Steroid regulation: An overlooked aspect of tolerance and chronic rejection in kidney transplantation

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Title: Steroid regulation: An overlooked aspect of tolerance and chronic rejection in kidney transplantation

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Declarations of interest:

M.H.F. and I.R.B currently are employees of UCB Celltech, a pharmaceutical company. Their involvement in the conduct of this research was solely in their capacity as academics at King's College London. The rest of the authors of this manuscript have no conflicts of interest to disclose.

Abbreviations

11β-HSD – 11β-Hydroxysteroid dehydrogenase

C1s – Complement component 1, s subcomponent gene

CISH – Cytokine inducible SH2-containing protein gene

CNI – calcineurin inhibitors

CR – chronic rejection

CV.AUC – cross-validated area under the receiver operating characteristic curve

CvS – comparison between chronic rejectors and stable patients

FOXP3 – forkhead box P3 gene

GAMBIT – Genetic Analysis of Molecular Biomarkers of Immunological Tolerance study

GC – glucocorticoid(s)

GR – glucocorticoid receptor (*NR3C1* gene)

H6PD – hexose-6-phosphate dehydrogenase gene

HC – healthy controls

HPRT – hypoxanthine phosphoribosyltransferase 1 gene

HSD11B1* and *HSD11B2 – hydroxysteroid (11-beta) dehydrogenase 1 and 2 genes

IQR – interquartile range

KTR – kidney transplant recipients

MAPK8 – mitogen-activated protein kinase 8 gene

MR – mineralocorticoid receptor (*NR3C2* gene)

NR3C1 – nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) gene

NR3C2 – nuclear receptor subfamily 3, group C, member 2 (mineralocorticoid receptor) gene

PBMC – peripheral blood mononuclear cells

PNOC – prepronociceptin gene

PPIF – peptidylprolyl isomerase F gene

RT-qPCR – quantitative real-time reverse transcriptase polymerase chain reaction

STAT1 - signal transducer and activator of transcription 1 gene

T-reg – regulatory T-cells

TSC22D3 – TSC22 domain family, member 3 gene

TvS – comparison between tolerant and stable patients

Abstract

Steroid conversion (*HSD11B1, HSD11B2, H6PD*) and receptor genes (*NR3C1*, *NR3C2*) were examined in kidney-transplant recipients with “operational tolerance” and chronic rejection (CR), independently and within the context of 88 tolerance-associated genes. Associations with cellular types were explored. Peripheral whole-blood gene-expression levels (RT-qPCR-based) and cell counts were adjusted for immunosuppressant drug intake. Tolerant (n=17), stable (n=190) and CR patients (n=37) were compared. Healthy controls (n=14) were used as reference. The anti-inflammatory glucocorticoid receptor (*NR3C1*) and the cortisol-activating *HSD11B1* and *H6PD* genes were up-regulated in CR and were lowest in tolerant patients. The pro-inflammatory mineralocorticoid gene (*NR3C2*) was downregulated in stable and CR patients. *NR3C1* was associated with neutrophils and *NR3C2* with T-cells. Steroid conversion and receptor genes, alone, enabled classification of tolerant patients and were major contributors to gene-expression signatures of both, tolerance and CR, alongside known tolerance-associated genes, revealing a key role of steroid regulation and response in kidney transplantation.

Keywords

steroid receptor genes; steroid conversion; transplantation; kidney; tolerance; chronic rejection

1 Introduction

Glucocorticoids (GC) act as immunosuppressants (IS) when administered in high, pharmacological doses, but play a complex role in the regulation of the innate and allogeneic immune response in lower, physiological doses. They show immunomodulatory effects and activate regulatory T-cells (Treg) (Dimeloe et al., 2010), which are involved in the establishment and maintenance of graft tolerance. Although graft tolerance is largely maintained by IS treatment, in extremely favourable cases IS can be completely withdrawn and graft tolerance retained ("operational tolerance") (Lerut and Sanchez-Fueyo, 2006,Roussey-Kesler et al., 2006). On the other hand, despite being maintained on IS, kidney transplant recipients (KTR) frequently develop features of chronic rejection (CR), which is associated with graft dysfunction and leads to graft failure (Heemann and Lutz, 2013).

Exogenous GC are fundamental for IS regimens in KTR. Despite refraining from high doses due to metabolic side effects, the benefits of withdrawal from low GC doses remain debatable (Steiner and Awdishu, 2011). Systematic clinical trials have demonstrated little benefit in steroid withdrawal, whilst increasing the risks of rejection (Haller et al., 2016). GC effects have, traditionally, been associated with their levels in the circulation, but the importance of local intracellular regulation is gaining appreciation (Hardy et al., 2012). 11β-Hydroxysteroid dehydrogenases (11β-HSDs) play a key part in the intracellular regulation of pre-receptor GC bioavailability via interconversion of the active cortisol to the inactive cortisone. 11β-HSD1 (*HSD11B1* gene) activates GC and, due to its intra-luminal orientation in the endoplasmic reticulum, is regulated by the availability of the proton donor, supplied by the endoluminal hexose-6-phosphate dehydrogenase (*H6PD*) (Odermatt et al., 2006). 11β-HSD2 (*HSD11B2*) inactivates GC and, due to its cytoplasmic orientation on the endoplasmic reticulum membrane, ensures protection of the mineralocorticoid receptor (MR) from GC action (Odermatt et al., 2001). GC regulation by 11β-HSDs has been demonstrated in lymphoid organs (Hennebold et al., 1996) and lymphocytes (Zhang et al., 2005). These enzymes also regulate

the interconversion of prednisolone and prednisone (Diederich et al., 2002) and can affect clinical outcomes by contributing to GC resistance (Sai et al., 2011).

GC exert their main action via the glucocorticoid receptor (GR) (*NR3C1*), which plays a fundamental anti-inflammatory role (Baschant and Tuckermann, 2010) and interacts via tethering with immunoregulatory transcription factors (Ratman et al., 2013). There is a clear evidence that hindered *in vitro* response to exogenous GC and GC resistance in dialysis patients with chronic kidney disease pre-transplantation are associated, in the long-term (Frezza et al., 2014), as well as in the short-term (De Antonio et al., 2008), with higher incidence of acute rejection and poor allograft outcomes post-transplantation. Conversely to the anti-inflammatory role of GR, a pro-inflammatory role of the MR (*NR3C2*) is becoming apparent (Bene et al., 2014). MR activation has been associated with activation of pro-inflammatory Th17 cells and a downregulation of the tolerance and T-reg-associated *FOXP3* gene (forkhead box P3*J* in peripheral blood mononuclear cells (PBMC) and kidney and a decrease of *FOXP3*-positive $CD4^+$ cells (Amador et al., 2014).

Given the high importance of GC action for transplantation outcomes and the importance of endogenous GC conversion for GC action, it is surprising that studies on the role of endogenous GC conversion and GC response independent of exogenous GC administration are scarce in transplantation research and that largely only the effects of exogenous steroids on lymphocyte populations have been considered (Berki et al., 2002,Ribarac-Stepic et al., 2001).

We, therefore, set to examine the role of endogenous GC conversion and steroid response in patients with established kidney grafts for several years post transplantation. We hypothesised that the state of “operational tolerance” is maintained via activated anti-inflammatory processes and hindered pro-inflammatory processes, which is reflected in upregulated GR and GC activation and downregulated MR and GC inactivation in tolerant compared to stable KTR. In CR, as a process physiologically opposite to tolerance, with a maintained pro-inflammatory state despite IS

treatment, we expected changes in the opposite direction, i.e. domination of pro-inflammatory processed and hindered or absent anti-inflammatory responses.

We examined the expression of steroid conversion (*HSD11B2, HSD11B1, H6PD*) and receptor genes (*NR3C1, NR3C2*) in peripheral whole blood of KTR with "operational tolerance" or with CR and in clinically stable patients maintained on IS according to local clinical practice. We evaluated whether differences in the expression of steroid conversion and receptor genes would be sufficiently large to enable discrimination between clinical groups of transplanted patients and whether these genes would remain informative within the context of known tolerance-associated genes and, hence, could contribute to clinically-applicable gene-expression signatures of tolerance and CR. To account for the effect of exogenous drug administration, i.e. to evaluate features associated with tolerance, as opposed to differences arising from the absence of IS medication in patients with "operational tolerance", and to evaluate features of CR, as opposed to differences driven by the IS regimens, we used drug-adjusted gene-expression levels, as previously (Rebollo-Mesa et al., 2016), and extended this approach to cell counts, after exploring the influence of prednisolone treatment on steroid conversion and receptor genes and demonstrating the effect of drug-adjustment.

Finally, since the main molecular assay that we used was indiscriminate to GR isoforms (α+β), we examined separately mRNA expression of GRβ. Whilst GRβ does not directly regulate GC-responsive genes, when co-expressed with GRα it functions as a dominant-negative inhibitor of the effects of GRα on GC-regulated genes (Oakley and Cidlowski, 2011) and, thus, is involved in the development of GC-resistance. It was of interest to examine whether there is evidence of GC-resistance or lack of GC response in CR, as this might explain why IS treatment, which often includes an exodenous GC, fails to suppress pro-inflammatory processes in CR.

2 Materials and Methods

2.1 Patients and Samples

Samples originated from KTR in the GAMBIT study (Genetic Analysis of Molecular Biomarkers of Immunological Tolerance, Research Ethics Reference: 09/H0713/12). The cohort included four clinical groups: tolerant, stable and CR patients and healthy controls (HC). The rational for patient classification, patient characteristics and IS regimens have previously been described (Rebollo-Mesa et al., 2016). Only patients treated with any of the following IS drugs were included: prednisolone, calcineurin inhibitor (CNI) (cyclosporine of tacrolimus), anti-proliferative agent (azathioprine or mycophenolate-mofetil (MMF)). Patients receiving other IS drugs were few and were excluded from the selection, to enable a more robust drug-adjustment.

Steroid receptor and conversion genes were examined in a total of 258 individuals (timepoint 1 (T1 cohort) samples): 17 tolerant (5 additional to our previous study), 190 stable, 37 CR patients and 14 HC. A second set of samples (timepoint 2 (T2 cohort)) (collected after a median of 182 days following the first sample, inter-quartile range (IQR) 126 – 233 days) was available for 82 of these individuals (13 tolerant, 45 stable, 16 CR patients and 8 HC). Glucocorticoid receptor β-isoform mRNA expression was examined in T1 samples from 167 individuals (12 tolerant, 123 stable, 24, CR patients and 8 HC). Tolerance-associated genes were analysed in T1 samples for 229 of the individuals (13 tolerant, 171 stable, 34 CR patients and 11 HC) and in 52 of those (10 tolerant, 26 stable and 16 CR patients) also in T2 samples. Blood cell counts were available for T1 samples of KTR as follows: neutrophil counts in 186 patients (9 tolerant, 144 stable, 33 CR); T-cell counts in 96 patients (8 tolerant, 65 stable, 23 CR); B-cell counts in 87 patients (10 tolerant, 58 stable, 19 CR).

An independent steroid-withdrawal cohort comprised 28 patients providing a sample prior to initiation of prednisolone withdrawal and after a median of 209 days (IQR 131 – 254 days) post withdrawal completion. The withdrawal was conducted due to clinical reasons or according to local standard clinical practice and took place over a median period of 115 days (IQR 75 – 164 days). The median prednisolone dose was 3 mg per day (IQR 3 – 5.6 mg) and the accompanying IS drugs were

MMF (both timepoints) and CNI (12 cyclosporine and 15 tacrolimus at both timepoints, one patient switched from cyclosporine to tacrolimus). Neutrophil counts were available for 22 of these patients.

2.2 Gene-expression and flow-cytometry analysis

Peripheral vein blood was collected into Tempus™ Blood RNA Tubes (Life-Technologies). Steroid conversion and receptor genes were analysed with RT-qPCR (Applied Biosystems). Fluidigm (BioMark) RT-qPCR platform was used, with a pre-amplification step, to analyse 3 endogenous reference genes and 93 tolerance-associated genes (Table S1). These were compiled from our previous analysis of microarrays (28 genes, which contributed to the development of our-previously published signature of tolerance (Rebollo-Mesa et al., 2016)) and from literature sources (65 additional tolerance-associated genes). Neutrophil counts were obtained from routine haematological differential blood-cell counts. Lymphocyte subset counts were derived from flow-cytometry analysis of PBMC. Sample storage, RNA isolation, cDNA synthesis, RT-qPCR and Fluidigm conditions, antibody panels and acquisition details for flow cytometry have previously been previously described in detail (Rebollo-Mesa et al., 2016). Samples from a given clinical group and IS regimen were distributed over all analytical plates, to avoid batch effects determining group or drug differences. Pre and post withdrawal samples were analysed in the same plate. Five tolerance-associated genes with unsatisfactory reproducibility in Fluidigm analysis (missing in over 25% of the samples) were excluded. *HSD11B2* was detected in 88.9% of the samples, albeit above the conventional threshold for positive RT-qPCR determination of 35 Ct. GRβ mRNA expression was also above 35 Ct in the examined samples. Relative gene-expression values were calculated on log2 scale with the $-\Delta$Ct method (Schmittgen and Livak, 2008) relative to *HPRT* (hypoxanthine phosphoribosyltransferase), used as reference gene in steroid (Bisschop et al., 2013) and transplantation research (Rebollo-Mesa et al., 2016).

2.3 Statistical analysis

Statistical analysis was performed in R version 3.2.2 (RCoreTeam, 2015). Outliers were recoded to the next highest or lowest value prior to parametric analyses. Missing gene expression data were imputed with K-nearest neighbour for microarrays (Troyanskaya et al., 2001) (package *impute* (Hastie, 2015)) prior to multivariable analyses. P-values were interpreted as an estimate of the strength of evidence rather than in a relation to a significance cut-off.

2.3.1 Drug-adjustment strategy

We have previously demonstrated that intake of IS drugs affects gene-expression levels and signatures and warrants adjustment of gene-expression values for IS drug intake. Cell counts can also be influenced by drugs (Nakagawa et al., 1998,Tareyeva et al., 1980) and would, similarly, require adjustment for immunosuppressant intake. We used a binary indicator for prednisolone intake and categorical indicators for intake of CNI (off, cyclosporine, tacrolimus) and anti-proliferative drugs (off, azathioprine, MMF).

In order to differentiate the effects of exogenous steroids from the endogenous mechanisms involved in tolerance in CR, we first examined the effect of prednisolone on unadjusted gene-expression values and cell counts, taking into account the intake of other IS drugs. We then regressed-out the effects of all IS drugs and generated drug-adjusted residuals using linear regression models based only on stable patients, to avoid obliterating differences with tolerant or CR patients if analysed jointly. Drug adjustment resulted in centring the data relative to the mean in stable patients, whilst retaining the original unit of measurement.

To compare unrelated patients on and off prednisolone, adjusting for confounding by drugs other than prednisolone, we used linear regression models with categorical indicators for drug intake. To compare samples pre and post prednisolone withdrawal in patients from the steroid-withdrawal cohort, adjusting for confounding by drugs other than prednisolone, we used linear mixed-effects models with a random intercept and fixed effects for the indicators of drug intake.

2.3.2 Comparison between clinical groups

To compare individual variables between clinical groups, we used Wilcoxon-Mann-Whitney test. While single-variable comparisons are easy to visualise and appear more intuitive to the biomedical audience, they consider individual components in isolation. Therefore, to account for the fact that genes act in combination within the organism, i.e. conditional on the levels of expression of each other, and to accommodate multiple, potentially correlated genes in one group-discrimination model, we used multivariable penalised logistic regression with an elastic net penalty (package *glmnet* (Friedman et al., 2010)). Within a multivariable model, the perturbations in the expression levels of a given gene between two groups are compared among individuals with equal levels of expression of the other examined genes, i.e. accounting for the effect of the rest of the genes included in the model. Further, the elastic net penalty enables a statistical selection of genes relevant to a given association and exclusion (by shrinking of the regression coefficients to zero) of genes with minimal or no contribution to discrimination between clinical groups, while at the same time allowing the inclusion of groups of strongly-correlated genes in the gene-expression signatures (models). Consequently, to evaluate the importance of each gene, conditional on the expression of all examined genes, we used the absolute value of the penalised regression coefficients. Only genes selected by the penalised regression procedure were considered informative for group discrimination ("classification-informative"). We set the penalty parameter alpha to 0.7 which, in our experience, gives a good balance between the number of classification-informative and strongly-correlated genes selected in a signature. The second penalty parameter (lambda) was determined as the median of 100 repeats of five-fold cross-validation cycles (function *cv.glmnet* (Friedman et al., 2010)).

To identify features of tolerance and CR we compared, correspondingly, tolerant with stable patients (TvS) and CR with stable patients (CvS). T1 samples provided the training dataset.

To minimise overfitting, we derived the regression coefficients of each of the final group-discrimination models ("median models") as the medians of the penalised regression coefficients of 50 group-stratified repeats of five-fold cross-validation cycles and summarised the variability with IQR and the 2.5^{th} - 97.5^{th} centile range. Unlike classical regression, modern statistical methods such as elastic net penalised regression do not provide p-values. However, evaluating the variability of regression coefficients with cross-validation provides a better measure of the generalisability of the findings. The interpretation of the summaries of regression coefficients derived via cross-validation is as follows: genes with non-zero IQR of the regression coefficients would be considered of particular importance for group discrimination, because these had been selected as informative (different from zero) in more than half of the multivariable gene-expression signatures derived from repeats of the cross-validation cycles. Furthermore, genes with a non-zero 2.5^{th} - 97.5^{th} centile range of the regression coefficients had been selected as informative in more than 95% of the models, hence the highest importance.

To evaluate model performance, we used the area under the receiver operating characteristics curve (AUC) for the predicted probability of tolerance or CR.

2.3.3 Validation strategy

To confirm observations of exogenous steroid effects based on stable patients on and off prednisolone, we compared the paired samples from the patients in the steroid-withdrawal cohort, as well as samples from CR patients on and off prednisolone.

To evaluate whether the drug-adjustment approach has accounted for exogenous steroid effects in samples unseen in the development of the drug-adjustment models (based only on stable patients), we compared the drug-adjusted residuals in the paired samples of the patients in the steroid-withdrawal cohort and in the CR patients on and off prednisolone.

To validate our gene-expression signatures, we used the following approaches:

First, we performed cross-validation with the training T1 datasets. A cross-validated AUC (CV.AUC) was calculated for the predicted probabilities of tolerance and CR for unseen data in five-fold cross-validation cycles, in which all steps of model development, including generation of drug-adjusted residuals, imputation of missing data and outlier correction, were performed with the training sub-set and the left-out unseen test subset was used solely for model evaluation. CV.AUC obtained in 50 repeats of the cross-validation cycles was summarised with median and 2.5^{th} - 97.5^{th} centile range.

Second, we used samples from the KTR groups not participating in the given group-discrimination model, i.e. samples from CR patients were unseen for the development of the tolerance signatures and samples from tolerant patients were unseen for the development of the CR signatures. Given that some stable KTRs may be potentially tolerant and others experiencing subclinical or early stages of CR, i.e. there is an overlap between the training clinical groups, we expected that a mechanistically relevant signature would achieve a better discrimination between tolerant and CR patients compared to the discrimination achieved between tolerant and stable patients or between CR and stable patients, as tolerant and CR patients would not be overlapping. Similarly, HC did not participate in the development of the group-discrimination models. These were compared only with tolerant patients as a drug-free and transplantation-free reference.

Third, we used the unseen samples from the T2 test dataset, which were analysed separately and had not participated in any model development.

2.3.4 Gene-gene-cell associations

To evaluate associations between steroid conversion and receptor genes, classification-informative tolerance-associated genes and absolute blood cell counts, we used Pearson regression coefficients and applied hierarchical cluster analysis to the gene-cell correlation matrix using Ward's minimum variance linkage method (Murtagh and Legendre, 2014). We also examined matches of classification-informative genes with gene sets included in the collections of the Molecular Signatures Database v5.1 (Broad Institute) (Subramanian et al., 2005).

3 Results and Discussion

3.1 Prednisolone intake affects gene expression and cell counts, which is accounted for in drug-adjusted residuals

To evaluate the effect of prednisolone, we compared the expression of steroid conversion and receptor genes in samples from stable patients on and off prednisolone, adjusting for CNI and anti-proliferative drug intake (Figure S1A-E). In prednisolone-treated patients *HSD11B1* (p=0.006) and *NR3C2* (p=0.027) expression were lower, *H6PD* expression was somewhat higher (p=0.098), whilst there was no evidence for a difference in *HSD11B2* (p=0.676) and *NR3C1* (p=0.510) expression, contrary to a reported down-regulation of GR binding sites in PBMC with pulse GC administration (Andreae et al., 2001). However, comparison of GR levels in kidney transplant patients has previously demonstrated that a reduction of GR levels is observed only in patients treated with high booster doses of GC and not in patients treated long-term with lower doses (Berki et al., 2002), which is in agreement with our findings of no difference in *NR3C1* expression off and on long-term prednisolone treatment. The comparison of paired samples on and off prednisolone from the steroid-withdrawal cohort confirmed our observations in stable patients (Figure S1F-J), with the exception of *HSD11B2* expression, which showed a tendency to be higher with prednisolone treatment (p=0.084). To examine whether the latter effect was driven by IS drugs other than prednisolone, we examined a subset of the T1 cohort with IS regimen matching that of the steroid-withdrawal cohort (on CNI and MMF). This confirmed the results of the complete set of stable patients, as did the comparison of CR patients on and off prednisolone (Figure S1K-O). A key difference between the steroid-withdrawal and the stable patients was the time post transplantation, with no overlap between the two groups (median 1.2 years (min 6 days – max 3.1 years) compared to median 13.0 years (4.2 – 36.7 years)) respectively, which could be responsible for the difference.

We then generated drug-adjusted residuals (as described in Section 2.3.1) and re-examined differences on and off prednisolone (p-values in brackets in Figure S1). Prednisolone effects were accounted for in all groups and for all genes, except *HSD11B2* expression in the steroid-withdrawal cohort, as this was not observed in the stable patients informing the drug-adjustment models (Figure S1C,H).

Further, we examined the effect of prednisolone on neutrophil cell counts, adjusting for CNI and anti-proliferative drug intake. As anticipated, based on literature reports (Nakagawa et al., 1998), there was evidence for an increase in neutrophil counts with prednisolone treatment in all patient groups (Figure S2), which was accounted for in the drug-adjusted counts (p-values in brackets Figure S2).

Having demonstrated that prednisolone effects are accounted for in drug-adjusted residuals, it was justifiable to proceed with interpreting differences between clinical groups observed in drug-adjusted gene-expression levels and cell counts as driven by endogenous processes related to tolerance and CR and not to exogenous steroid treatment. Confining the study to patients with prednisolone-free drug regimen would not have been appropriate, as other IS drugs also affect gene-expression and cell counts (Nakagawa et al., 1998,Rebollo-Mesa et al., 2016,Tareyeva et al., 1980) and, therefore, adjustment for all IS drugs is warranted. Similarly, using steroid-treated patients with a different condition would not have been appropriate, because these would have carried features of their underlying condition (most likely with an immunological inflammatory component, if it requires steroid treatment) in addition to features of prednisolone intake.

3.2 Expression of steroid conversion and receptor genes alone could differentiate tolerant and chronic rejection patients

We next examined differences in the drug-adjusted expression of individual steroid conversion and receptor genes (Figure 1) between clinical groups, in order to gain information on the net contribution of individual genes to group discrimination. There was evidence for higher expression

of *HSD11B1*, *H6PD* and *NR3C1* in stable and CR compared to tolerant patients (Figure 1A,B,D), while the *NC3R2* showed changes in the opposite direction and was low in CR and stable patients (Figure 1E). *NR3C1* upregulation and *NR3C2* downregulation was universal for KTR compared to HC and was especially pronounced in the relative expression NR3C1v2 (defined as the difference on a log2 scale, equivalent to the ratio on a linear scale), which was independent of the reference gene and was highest in CR and lowest in HC (Figure 1F).

To evaluate the joint contribution of the steroid conversion and receptor genes to clinical group discriminations, we trained multivariable penalised logistic regression models (as explained in Section 2.3.2.) (Figure 2, Table S2) and used the median models to predict the probabilities of tolerance and CR for all examined clinical groups (Figure 3). The multivariable models further emphasised the group differences observed for individual genes (Figure 2, Figure 1).

The tolerance signature achieved a very good discrimination of tolerant patients from each of the other clinical groups included in the validation (Figure 3B), as well as in the training dataset (Figure 3A,B) (Table 1). Although all 5-genes were retained in the median tolerance signature, it is worth pointing out that the 2.5^{th} -97.5^{th} centile intervals of the penalised regression coefficients for *HSD11B1* and the steroid receptor genes were well away from the gene-exclusion value of zero (Figure 2A). This means that the three genes were selected as classification-informative in at least 95% of the cross-validation models, emphasising that whilst there is some variability between the features of individual patients, differences in the expression of the three genes are of major importance in the majority of them.

The CR signature could separate, to an extent, CR from stable patients (Figure 3C,D), but the difference in the predicted probability of CR was insufficient to enable good discrimination between the clinical groups, although the performance of the CR signature in the validation dataset (T2) was better than in the training data (T1) (Table 1). Only *HSD11B2* and *NR3C1* were retained as classification-informative genes in the median CR signature (both higher in CR) (Figure 2B).

The predicted probabilities of tolerance and CR were more extreme in the clinical groups not participating in model development (Figure 3), i.e. the discrimination between tolerant and CR patients was excellent with the tolerance signature and very good even with the CR signature (Table 1).

3.3 Steroid conversion and receptor genes were main contributors to tolerance and chronic rejection signatures, alongside tolerance-associated genes

Further, we examined whether steroid conversion and receptor genes would continue to contribute to the discrimination of tolerant and CR patients after the addition of 88 tolerance-associated genes (Table S3) to the multivariable penalised logistic regression models. Genes contributing to the combined gene-expression signatures are shown in Figure 4 (listed in Table S3) and the predicted probabilities of tolerance and CR for all examined clinical groups are plotted in Figure 5 (group comparisons for the individual classification-informative tolerance-associated genes are plotted in Figure S3). The discrimination of tolerant from stable patients remained the same (Figure 5A,B), whilst the discrimination of CR from stable patients was considerably improved (Figure 5C,D) (Table 1). Remarkably, representatives of the steroid conversion and receptor genes had prominent places among the 17 genes informative for tolerance (Figure 4A) and the 15 genes informative for CR discrimination (Figure 4B). The discrimination between tolerant and CR patients with either signatures was excellent (CV.AUC above 0.930, Table 1). Reassuringly, gene-expression signatures were not influenced by prednisolone intake (Figure S4), re-affirming the merits of drug-adjustment. Among all classification-informative genes in the tolerance signature, *HSD11B1* had the largest and most consistent contribution to the discrimination between clinical groups (narrow 2.5^{th}-97.5^{th} centile interval) (Figure 4A), even larger than that of well-established tolerance-associated genes such as the T-reg-activation gene *FOXP3* (Sagoo et al., 2010) or the B-cell receptor genes *IGKV4-1* and *IGKV1D-13* (Moreso et al., 2014). In support of a mechanistic involvement of *HSD11B1* in tolerance, a change in the opposite direction (an increase in 11β-HSD1 enzyme activity) has been

associated with activation of CD4+ T-cells and polarisation into Th1 or Th2 cells (Zhang et al., 2005), a process which would be reduced in tolerance. In steroid target tissues *HSD11B1* is co-expressed with the GR-gene (*NR3C1*) (Tomlinson et al., 2004), coupling cortisol activation to GR availability. This agrees with our findings of concomitantly low *NR3C1, HSD11B1* and *H6PD* expression in tolerant compared to stable and CR patients (Figure 1, Figure 2). In addition, *HSD11B1* and *STAT1* (low in tolerant patients, Figure 4A), along with *CISH* and *C1s* (high in CR patients, Figure 4B), were among genes down-regulated in CD4:*FOXP3*+ T-reg compared to *FOXP3* knockout T-reg precursors (Samstein et al., 2012,Subramanian et al., 2005), suggesting that *HSD11B1* downregulation is coupled with T-reg activation. Further, GC treatment has been associated with increased *FOXP3* expression in *vivo* and, along with *IL-10*, *in vitro* (Karagiannidis et al., 2004). While *FOXP3* expression is not exclusively attributable to T-reg (Prado et al., 2011) and a transient state of T-reg functionality (with increased *FOXP3* and *IL-10* expression) is observed in all activated human T-cells (Pillai et al., 2007), higher *FOXP3* expression was a consistent feature of our tolerant patients (Figure 4), in which the immune responses would have reached a longer-term equilibration and, therefore, *FOXP3*-associated pathways would be derived from immune-suppressive T-reg cells. Therefore, given that "operationally tolerant" patients do not receive exogenous steroids, we can conclude that they have an adequate supply of endogenous cortisol.

Conversely, in CR patients, in which pro-inflammatory responses would be activated, steroid conversion and receptor genes showed changes in the opposite direction. *NR3C1* expression was upregulated (Figure 1D), along with that of *HSD11B2* (Figure 1C), and both genes were among the main contributors to the discrimination of CR patients (Figure 2B, Figure 4B). Strengthening the mechanistic argument, *HSD11B2* and *NR3C1* were part of a MAPK8-upregulated gene set (Subramanian et al., 2005,Yoshimura et al., 2005). MAPK8 plays a key role in T-cell proliferation, apoptosis and differentiation and assists polarized differentiation of pro-inflammatory Th1 cells (Arbour et al., 2002,Dong et al., 2001). At the same time, ligand-bound GR (*NR3C1*) interacts with the MAPK8 signaling pathway and modulates pro-inflammatory gene expression (Baschant and

Tuckermann, 2010), executing a negative feed-back regulation in situations of increased allo-antigen challenge. Given that gene expression in whole blood captures the net effect of multiple pathways, we would argue that *NR3C1* upregulation is part of an anti-inflammatory response, activated in CR concomitantly to the pro-inflammatory pathways. We interpret this as an evidence for increased or unmet GC demand, a concept compliant with an inappropriately low serum cortisol relative to the levels of inflammatory factors described in chronic inflammation (Straub et al., 2002). In this regard, *HSD11B2* upregulation in CR could be a response to pro-inflammatory cytokines leading to GC deactivation in immune cells in peripheral blood, but also in the kidney parenchyma, via induction from infiltrating cells, and this could be responsible for the development of GC resistance. Indeed, a larger proportion of CR patients already receive prednisolone (73% vs 43% in stable patients, p=0.002), yet they appear GC deficient. Another option for *HSD11B2* activation could be to ensure that the GC effects within the immune cells are confined to GR, since the 11β-HSD2 enzyme ensures cortisol inactivation within the vicinity of the pro-inflammatory MR (Odermatt et al., 2001). *NR3C2* itself was downregulated in CR and stable, compared to tolerant patients (Figure 1), which could reflect an attempt to re-gain tolerance, as MR induction has been associated with T-reg decrease (Amador et al., 2014), and to assist anti-inflammatory processes, as a macrophage-specific deletion of MR results in alternative activation and M2 polarisation of macrophages (Usher et al., 2010). A further argument in support of GC involvement in the activation of anti-inflammatory pathways in CR was the increased expression of *TSC22D3* (Figure 4), a key GC-induced regulator of inflammation (Beaulieu and Morand, 2011), commonly known as *GILZ* (glucocorticoid-induced leucine zipper protein gene). GILZ protein exerts anti-inflammatory properties by inhibiting T-cell activation and nuclear factor kappaB (Ayroldi et al., 2001) and directs the differentiation of antigen-specific T-reg (Hamdi et al., 2007), outlining a role of *GILZ* gene, along with its inductor GR, in tolerance. The expression of *GILZ (TSC22D3)* gene was fairly strongly positively associated with the expression of *NR3C1* gene (r=0.46, p=<0.001), more weakly with *HSD11B2* gene (r=0.20, p=0.004), effectively not associated with *HSD11B1* gene (r=0.03, p=0.70) and negatively associated with *NR3C2* gene

expression (r=-0.21, p=0.002). Although we were not able to measure the GR protein levels or binding capacity or the activity of 11β-HSDs, the fact that the increase of *NR3C1* gene expression was paralleled by an upregulation of the GC-inducible GR-responsive *GILZ* gene and that both genes were considered by the statistical algorithm important for CR discrimination (Figure 4B), indicates that there is a true activation of the GC cascade with the involvement of GR protein synthesis and a GR-mediated GC response, at least in some blood sell subtypes, in CR patients.

3.4 Gene-cell associations

Having unravelled an association of steroid conversion and receptor genes with tolerance and CR, we set to investigate possible associations between these and the classification-informative tolerance-associated genes, as well as with the main components of the cellular compartment of peripheral blood, using hierarchical cluster analysis of the Pearson correlation (r) matrix of drug-adjusted gene-expression and cell counts (Figure 6, Figure S6). While tolerance-associated genes were, overall, strongly correlated with each other and formed two distinct clusters, steroid conversion and receptor genes were fairly isolated in separate clusters. Contrary to the reports of co-expression of *NR3C1* and *HSD11B1* in steroid target tissues (Tomlinson et al., 2004), there was only a very weak positive association of *NR3C1* with *HSD11B1* (r=0.13, p=0.037), but a very strong one with *H6PD* (r=0.75, p<0.001) instead (Figure 6). Despite the opposite changes in the expression of the GR and MR gene in tolerant and CR patients (Figure 1D-F), there was no direct negative association between them (r=0.024, p=0.708). There was, however, a positive association of the expression of *NR3C1* with neutrophil counts (r=0.30, p<0.001) and a negative association with T-cell (r=-0.32, p=0.001) and total and transitional B-cell counts (both r=-0.34, p=0.001), while *NR3C2* expression was positively associated with T-cell (r=0.47, p<0.001) and less with total and transitional B-cell counts (r=0.21, p=0.046 and r=0.20, p=0.064) and negatively associated with neutrophil counts (r=-0.22, p=0.003), indicating an indirect link between *NR3C1* and *NR3C2* regulation. In addition, there was some decrease of T-cell counts in CR compared to stable patients (p=0.011, Figure S5B),

which may reflect an infiltration of a particular T-cell sub-set in the kidney, but there was no difference in neutrophil counts (p=0.892, Figure S5A). Drug-adjusted B-cell counts were higher in tolerant and lower in CR patients (Figure S5C,D) (similar to the un-adjusted counts (Rebollo-Mesa et al., 2016)) and were positively associated with the B-cell receptor genes *IGKV4-1* (r=0.50, p<0.001 for total and r=0.29, p=0.008 for transitional B-cell counts) and *IGKV1D-13* (r=0.42, p=0.001 and r=0.24, p=0.069) (Figure 6), as would be expected.

Published reports indicate that peripheral blood lymphocytes from CR patients show lower number of GR binding cites and lower GR affinity for GC compared to stable patients (Ribarac-Stepic et al., 2001), which would be in agreement with our findings of a negative association of *NR3C1* expression with T and B-cell counts. The positive association of *NR3C1* expression with neutrophil counts may explain why we have observed a net increase in *NR3C1* expression in whole blood samples from CR compared to stable patients (Figure 1D, Figure 2B). The adjustment for drug intake that we have used would take care of differences potentially arising from prednisolone contribution to granulocytosis (Nakagawa et al., 1998), as shown in Figure S2, and we would, therefore, expect that true differences in neutrophils exist between CR and stable patients.

3.5 Glucocorticoid resistance could be present in chronic rejection, related to upregulation of the GRβ isoform

Having established *NR3C1* as one of the main genes up-regulated in CR (based on GRα+β mRNA expression), we set to examine the relative contribution of the alternatively spliced inhibitor isoform GRβ. Whilst the absolute levels of GRβ mRNA expression were low (in agreement with literature reports (Oakley and Cidlowski, 2011)), evidencing a clear upregulation of the GRα isoform, the patterns of expression of the GRβ isoform followed those established for GRα+β mRNA - there was no evidence for prednisolone effect (Figure S7A, similar to Figure S1D,I,N) and the drug-adjusted GRβ mRNA expression was particularly high in CR, but also in stable, compared to tolerant patients

(Figure S7B, similar to Figure 1D). Liang et al. have reported a reduced mRNA expression and protein levels for GRα in PBMC for GC-resistant compared to GC-sensitive patients with immune thrombocytopenia and no difference in GRβ mRNA expression, which have led the authors to the conclusion that downregulation of GRα levels may be involved in GC-resistance in this condition (Liang et al., 2016). Our findings in whole blood samples from CR patients bear more similarity to ulcerative colitis, where an increase of GRβ-positive cells and GRβ mRNA expression in colonic mucosa has been reported in GC-resistant compared to GC-sensitive patients, along with an increase of GRα mRNA expression in both groups of patients compared to healthy controls, which have led these authors to propose that GRβ may be involved in GC-resistance in ulcerative colitis (Fujishima et al., 2009). Given that we have examined drug-adjusted gene expression values, which reveal endogenous, rather than prednisolone-driven processes (Section 3.1.), it is possible that pro-inflammatory transcription factors in CR contribute to the development of steroid resistance via the inhibitory effect of GRβ on the GC response of GRα. Indeed, a similar-fold increase in mRNA expression of both GRα and GRβ isoforms has been described in cell lines *in vitro* in response to tumour necrosis factor (TNFα), which resulted in a disproportionately larger increase in the steady-state levels of the GRβ protein isoform compared to GRα (Webster et al., 2001). Given that we have examined all blood cells jointly in a whole blood sample, it is also possible that GRα and GRβ mRNA upregulation does not co-localise in the same cells and that a GRβ-conferred GC resistance in a particular cellular sub-type, most likely induced by pro-inflammatory cytokines, hinders the anti-inflammatory attempts initiated by GRα upregulation in a different cellular subtype and leads to an overall inefficiency of IS treatment in CR. The quantitative importance of GRβ gene up-regulation in CR warrants further investigation, especially in the light of the increased GRα mRNA expression. A factor that could be contributing to GC resistance down-stream from GR gene expression is vitamin D, which when increased could inhibit the nuclear translocation of GR via alternative phosphorylation (Kassi et al., 2016). In kidney transplant recipients, however, vitamin D levels are often low (Cianciolo et al., 2016) and a vitamin-D-induced GC resistance may be of lesser importance

for them, unless high doses of exogenous vitamin D products are administered. Whist GC resistance developing during dialysis treatment prior to transplantation could also propagate into the post-transplantation period and increase the risk of CR (De Antonio et al., 2008,Frezza et al., 2014), no data were available on the pre-transplantation status of our patients as they were recruited several years post-transplantation when tolerant patients are usually identified.

4 Conclusions

The expression of steroid conversion and receptor genes in peripheral blood of KTR showed differences in opposite directions to our original hypotheses. Increased steroid activation and response paralleled an anti-inflammatory demand, rather than indicated an advantageously high constitutive anti-inflammatory predisposition (*HSD11B1*, *H6PD* and *NR3C1* genes were upregulated in CR and stable compared to tolerant patients). Conversely, the expression of the MR-gene reflected a suppression of pro-inflammatory processes in conditions of increased immunological challenge, rather than indicated an advantageously low constitutive pro-inflammatory state in "operational tolerance" (*NR3C2* was downregulated in CR and stable compared to tolerant patients) (Figures 1,2,4). Our findings suggest that "operational tolerance", rather than being a condition in which active pro-inflammatory processes are efficiently suppressed by GC-related anti-inflammatory responses, is a state in which the immune system does not appear to identify the graft as "foreign" and, hence, neither activation of the GC-related anti-inflammatory response nor suppression of the pro-inflammatory MR are required. Further, our data suggest that in some peripheral blood cells in CR GC-related anti-inflammatory responses are operating and the MR is suppressed and, hence, is less likely to contribute to the pro-inflammatory processes, but also, that in other cell types there is GC-resistance related to up-regulation of the suppressive GRβ isoform or the *HSD11B2* gene involved in intracellular GC-inactivation.

Using penalised logistic regression as a statistical learning tool we have demonstrated that steroid conversion and receptor genes have a place right into the heart of the immunological mechanisms

involved in tolerance and CR in KTR. It is impressive that tolerance signatures based only on steroid conversion and receptor genes showed similar performance at cross-validation to the gene signatures derived after addition of 88 tolerance-associated genes (Table 1). Expression of steroid conversion and receptor genes was poorly associated with other classification-relevant genes (Figure 6), indicating their involvement within unexamined immunological networks. While these findings should not be unexpected, considering the central role that steroids play in immunoregulation and the anti-inflammatory response and the fact that that steroid treatment is fundamental to the maintenance IS regimens in KTR, it is surprising that studies of endogenous steroid conversion and response have not yet found a deserved place in the context of transplantation research. The role of GC conversion and the constitutive intra-organ bioavailability of cortisol to tolerance remains currently unexplored. Given that the activating 11β-HSD1 enzyme is preferentially expressed in liver and the inactivating 11β-HSD2 enzyme in kidney (Hardy et al., 2012), it may not be coincidental that "operational tolerance" is far more frequent in liver than in kidney recipients (Lerut and Sanchez-Fueyo, 2006). It is, therefore, imperative that the intracrine aspects of steroid metabolism and action are further evaluated in solid-organ transplantation.

Our study has examined differences between KTR in whole blood at gene-expression level. It should be noted, however, that alterations in mRNA levels may not automatically translate into altered protein levels and enzyme activity and that factors involved in the regulation of the GC response extend into posttranslational protein modifications, GR affinity to GC, availability of chaperons inactivating GR in the cytoplasm, GR translocation into the nucleus and GR interactions with other transcription factors involved in the pro- and anti-inflammatory processes. Nevertheless, our study reveals that differences in steroid conversion and receptor genes between tolerant, CR and stable patients are comparable or even larger than the differences for well-established tolerance associated genes such as *FOXP3* and the B-cell receptor genes *IGKV4-1* and *IGKV1D-13* (Figure 4), indicating that steroid conversion and receptor genes should be taken into consideration when genes are selected for clinically-relevant gene-expression signatures.

Our work also directs future studies in cell subsets based on the demonstrated association of the GR-gene (NR3C1) with neutrophils and of the MR-gene (NR3C2) with T-cells (Figure 6), locations previously noted (Armanini et al., 1988,Nakagawa et al., 1998), but with insufficiently unexplored role in transplantation. Immune cell functional studies in transplantation are often confined to PBMC, due to their analytical stability, disregarding a potential contribution of neutrophils and the innate immune response to the immunological processes involved in tolerance and rejection. Our work highlights the need to address this gap. It also illustrates how analysis of peripheral whole blood can give an *in vivo* insight into pathological states in the human with minimally invasive means, reflecting the net effect of simultaneously activated immunological pathways.

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Figure Captions

Figure 1 Comparison of drug-adjusted expression of individual steroid conversion and receptor genes between clinical groups

Drug-adjusted gene expression (fold difference on log2 scale) was derived as the difference between the observed $-\Delta$Ct values (relative to hypoxanthine phosphoribosyltransferase (*HPRT*)) and the values predicted by linear regression models, based on stable patients, with a binary indicator for prednisolone intake and categorical indicators for intake of calcineurin inhibitors (CNI) (off, cyclosporine, tacrolimus) and anti-proliferative drugs (off, azathioprine, mycophenolate mofetil/mycophenolic acid); **Tolerant** – tolerant patients (n=17); **Stable** – stable patients (n=190); **CR** – chronic rejector patients (n=37); **HC** – healthy controls (n=14); **Black dots** – patients receiving prednisolone; **Gene symbols** are explained with full gene names in Table S1; **NR3C1v2** – difference between the expression of *NR3C1* and *NR3C2* on log2 scale (equivalent to the ratio on a linear scale); **p-values** were derived from Wilcoxon-Mann-Whitney tests.

Figure 2 Regression coefficients for steroid conversion and receptor genes jointly examined in multivariable models

Group comparisons were based on penalised logistic regression (elastic net penalty, alpha 0.7); **A.** – a model comparing tolerant (n=17) with stable (n=190) patients; **B.** – a model comparing chronic rejector (CR) (n=37) with stable patients; **Regression coefficients** represent summaries (median, interquartile range (**grey box**), 2.5th - 97.5th centile (**vertical segments**)) of 50 repeats of five-fold cross-validation cycles (numeric details in Table S2) (absolute values indicate gene importance, for further details on the interpretation of the centile ranges see Section 2.3.2.); **Gene symbols** are explained with full gene names in Table S1.

Figure 3 Predicted probability of tolerance and chronic rejection based on steroid conversion and receptor gene signatures

Predicted probabilities were derived from multivariable penalised logistic regression models (elastic net penalty, alpha 0.7) with the training groups for each comparison between the clinical groups indicated in the plot title (the remaining two groups served as validation sets) and regression coefficients (Figure 2) derived as the median of 50 repeats of five-fold cross-validation cycles; **Tolerant** – tolerant patients (n=17); **Stable** – stable patients (n=190); **CR** – chronic rejector patients (n=37); **HC** – healthy controls (n=14); **black dots** – patients receiving prednisolone; **T1** –timepoint 1 training dataset (**A,C**) ; **T2** – timepoint 2 validation dataset (**B,D**); **p-values** were derived from Wilcoxon-Mann-Whitney tests.

Figure 4 Regression coefficients for steroid conversion and receptor genes and tolerance-associated genes jointly examined in multivariable models

Comparisons between clinical groups were based on penalised logistic regression (elastic net penalty, alpha 0.7), including 95 genes (5 steroid conversion and receptor genes, 88 tolerance-associated genes, 2 genes used as reference in the literature); **A.** – a model comparing tolerant (n=13) vs stable (n=171) patients; **B.** – a model comparing chronic rejectors (CR) (n=34) with stable patients; **Regression coefficients** represent summaries (median, interquartile range (**grey box**), 2.5^{th} - 97.5^{th} centile (**vertical segments**)) of 50 repeats of five-fold cross-validation cycles (numeric details in Table S3) (absolute values indicate gene importance, for further details on the interpretation of the centile ranges see Section 2.3.2.); Only classification-relevant genes are labelled for each group comparison; **Gene symbols** are explained with full gene names in Table S1 (steroid conversion and receptor genes have been listed first).

Figure 5 Predicted probability of tolerance or chronic rejection based on steroid conversion and receptor and tolerance-associated gene signatures

Predicted probabilities are based on multivariable penalised logistic regression models (elastic net penalty, alpha 0.7) with the training groups for each comparison between the clinical groups indicated in the plot title (the remaining groups served as validation sets) and regression coefficients (Figure 4) derived as the median of 50 repeats of five-fold cross-validation cycles; **Tolerant** – tolerant patients (n=13); **Stable** – stable patients (n=171); **CR** – chronic rejector patients (n=34); **HC** – healthy controls (n=11); **black dots** – patients receiving prednisolone; **T1** – timepoint 1 training dataset (**A,C**) ; **T2** – timepoint 2 validation dataset (**B,D**); **p-values** were derived from Wilcoxon-Mann-Whitney tests.

Figure 6 Correlation heatmap of drug-adjusted expression of steroid conversion and receptor genes, classification-relevant tolerance-associated genes, neutrophils and lymphocyte counts

Drug-adjusted gene expression (fold difference on log2 scale) and **drug-adjusted cell counts** were derived as the difference between the observed values and the values predicted by linear regression models, based on stable patients, with a binary indicator for prednisolone intake and categorical indicators for intake of calcineurin inhibitors (CNI) (off, cyclosporine, tacrolimus) and anti-proliferative drugs (off, azathioprine, mycophenolate mofetil/mycophenolic acid); the dataset comprised tolerant, stable and chronic rejector patients at timepoint 1; the correlation matrix was based on pairwise Pearson correlation coefficients; * blood cell subtypes; ** steroid conversion and receptor genes; **Gene symbols** are explained with full gene names in Table S1.

Table 1. **Multivariable model performance evaluated with AUC**

Time point	Signatures / Comparison	Tolerance g5	Tolerance g17	Chronic rejection g2	Chronic rejection g15
T1	Tolerant/CR* vs Stable (train)	0.87 (0.79 - 0.94)	0.98 (0.96 – 1.00)	0.65 (0.55 - 0.75)	0.88 (0.83 - 0.94)
T1	Tolerant/CR* vs Stable (cross-validation)**	0.82 (0.78 - 0.85)	0.82 (0.73 - 0.89)	0.57 (0.50 - 0.62)	0.73 (0.68 - 0.77)
T2	Tolerant/CR* vs Stable (test)	0.78 (0.66 - 0.90)	0.95 (0.88 – 1.00)	0.75 (0.61 - 0.89)	0.90 (0.80 – 1.00)
T1	Tolerant vs CR (train+test)	0.86 (0.77 - 0.96)	0.96 (0.91 – 1.00)	0.77 (0.64 - 0.90)	0.93 (0.82 – 1.00)
T2	Tolerant vs CR (test)	0.94 (0.86 – 1.00)	0.98 (0.93 – 1.00)	0.85 (0.70 – 1.00)	0.96 (0.89 – 1.00)

*****- comparison of tolerant vs stable patients for the tolerance signatures and chronic rejector (**CR**) vs stable patients for the CR signatures; **AUC** - area under the ROC (receiver operating characteristics) curve (95% DeLong confidence interval, except for cross-validation (see below)); **Signature** – gene-expression signature based on penalised logistic regression with elastic net penalty (alpha 0.7) (gene selection was based on a median regression coefficient >0.001 or <-0.001 from 50 group-stratified five-fold cross-validation repeats); **g2** – 2-gene signature of CR based on *HSD11B2* and *NR3C1* expression (Figure 2B); **g5** - 5-gene signature of tolerance, based on *H6PD, HSD11B1, HSD11B2, NR3C1* and *NR3C2* expression (Figure 2A); **g15** – 15-gene signature of CR based on the expression of *HSD11B2, NR3C1* and a selection of 13 tolerance-associated genes (Figure 4B); **g17** – 17-gene signature of tolerance based on the expression of *HSD11B1, NR3C1* and *NR3C2* and a selection of 14 tolerance-associated genes (Figure 4A); **Gene symbols** are explained with full gene names in Table S1; **train** – AUC of predicted probability of tolerance (tolerance signature) or CR (CR signature) for the training dataset – timepoint 1 (**T1**); **cross-validation** – median AUC (2.5^{th} – 97.5^{th} centile) from 50 repeats of five-fold cross-validation cycles with the training data; **test** - AUC of the predicted probability of tolerance or CR for the validation dataset – timepoint 2 (**T2**); **train+test** – AUC of the predicted probability of tolerance comparing (at timepoint 1) the training tolerant patients with the CR patients (which do not participate in the tolerance signature development) and AUC of the

predicted probability of CR comparing (at timepoint 1) the tolerant patients (which do not participate in the CR signature development) with the training CR patients.