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Clinical potential of DNA methylation in organ transplantation

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Abstract

Identification of patients at risk for post-transplant complications is a major challenge and will improve clinical care and patient health after organ transplantation. The poor predictive values of current biomarkers strengthens the need to explore novel and innovative methods such as epigenetics for the discovery of biomarkers. Cell differentiation and function of immune cells is dependent on epigenetic mechanisms, which regulate gene expression without altering the original DNA sequence. These epigenetic mechanisms are dynamic, potentially heritable, change with age and can be regulated and influenced by environmental conditions. One of the most well-known epigenetic mechanism is DNA methylation, which comprises the methylation of a cytosine (C) next to a guanine (G; CpG dinucleotides). Aberrant DNA methylation is increasingly associated with diseases, including immune-mediated diseases, and these alterations precede the clinical phenotype. The impact of DNA methylation profiles on transplant acceptance and rejection as well as on other post-transplant complications is unknown. Here we will discuss the current evidence of the functional role of recipient and donor DNA methylation on outcome after organ transplantation. Changes in DNA methylation may predict the risk of developing post-transplant complications including infections, malignancies and allograft rejection. We speculate that identification of these changes in DNA methylation contributes to the earlier diagnosis and prevention of post-transplant complications leading to improved patient care.

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Introduction

A major challenge in organ transplantation today is the identification of patients at risk for post-transplant complications. The current method for diagnosing rejection in heart transplantation is invasive and large variability is observed between pathologists in biopsy interpretation (1, 2). Crespo-Leiro et al. (3) evaluated the ISHLT 2004 acute cellular rejection (ACR) grading scheme within the CARGO II pathology panel and found low all-grade agreement, strengthening the need to discover and develop novel non-invasive methods to monitor the allograft. Ideally, rejection and other complications can be diagnosed and possibly predicted non-invasively from markers present in the peripheral blood or urine.

Several methods have been investigated to assess their potential to identify patients at risk for post-transplant complications. Immune-related chemokines and cytokines in the peripheral blood have been studied as non-invasive markers for acute and chronic rejection (4, 5). The Clinical Trials in Organ Transplantation (CTOT)-05 is a recently published multicenter cohort study which analyzes the potential of several biomarkers previously studied in both heart and kidney transplant recipients (6). Their results show no significant associations between the majority of tested biomarkers and biopsy-proven acute rejection (BPAR) or CAV, including reactive T cell panels (interferon-gamma (IFNγ) Enzyme-Linked Immuno Spot (ELISPOT)), anti-Human Leukocyte Antigen (HLA) class II or anti-Donor-Specific Antibodies (DSA), and gene expression of 6 specific genes in blood. The results of this study do not support routine use of the studied assays to predict BPAR or CAV, however the authors point out the heterogeneity due to different clinical practice among centers as a weakness of the study. Even though Starling et al. (6) found no associations, other studies show promising results when analyzing gene expression in heart transplant patients, possibly due to a wider range of genes studied. Allomap® is a commercially available blood-based test to diagnose cardiac allograft rejection, based on gene expression levels of 11 genes (7). It is developed based on results from the IMAGE (Invasive Monitoring Attenuation through Gene Expression) (8) and CARGO (Cardiac Allograft Rejection Gene Expression Observational Study) trials (9). In both studies gene expression analysis resulted in significantly fewer biopsies taken to monitor the allograft. However, its results are also critically discussed (10) and we should be careful when extrapolating these results to high-risk patients. Another promising approach is the analysis of cell-free donor derived DNA (cfdDNA) in the serum of transplant patients, which is based on the assumption that the concentration of the cfdDNA correlates with the severity of the cardiomyocyte and endothelial damage (11). Levels of cfdDNA were significantly higher in patients during acute rejection compared to

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stable transplant patients (12, 13). The predictive value of these assays is now explored and additional validation is necessary before clinical implementation (4, 14).

Biomarkers for the diagnosis and/or prediction of post-transplant complications should be accurate with high specificity, high sensitivity and its results should be reproducible. The goal is to find biomarkers that display changes at the molecular level as early indicator for these post-transplant complications, before the clinical signs appear, as this may prevent irreversible tissue damage (15). In addition to the gene expression studies, mechanisms that regulate gene expression, the so-called epigenetic mechanisms, can be studied as well. Genome-wide analysis of epigenetic features in transplant recipients may lead to the discovery of new biomarkers for the identification of patients at risk for post-transplant complications. This will provide tools to improve diagnostics and current treatment strategies. The first indication that epigenetics can be used for the diagnosis of rejection comes from studies by Mehta et al. (16). The authors propose hypermethylation of the *CALCA* gene in urine of kidney transplant recipients as a biomarker for acute kidney injury. Nevertheless, more research should be performed to explore the potential of epigenetics in transplantation (17). Integration of molecular techniques to provide biomarkers for complications after organ transplantation holds potential to improve the currently used diagnostics (7, 11, 15).

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Epigenetic mechanism: DNA methylation

There are several epigenetic mechanisms which influence the condensation of the chromatin to make specific genes accessible or inaccessible to transcription factors, and thereby determine which genes are transcribed (18). Histone modifications such as methylation or acetylation, non-coding RNA molecules which bind the DNA (siRNAs, lncRNAs, microRNAs) and methylation of DNA are the main epigenetic mechanisms (19) which are nicely illustrated in an extensive review by Portela et al. (20). In the current review, we focus on DNA methylation which is the methylation of a cytosine (C) followed by a guanine (G), a CpG dinucleotide (Figure 1A). A methyl group is added to the DNA by DNA methyltransferases (DNMT) (Figure 1B), these are specific enzymes. CpG dinucleotides are unequally distributed across the genome and regions with a high number of CpG sites are called CpG islands which are mostly located in promoter regions (21). High methylation of promoter CpG sites is associated with a tight, closed

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chromatin structure and transcriptional silencing of the associated gene (Figure 1C) (22). DNA methylation is thereby responsible for the fine control of different cellular functions, including T cell differentiation during an immune response (23, 24). It is a dynamic feature, susceptible to cues from the environment (25), infections, chemical agents and drugs are examples of external factors that can influence methylation. Also internal stimuli like cytokines and hormones influence DNA methylation profiles (26, 27).

Recent evidence indicates that functional DNA methylation also occurs outside of promoters in intra- and intergenic regions (28, 29), affecting the three-dimensional organization of the DNA (30). This is especially interesting because changes in methylation on CpG sites outside promoters might be associated with a specific disease, highlighting their potential as biomarker (21, 31). Smyth et al. (27) found intragenic CpG sites of which the DNA methylation was associated with chronic kidney disease without a significant change in the gene expression, suggesting a more distant function of the DNA methylation.

DNA methylation is one of the most studied epigenetic mechanism since it can be quantified relatively easy (32). Bisulfite sequencing is currently the golden standard and a costly but more comprehensive approach is provided by Illumina for genome-wide DNA methylation analysis (33). The advantage of studying DNA methylation over the whole epigenome is that not only DNA methylation on CpG islands and promoter regions is quantified but also intra- and intergenic regions are represented on the microarray. Due to cell-specific DNA methylation profiles it is recommended to focus DNA methylation studies on a specific cell type and not on a heterogeneous cell population as for example peripheral blood mononuclear cells (PBMCs) (34-36).

DNA methylation in disease

Aberrant DNA methylation profiles have been associated with different types of complex diseases (27, 37, 38). This was first discovered in oncology where they found global loss of DNA methylation and regional increase in methylation (39). To date the use of epigenetic biomarkers is well established for detection and diagnosis of several cancers (40). Some of these markers can be detected not only by analyzing the affected tissue but also by other, less invasive methods, e.g. detection in urine or

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peripheral blood (41, 42). Already in 1999 Esteller et al. discovered hypermethylation of tumor suppressor genes in serum DNA as diagnostic marker for small-cell lung cancer (43).

In patients with immune disease such as systemic lupus erythematosus (SLE), diabetes, rheumatoid arthritis (RA) abnormal DNA methylation of immune cells is observed (44-46). Heart failure is also associated with epigenetic changes and DNA methylation (47, 48). Differential DNA methylation of peripheral blood leukocytes (PBL) in repetitive elements *ALU*, *Satellite 2* and *LINE-1*, which is a measure for global DNA methylation, showed to be associated with ischemic heart disease and its risk factors (49, 50). However, exact cell composition was unknown in these PBL samples and the association was only significant in male subjects.

Discovery of a comprehensive set of biomarkers for diagnostic purposes improves (early) detection of the disease but also opens up the possibility to use them as therapeutic targets (39, 51). In oncology, epigenetic drugs such as demethylating agents 5'-azacitidine and decitabine, have proven to be an effective treatment and are both FDA approved (52). Costa et al. (53) showed that 5'-azacitidine is effective in chronic myelomonocytic leukemia by decreasing activity of DNA methyltransferase, however its function is global and unspecific. For patients with cancer an epigenetic drug might be the last possible treatment, though the potential of these drugs for modulating DNA methylation in a more specific way should be carefully studied in a wider range of diseases.

DNA methylation in organ transplantation

Successful organ transplantation is the net-result of the overall cumulative injury caused by several events in the donor e.g. age, life-style, ischemia/reperfusion injury (IRI) and the immune response in the recipient (54-57). Research shows that IRI causes epigenetic changes in the donor organ. Specifically the promoter region of the *C3* gene becomes demethylated in the kidney, which is associated with chronic nephropathy post-transplantation (58, 59), a similar change in DNA methylation might occur during heart transplantation. The initial immune response by the recipient towards the transplanted organ largely depends on the IRI induced changes in the donor organ (60). DNA methylation is a large contributor to the balanced immune response towards the graft as it regulates the function of cells of the immune system (26). B cells, NK cells, T cells and other immune cells are established during

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differentiation of hematopoietic stem cells resulting in distinct DNA methylation profiles for each cell type (61, 62). As in organ transplantation T cells play prominent roles in alloreactivity and are the key target for immunosuppressive drugs, we focus in this review on DNA methylation in T cells.

A direct link between DNA methylation profiles and organ rejection is not yet established, however several genes involved in alloreactivity are under regulation of DNA methylation. A summary of relevant genes, which can potentially serve as biomarkers for acute cellular rejection and immune activation in organ transplant recipients, is provided in Table 1. During an immune response the T cell differentiation from naïve to memory cells is controlled by DNA methylation. When genes encoding effector molecules become transcriptionally upregulated, repressive epigenetic marks as DNA methylation are lost (24, 26, 63). The presence of memory T cells is both associated with acute and chronic rejection after organ transplantation (63-65). Memory T cells express chemokine receptor type 6 (CCR6) at a higher level compared to naïve T cells, which is the consequence of hypomethylation of *CCR6* (66). In CD8+ cytotoxic T cells the differentiation from naïve to effector cells is established by demethylation of the promoter region of specific effector genes as granzyme B and *IFNγ* (67). The ability of memory CD8+ cells to rapidly demethylate effector genes on antigen re-exposure is most likely an interplay between DNA methylation and histone modifications (67). The CD4+ T cell differentiation is more complex due to the T helper cell lineage diversity within the effector population (23, 24, 68). For example, the promoter region of *IFNγ* is methylated in naïve CD4+ T cells and upon antigen stimulation, in the presence of Thelper1 (Th1) polarizing cytokines (e.g. Interleukin (IL)-12), the promoter region of *IFNγ* becomes demethylated enabling expression of *IFNγ* in CD4+ Th1 cells. Consequently, cellular instability and plasticity (69) are both under control of DNA methylation. Cellular instability is a major concern in organ transplantation while considering regulatory T cell therapy. Under inflammatory conditions regulatory T cells can earn effector functions as IFNγ production (70) and IL-17 production through demethylation (71, 72).

Important regulators of the immune response are regulatory T cells (Treg; CD4+FOXP3+) (73). The role of these Treg in the induction and maintenance of tolerance in organ transplantation has been demonstrated in several experimental models of transplantation (74). In humans high urinary mRNA levels of *FOXP3* have been associated with rejection reversal (75) though on the contrary high *FOXP3* expression in the allograft is found during acute rejection in transplanted organs (76 , 77, 78). Treg develop in the thymus, natural Treg (nTreg), and in addition Treg develop in the periphery (induced

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Treg; iTreg) under a variety of conditions including antigen stimulation in the presence of IL-2 and TGFβ (79, 80). The transcription factor FOXP3 is essential for the maintenance and immune suppressive function of Treg and its expression is controlled by DNA methylation (81, 82). The *FOXP3* gene contains one region, the Treg Specific Demethylated Region (TSDR), which is demethylated in nTreg and methylated in other peripheral blood leukocyte subtypes, including iTreg and recently activated T cells. Demethylation of this region results in a stable, constitutive expression of *FOXP3* and is used as a marker to identify nTreg (83, 84).

Recently, we studied demethylation of the *FOXP3* gene in endomyocardial biopsies (EMB) after heart transplantation (85). According to the International Society for Heart and Lung Transplantation (ISHLT) definition of rejection, only patients with EMB scored ≥ 2R are considered to experience a clinical relevant rejection requiring therapy (86). Multiple EMB were analyzed of both patients who remained free from rejection (non-rejectors; 1R EMB) and patients who developed a histologically proven acute rejection (rejectors; 2R EMB and 1R EMB sampled 8 days before the 2R EMB). The percentage of demethylated *FOXP3* was significantly higher in the 1R EMB collected before rejection compared to the 1R EMB of the non-rejectors (Figure 2A). The question is whether this difference can be used to predict rejection which enables timely intensifying of the immunosuppressive therapy and possibly prevention of tissue damage. ROC analysis demonstrated a relatively good discrimination between the rejectors and non-rejectors (Figure 2B, area: 0.79, 95% confidence interval: 0.65-0.93). Nevertheless, the overlap between both groups is that large that at this moment a true cut-off point of % demethylated *FOXP3* to predict rejection is impossible to identify. By studying larger cohorts of 1R EMB or in combination with other, to be identified, predictive markers, the % of demethylated *FOXP3* might be proven to be predictive for a clinical relevant rejection in the future.

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Table 1: Genes involved in T cell alloreactivity under control of DNA methylation.

Gene product	Gene	T cell source	Reference
Cytokines	IFNγ	CD4 (Th1)/CD8	(87-91)
	IL2	T cells	(92, 93)
	IL4	CD4 (Th2)	(87, 94, 95)
	IL10	Treg	(87, 96)
	IL17	CD4 (Th17)/CD8	(97)
Costimulatory molecules	PD1	CD8	(98)
	CD40L	CD4	(99, 100)
Cytotoxic proteins	Granzyme B	CD4/CD8	(101) in mice
	Perforin 1	CD8	(102, 103)
Transcription factors	FOXP3	Treg	(78, 81-85)
	RORC (RORγt)	CD4 (Th17)	(72, 91)
	TBX21 (T-bet)	CD4 (Th1)	(104, 105)
	GATA3	CD4 (Th2)	(106, 107)
Receptor	CCR6	Memory T cells	(66)

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Currently prescribed immunosuppressive drugs prevent the occurrence of rejection, however long-term complications of life-long use of these immunosuppressive drugs has become a major problem (108). Demethylation of *FOXP3* is not only studied for predicting rejection but also for predicting long-term complications. Organ transplant recipients are 200 times more likely to develop a cutaneous squamous cell carcinoma (cSCC) (109), a specific type of skin cancer, for which immunosuppressive medication seems to be a large risk factor. Sherston et al. (110) studied demethylation of the TSDR as a marker for cSCC in kidney transplant recipients. They followed a cohort of 58 kidney transplant recipients in time and found a significant increase in the proportion of demthylated CD4+ FOXP3+ cells in patients who had previously developed an cSCC. The immune phenotype was stable in time, emphasizing its potential as biomarker for cSCC post-transplantation (110).

After heart transplantation cardiac allograft vasculopathy (CAV) is a common reason for retransplantation (111) and a common cause of death after 3 years, together with malignancy and renal failure (112). Once CAV has been initiated in a patient it cannot be reversed, therefore early detection or

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prediction is important (113). To date no reliable biomarkers have been found to assess the risk for developing CAV (6), possibly the study of epigenetics in patients with CAV could complement or improve current diagnostics and hopefully provide therapeutic targets (114).

Conclusion

Epigenetics is part of the molecular mechanisms underlying the functional processes we have been studying in the past. Studying epigenetics over the whole epigenome will unravel the clinical potential of DNA methylation in organ transplant recipients. Most likely these studies will lead to the discovery of novel biomarkers for the identification of patients at risk for post-transplant complications. The advantage of studying DNA methylation over the whole epigenome is that not only DNA methylation in promoter regions is quantified but also intra- and intergenic regions are included, the exact function of DNA methylation in these regions is not entirely known yet. CpG sites outside of the promoter, which do not directly have an influence on gene expression, might be a discriminating biomarker for certain diseases. We speculate that DNA methylation analysis in organ transplant recipients will contribute to improved diagnostics of post-transplant complications with earlier detection, prediction and possibly prevention.

In Figure 3 we illustrate three hypothetical situations where several risk factors are assessed by quantifying DNA methylation on specific places in the genome. When risk factors can be estimated for a patient their treatment strategy can be adjusted, not only by altering medication but also by providing more specific lifestyle advices. Hopefully treatment of transplant patients can be customized in the future, moving more towards personalized medicine. The discovery of new epigenetic biomarkers also opens the possibility to use them as therapeutic targets, epigenetic drugs are becoming available in oncology and these should be studied to explore their application in transplantation.

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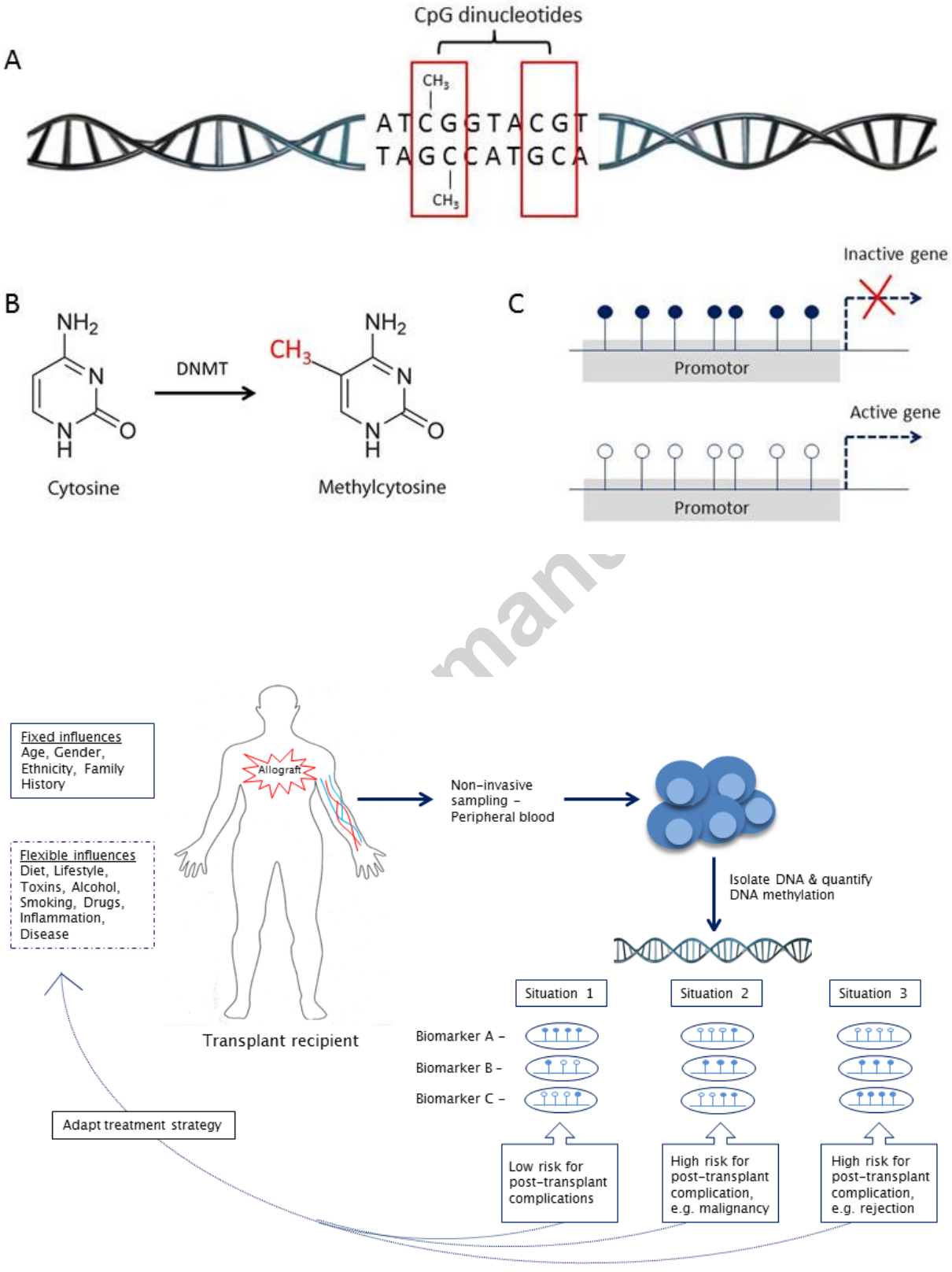
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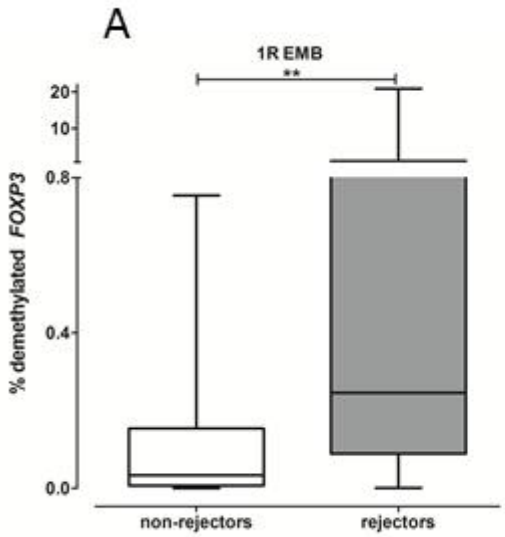
Figure 1. DNA methylation A: A DNA strand with the nucleotides Adenosine (A), Thymidine (T), Cytosine (C) and Guanine (G). The red boxes highlight the CpG dinucleotides where the left is methylated and the right is demethylated. **B:** The addition of a methyl group to a cytosine is catalyzed by the enzyme DNA methyltransferase (DNMT). **C:** Two promoter regions where filled spots represent CpG sites which are methylated and empty spots represent CpG sites which are demethylated.

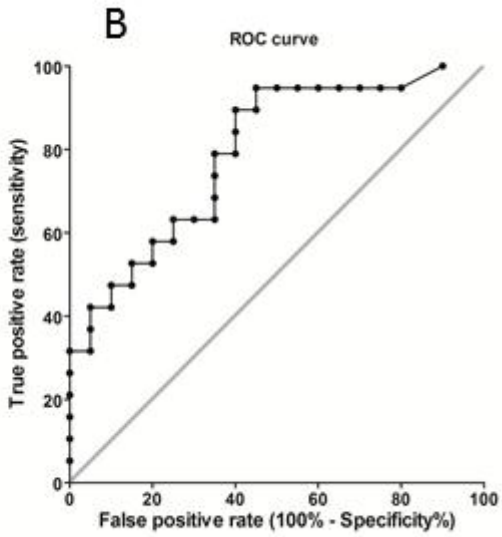
Figure 2. *FOXP3* demethylation in endomyocardial biopsies A: The percentage of demethylated *FOXP3* is significantly higher in 1R EMB of rejectors collected before rejection (n=19) compared to 1R EMB of non-rejectors (n=20) **B:** Receiver operating characteristic (ROC) curve of the ability to predict rejection. Area under the ROC curve: 0.79 and the optimal threshold for predicting rejection is 0.088% of demethylated *FOXP3* with 78,9% sensitivity and 65% specificity. ** indicates $p<0.005$

Figure 3. DNA methylation as biomarker in transplant patients. Illustration of the workflow when biomarkers would be used to assess risk factors in transplant patients. The use of a non-invasive sampling method and a specific cell population to infer DNA methylation on specific locations in the genome. When risk factors are assessed the treatment strategy could be adjusted.

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A
CpG dinucleotides
CH3
ATCGGTACGT
TAGCCATGCA
CH3
B
NH2
N
N
H
O
DNMT
CH3
NH2
N
N
H
O
Cytosine
Methylcytosine
C
Inactive gene
Promotor
Active gene
Promotor
Fixed influences
Age, Gender, Ethnicity, Family History
Flexible influences
Diet, Lifestyle, Toxins, Alcohol, Smoking, Drugs, Inflammation, Disease
Allograft
Transplant recipient
Non-invasive sampling – Peripheral blood
Isolate DNA & quantify DNA methylation
Situation 1
Situation 2
Situation 3
Biomarker A –
Biomarker B –
Biomarker C –
Low risk for post-transplant complications
High risk for post-transplant complication, e.g. malignancy
High risk for post-transplant complication, e.g. rejection
Adapt treatment strategy





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