapses and the general recovery, fatigue and quality of life many years after the initial disease. In this study we therefore addressed the following research questions:

- Is relapse or deterioration associated with vaccinations?
- What is the long-term outcome of GBS/CIDP or the final degree of recovery?

Methods

We sent a self-designed questionnaire to 461 members of the Dutch society of neuromuscular disorders with questions on vaccinations and relapses. It also contained various standardized and well validated sub-questionnaires like the numeric pain rating scale (NPRS), the fatigue severity scale (FSS), the Rotterdam Disability scale (RDS) and the short form health survey (SF-36), for quality of life. From their responses, we determined the frequency of recurrent GBS, whether vaccinations were associated with recurrences of GBS or deteriorations in CIDP, and we assessed the prevalence of common autoimmune disorders, pain, fatigue and the impact on quality of life after GBS and CIDP.

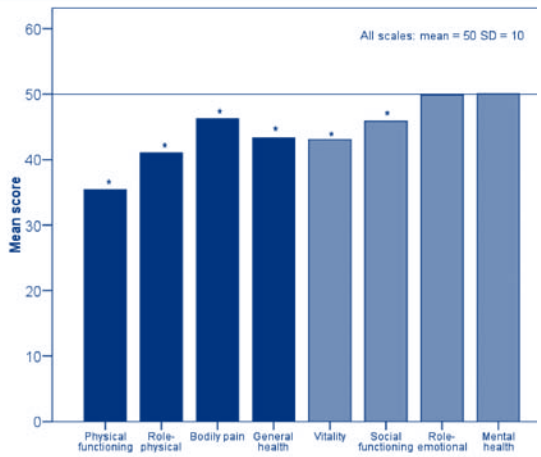
Results

Questionnaires were returned by 245 GBS patients and 76 CIDP patients (response rate 70%). Of the respondents, 9 patients reported confirmed recurrent GBS, 2 reported both GBS and CIDP and 9 patients reported having a relative with an immune-mediated polyneuropathy. Common auto-immune diseases were reported by 9% of GBS patients and 5% of CIDP patients. None of the 106 GBS patients who received a flu vaccination (total of 775 vaccinations with a range of 1-37 vaccinations per patient) reported a recurrence of GBS. Of the CIDP patients who received a flu-vaccination, 5 out of 24 reported an increase in symptoms (range 1-17 vaccinations per patient). Pain or severe fatigue were reported in about 70% of patients after GBS (median 10 years) or CIDP (median 6 years). Quality of life was significantly reduced even many years after the onset of GBS or CIDP (Figure 1).

Discussion

The occurrence of relapses in GBS patients, the combination of GBS and CIDP in patients or in families and the presence of other common auto-immune diseases may indicate a genetic susceptibility factor. Our study suggests that the seasonal flu vaccinations seems safe in patients who have had GBS. Furthermore, relapses in GBS patients do not seem to be associated with the yearly flu vaccination. In CIDP patients, however, flu vaccinations might induce a minor risk of deterioration. Several years after the diagnosis of GBS or CIDP, a significant number of patients still reported residual symptoms, such as pain and severe fatigue, as well as reduced quality of life. From this study we conclude that patients should not be advised to avoid vaccinations after experiencing GBS/CIDP; this is an important recommendation for daily practice. Furthermore, to increase the degree of recovery and outcome over the long term, additional research should be done to improve treatment options.

Figure 1
SF-36 health survey of GBS and CIDP patients (norm-based scoring)



Cross-sectional study: Median 10 years after diagnosis of GBS and 6 years after onset of CIDP

Based on:
Recurrences, vaccinations and long- term symptoms in GBS and CIDP, K. Kuitwaard, M.E. Bos Eyssen, P. Blomkwist-Markens and, P.A. van Doorn. J Peripheral Nerve Syst. December 2009

Cerebellar Contributions to the Processing of Saccadic Errors

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Introduction
Saccades are fast eye movements that direct the line of sight to a target in the visual field. Repeated post-saccadic visual errors can induce modifications of the amplitude of these saccades, a process known as saccadic adaptation. An essential part of saccadic adaptation is the assessment that a saccadic error has occurred. The anatomical substrate of saccadic error processing has yet to be elucidated. We performed two functional magnetic resonance imaging experiments using the same experimental paradigm. The first experiment focused on activity in the cerebral cortex while processing saccadic errors, the second experiment focused on the cerebellum.

Materials and methods
Data were acquired on a 1.5T MRI- scanner. Simultaneous in-scanner stimulus presentation and eye movement recording was performed with Avotec SV 7021 fiber optic glasses. The same block design paradigm was used in both experiments. Subjects were instructed to look at a yellow dot, i.e. the target. In the first active condition, the target jumped horizontally back and forth from 9 degrees eccentric to the left to 9 degrees eccentric to the right. In the second active condition, the target on the right was shifted from 9 degrees eccentric to a variable position between 5.1 and 12.9 degrees on the right during the saccade towards it. These random target shifts induced random saccadic errors while preventing saccadic adaptation to occur. In the baseline condition, subjects looked at a stationary dot.

Results
The condition in which the subjects made random saccadic errors predominantly produced significantly more cerebellar activation (vermis VIII, lobules VIII-X, left lobule VIIb).

Discussion
These results suggest a possible role for areas outside the oculomotor vermis of the cerebellum in the processing of saccadic errors. Future studies of these areas with, e.g., electrophysiological recordings, are warranted to reveal the nature of the error signals that drive the amplitude modifica¬tion of saccadic eye movements.

Full text in The Cerebellum, 2009, Sep;8(3):403-15.
http://www.springerlink.com/content/xu20664672471555/?p=daa3b622704b4698ad19841ac1141f24&pi=28

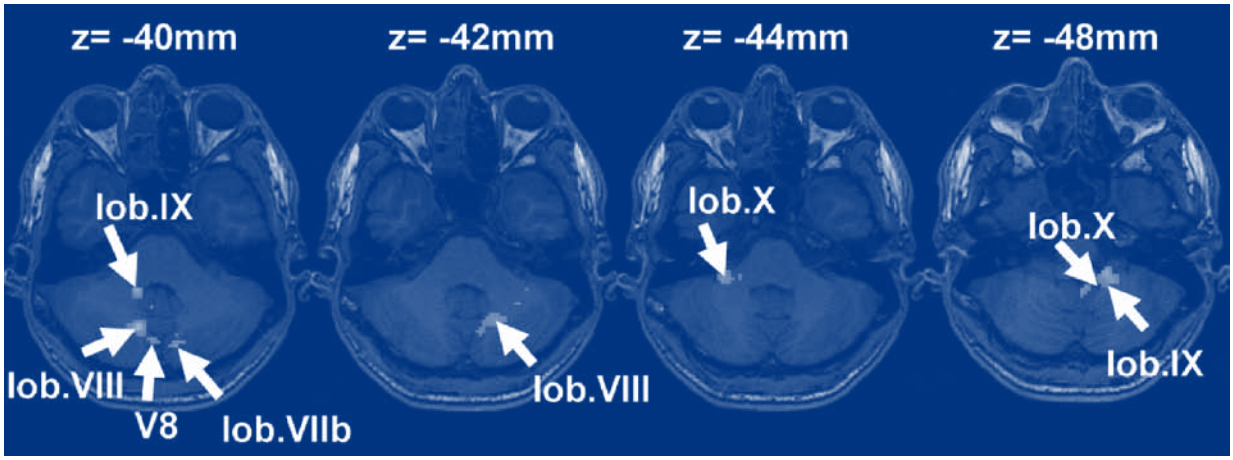

Figure 1
Four axial slices showing areas of activation of the direct comparison ([random-step > baseline] > [no-step > baseline]) in the cerebellum experiment (lob. = lobule; V8 = vermis VIII). All areas were thresholded at $p < 0.05$ corrected for multiple comparisons at cluster level and a minimum cluster size of 10 voxels.

Abstract

Baseline characteristics and statistical power in randomized controlled trials: selection, prognostic targeting, or covariate adjustment?

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** IMPACT: International Mission on Prognosis and Analysis of Clinical Trials in TBI*

Key Words
covariate adjustment, heterogeneity, randomized controlled trials, selection criteria, statistical power, traumatic brain injury

Previous Publication: **The full manuscript describing this research work was previously published in the journal Critical *Care Medicine:* Roozenbeek B et al., Baseline characteristics and statistical power in randomized controlled trials: selection, prognostic targeting, or covariate adjustment? Crit Care Med. 2009 Oct; 37(10): 2683-90.**

Background and Objective
Heterogeneity is common among patients selected for randomized controlled trials (RCTs). This heterogeneity may be caused by differences in baseline characteristics (e.g., age, gender or race), disease severity (e.g., mild vs. severe injury) and pathophysiology (e.g., intracranial hematoma vs. diffuse axonal injury). Treatment effects may be undetected due to lack of statistical power. We aimed to investigate the potential benefits of different approaches for dealing with heterogeneity in traumatic brain injury (TBI) studies.

Methods
We performed simulation studies on individual TBI patient data of 3 observational studies and 6 RCTs from the IMPACT database (n=8033). We investigated the statistical power and efficiency of RCTs in relation to [1] strict selection according to baseline characteristics (time window ≤ 8 hours; age ≤ 65; ≥ 1 reactive pupil; motor score > 1; GCS ≤ 8), [2] prognostic targeting (i.e., excluding those with a relatively extreme prognosis), and [3] covariate-adjusted analysis (i.e., adjustment for 3 baseline characteristics). Statistical power was expressed as the required sample size for obtaining 80% power. Efficiency was expressed as the relative change in study duration, reflecting both gains in power and adverse effects on recruitment. Treatment effects were simulated for 6-month unfavorable outcome, according to the Glasgow Outcome Scale, both uniform (treatment effective in all patients) and targeted (treatment only effective in those with a 20-80% risk for unfavorable outcome).

Results
For a uniform treatment effect, selection resulted in a sample size reduction of 33% in the observational studies and 5% in the RCTs, but decreased recruitment by 65% and 41% respectively (Figure). Hence, the relative study duration was prolonged (observational studies: +95%, RCTs: +60%). Prognostic targeting resulted in sample size reductions of 28% and 17%, and increased relative study duration by +5% in observational studies and +11% in the RCTs. Covariate adjustment reduced sample sizes by 30% and 16% respectively and did not affect recruitment. A targeted treatment effect led to larger sample size reductions by selection (observational studies: 47%; RCTs: 20%) and prognostic targeting (observational studies: 49%; RCTs: 41%) and smaller adverse effects on recruitment.

Conclusion
The benefits of selection and prognostic targeting in terms of statistical power of RCTs are reversed by adverse effects on recruitment. Covariate adjusted analysis in a broadly selected group of patients is advisable if a uniform treatment effect is assumed, since there is no decrease in recruitment.

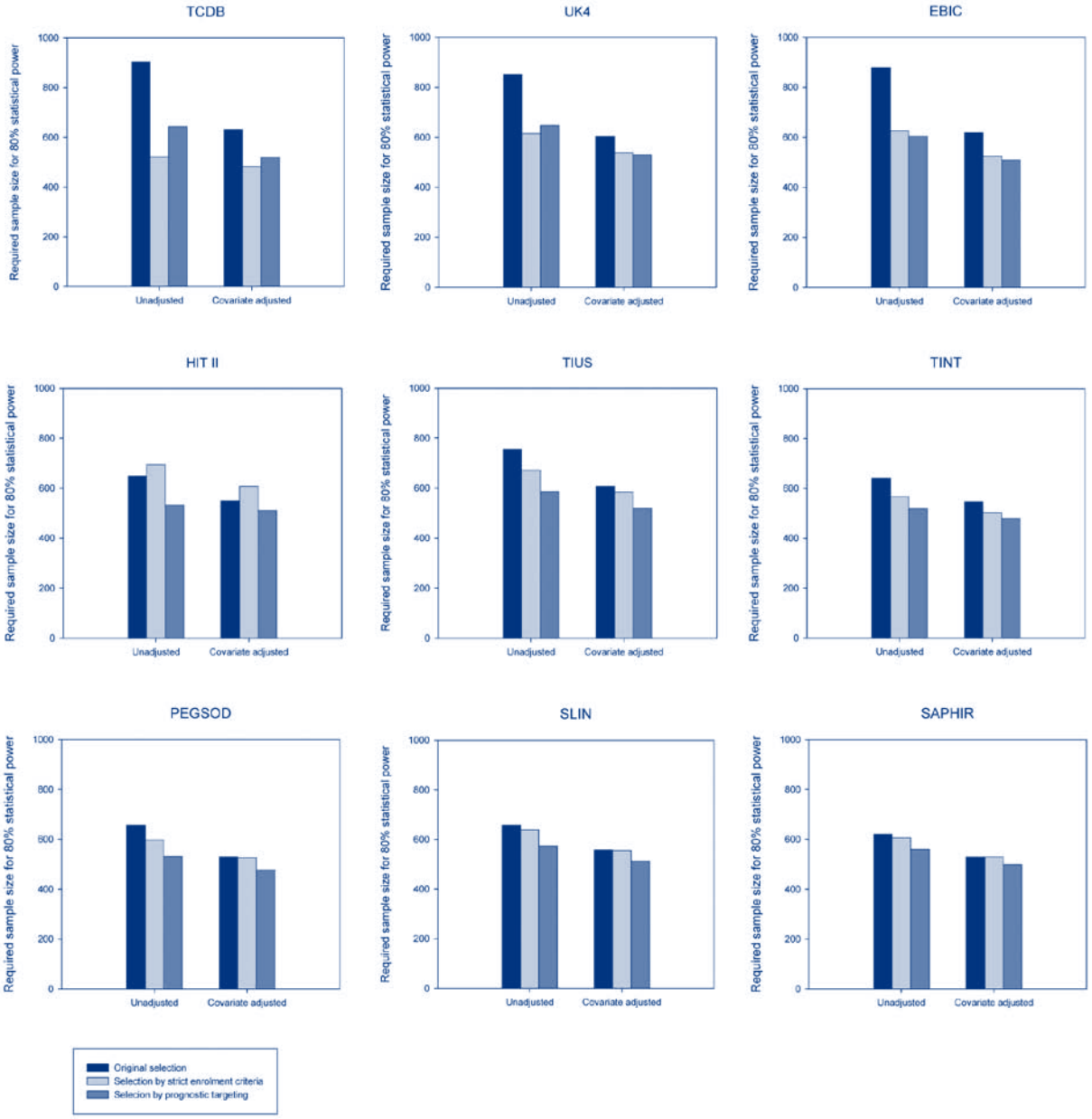

Figure 1
Required sample sizes for 80% statistical power in three observational studies (TCDB, UK4 and EBIC) and six RCTs (others) of the IMPACT database. Required sample size was calculated based on both the unadjusted and the covariate adjusted model. For both models, the required sample size was depicted for the original study population, the strictly selected population (time window ≤ 8 hours; age at injury ≤ 65 years; ≥ 1 reactive pupil; motor score > 1; GCS ≤ 8) and the prognostic targeted population (20-80% risk for unfavorable outcome). Estimates based on 1000 simulations assuming a uniform treatment effect.

Research news

Hand Surgery and Rehabilitation

"Hand Surgery and Rehabilitation" is a research group shared between two departments of the Erasmus MC: the Department of Rehabilitation Medicine and the Department of Plastic & Reconstructive Surgery. Both of these departments have a strong track record in research and clinical care in the area of hand surgery and rehabilitation. The research related to hand surgery and rehabilitation of both departments has been fully integrated to create a large and strong research team with knowledge in both hand surgery and hand therapy. The research group consists of hand surgeons, rehabilitation physicians, hand therapists, human movement scientists and engineers.

Our research focuses on a wide range of aspects relevant to hand function and the treatment of patients with hand disorders. In particular, we have ongoing research targeted at 1) Evaluating new interventions for patients with hand disorders, 2) Developing and evaluating assessment tools for hand functions, such as the assessment and quantification of hand strength, mobility and cold intolerance, and 3) Improving our understanding of the underlying mechanisms of diseases and interventions. From a patient perspective, our research efforts are focused mainly on nerve pathologies, tendon pathologies, congenital hand deformities, pain disorders, osteoarthritis and stroke.

At the moment out research group consists of a group leader, 2 post-docs, and 9 PhD students and 6 residents. Additionally, we are always on the look-out for talented students who are willing to support our research effort during an internship. So if you are looking for a exiting internship on the border of scientific research and clinical care and you are well-organized and hard working, please contact us!

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www.erasmusmc.nl/revalidatie/research/hand

Grown-up Congenital Heart Disease

Congenital heart disease with an incidence of around 8 cases per 1000 live births is the most prevalent form of congenital abnormality. The number of adults with congenital heart disease is steadily increasing due to the success of paediatric cardiology and open heart surgery. Open heart surgery using cardiopulmonary bypass started in Rotterdam in 1968. Before the era of open heart surgery 85% of the children died before reaching adulthood. Now > 90% of patients survive, resulting in a steadily growing population of adults with congenital heart disease. It is estimated that now + 30.000 adult patients with congenital heart disease are alive in the Netherlands. In Rotterdam we have a large population of about 2000 adults with congenital heart disease seen in our outpatient clinic every year. Total correction of congenital heart disease is rare and most patients have residual lesions and sequels. Observational studies have taught us that at some point a patient with a cardiac malformation will experience a decline in cardiac function because of emerging complications. Well-known and feared complications in these patients are arrhythmias, heartfailure, endocarditis, valvular lesions, thrombo-embolic complications and pulmonary hypertension, which all affect the physical condition of the patient

Research in this new area of Cardiology focuses on 1) Long-term outcome of specific groups of patients, for instance patients with tetralogy of Fallot or atrial septal defect, and on 2) Quality of life with extensive psychological questionnaires.
Other research topics are 3) Pregnancy and heart disease (most heart patients reach childbearing age and wants to become pregnant, however, pregnancy is a hemodynamic burden), 4) Pacemaker therapy and 5) New echocardiographic techniques (3D echo, speckle tracking). Furthermore a large study is currently set up to investigate 6) Biomarkers in adults with congenital heart disease.

If you are interested to do research in any of these fields please contact Prof Dr JW Roos-Hesselink, cardiologist, email: j.roos@erasmusmc.nl

Erasmus Journal of Medicine