Protein and Metabolite Biomarkers of Graft Injuries in Renal Transplantation

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UMR 850 INSERM/Univ Limoges/CHU Limoges
DHU SUPORT
FP7 BIOMARGIN
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<tr>
<th>Type of Financial Interest within last 12 months</th>
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<td>☐ Grants/Research Funding</td>
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<td>☐ Stock Shareholder</td>
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<td>☐ Consulting Fees</td>
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<td>☐ Other (Receipt of Intellectual Property Rights/Patent Holder, Speaker’s Bureau)</td>
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Clinical problems & needs in organ transplantation

- Large variability in patients and graft survival
- Little improvement in long-term survival
- Many possible causes, including ischemia-reperfusion, immune reactions, comorbidities, nephrotoxic drugs...

Need for early biomarkers of:
- Pre- and post-transplantation graft injuries
- Immunosuppressive drug efficacy or toxicity

![Kidney](image)

![Liver](image)

![Heart](image)

![Lung](image)
Possible biomarkers in Tx and associations

Types of biomarkers
- Nucleic acids (DNA, mRNA, micro-RNA)
- Proteins, peptides
- Lipids, metabolites

Matrix: preservation solution, machine perfusate, urine, blood … or biopsy

Possible association with:
- specific lesions (including ischemic lesions) in the graft
- primary non-function (PNF)
- delayed graft function (DGF)
- graft function in the stable phase
- graft survival (GS) or graft failure (GF)
1 – Pre-transplantation biomarkers
Characterization of the perfusate proteome of human donor kidneys


- Study of 18 kidney grafts kept in hypothermic conditions in a perfusion machine
  - Samples collected after 1h perfusion
  - 2D-DIGE separation of proteins
  - In-gel trypsin digestion
  - MALDI-TOF/TOF analysis
  - Mass spectra interpretation with MASCOT® algorithm and comparison with the Swissprot® database
  - 32 protein spots $\rightarrow$ 19 proteins identified
In kidneys with vs. without ischaemic lesions
- 2 unidentified protein spots significantly up-regulated
- haptoglobin significantly down-regulated

In kidneys with vs. without primary non-function:
- 2 unidentified protein spots up-regulated

In kidneys with vs. without delayed graft function:
- $\alpha_1$-antitrypsin up-regulated
Targeted analysis of 6 biomarkers in the perfusate of 306 renal grafts

[Moers et al. *Transplantation* 2010;90: 966–973]

- Kidneys from donation after brain death (231) or cardiac death (75)
- 230 kidneys developed immediate function, 7 PNF and 76 DGF
- Targeted biomarkers: LDH, ASAT, total GST, Ala-AP, NAG, H-FABP (i.e., proteins)
- Enzymatic and ELISA assays
Evolution of each biomarker’s perfusate concentration in time
Statistical analysis adjusted on known risk factors

Only GST, NAG and H-FABP concentrations measured at the end of machine perfusion (*but no sooner*) were independent predictors of delayed graft function

- However, all AUROC < 0.7
- Poor sensitivity & specificity
- No clear cut-off values

None of the 6 biomarkers could predict graft failure within the first year post-transplant
Targeted analysis of 6 biomarkers in the perfusate of 335 renal grafts

[Hoogland et al; Transplantation 2013;95: 603-610]

- Partly the same team as in previous study
- Partly same kidneys? Observation period 1997-2008
- All kidneys from donation after cardiac death (DCD)
- 63 kidneys developed immediate function, 67 primary non-function and 205 delayed function
- Targeted biomarkers: LDH, total GST, H-FABP, redox-active iron, IL-18 and NGAL (i.e., proteins and one metabolite)
- Enzymatic and ELISA assays for the proteins, “bleomycine detectable iron assay” for redox-active iron
**Biomarker concentrations at T4h MP**

<table>
<thead>
<tr>
<th></th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Risk of PNF (biomarker concentration at T_4)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>1.004 (0.998–1.009)</td>
<td>0.161</td>
</tr>
<tr>
<td>LDH</td>
<td>1.001 (1.000–1.002)</td>
<td>0.005</td>
</tr>
<tr>
<td>H-FABP</td>
<td>1.002 (0.998–1.007)</td>
<td>0.280</td>
</tr>
<tr>
<td>Redox-active iron</td>
<td>1.462 (0.974–2.195)</td>
<td>0.067</td>
</tr>
<tr>
<td><strong>IL-18</strong></td>
<td>1.001 (1.000–1.002)</td>
<td>0.003</td>
</tr>
<tr>
<td>NGAL</td>
<td>0.999 (0.980–1.018)</td>
<td>0.919</td>
</tr>
<tr>
<td><strong>Risk of DGF (biomarker concentration at T_4)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>1.006 (0.999–1.013)</td>
<td>0.112</td>
</tr>
<tr>
<td>LDH</td>
<td>1.002 (1.001–1.004)</td>
<td>0.007</td>
</tr>
<tr>
<td>H-FABP</td>
<td>1.007 (1.000–1.014)</td>
<td>0.064</td>
</tr>
<tr>
<td>Redox-active iron</td>
<td>1.532 (1.045–2.245)</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-18</td>
<td>1.003 (1.001–1.004)</td>
<td>0.002</td>
</tr>
<tr>
<td>NGAL</td>
<td>1.000 (0.982–1.018)</td>
<td>0.994</td>
</tr>
</tbody>
</table>
Kaplan-Meier graft survival curves

- GST, LDH & H-FABP significantly linked with graft survival

- Not redox-active iron, IL-18 or NGAL
In Summary: pre-transplantation biomarkers

- 1 very preliminary untargeted study of the proteome

- **Candidate biomarkers:**
  - **Ischemic lesions:** haptoglobin
  - **PNF:** LDH, GST ± redox iron and IL-18 after 4h MP
  - **DGF:** LDH, GST ± NAG and H-FABP after 12h MP, redox iron and IL-18 after 4h MP ± α1-antitrypsin
  - **Plasma creatinine level at M3:** Glutamate after 4h MP; Valine, alanine, glycine, glutamate and total glutathione after 12h MP
  - **Graft survival:** LDH ± GST & H-FABP after 4h MP
2 – Post-transplantation biomarkers
Several teams have searched for biomarkers of renal graft lesions, such as:
- mRNAs, micro-RNAs
- peptides, proteins
- (metabolites)

Urine > plasma > graft

Mostly biomarkers of “acute rejection” (vs. “normal”)

Little cross-fecundation of these different “omics” approaches
Regarding proteomics/peptidomics:

– **Untargeted approaches** → large diversity of mass spectrometry settings (historically SELDI-TOF, moving to MALDI-TOF/TOF, electrospray-orbitrap, ESI-Q/TOF, etc.)

– **Targeted approaches** → mostly immunoassays (e.g., ELISA), sometimes LC-MS/MS in the MRM mode
## Some untargeted proteomic results

<table>
<thead>
<tr>
<th>Reference</th>
<th>Biomarker candidates</th>
<th>Sample type</th>
<th>Method</th>
<th>Sample numbers</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quintana et al. 2009</td>
<td>14 ions in urine (proteins and peptides not identified)</td>
<td>Urine</td>
<td>MALDI-TOF</td>
<td>50</td>
<td>IFTA, AbMR</td>
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<tr>
<td>Bañón-Maneus et al. 2010</td>
<td>SERPINA1, α1BG, AGT, TFR2, GSN, anti-TNFα antibody light chain, B2M, heparin sulfate proteoglycan, leucine-rich, α-2-glycoprotein-1</td>
<td>Urine</td>
<td>2D-DIGE + MALDI-TOF and CE-MS/MS</td>
<td>24 training set</td>
<td>IFTA</td>
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<tr>
<td>Sigdel et al. 2010</td>
<td>UMOD, PEDF and CD44, among 22 up-regulated and 23 down-regulated proteins</td>
<td>Urine</td>
<td>Capillary LC-ESI-linear ion trap MS</td>
<td>60</td>
<td>AR</td>
</tr>
<tr>
<td>Wu et al. 2011</td>
<td>NF-κB, STAT1, STAT3 and 63 other proteins</td>
<td>Plasma</td>
<td>iTRAQ + preparative HPLC + offline ESI-Q/TOF</td>
<td>13</td>
<td>AR</td>
</tr>
<tr>
<td>Loftheim et al. 2012</td>
<td>IGFBP7, VASN, EGF, LG3BP</td>
<td>Urine</td>
<td>2D-LC-ESI-Orbitrap (IDA mode)</td>
<td>12</td>
<td>AR</td>
</tr>
<tr>
<td>Sigdel et al. 2014</td>
<td><strong>AR</strong>: HLA-DRB1, FGB, FGA, FGG, KRT14, HIST1H4B, ACTB, KRT7, DPP4, <strong>BKVN</strong>: KRT18, SUMO2, STMN1, CFHR2, KRT8, KRT19, RPL18, KRT75, FAM3C, HIST1H2BA <strong>CAI</strong>: CALR, FAM151A, SERPINA2P, FAM3C, DAG1, KITLG, LUM, FABP4, AGT, LRG1</td>
<td>Urine</td>
<td>iTRAQ + 2D-LC-ESI-LTQ-Orbitrap</td>
<td>108 discovery set</td>
<td>AR, BKVN, CAI</td>
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### Some targeted proteomic results

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Sample type</th>
<th>Sample numbers</th>
<th>Outcome</th>
<th>Method</th>
<th>AUROC</th>
<th>Reference</th>
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<tr>
<td>NGAL</td>
<td>Urine</td>
<td>91</td>
<td>DGF</td>
<td>Immunoassay</td>
<td>0.82</td>
<td>Hall et al. 2010</td>
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<tr>
<td></td>
<td>Urine</td>
<td>182 and 11 for validation</td>
<td>AKI</td>
<td>Immunoassay (screening and validation)</td>
<td>0.92</td>
<td>Heyne et al. 2012</td>
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<tr>
<td></td>
<td>Urine</td>
<td>577</td>
<td>Graft failure</td>
<td>Immunoassay</td>
<td>0.63</td>
<td>Nauta et al. 2011</td>
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<td>CXCL9</td>
<td>Urine</td>
<td>91</td>
<td>Subclinical tubulitis</td>
<td>Immunoassay</td>
<td>0.78</td>
<td>Schaub et al. 2009</td>
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<td></td>
<td>Urine</td>
<td>204</td>
<td>AR</td>
<td>q-PCR (for mRNA) Immunoassay (for proteins)</td>
<td>0.856</td>
<td>Hricik et al. 2013</td>
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<td></td>
<td>Urine</td>
<td>156</td>
<td>AR and BKVI</td>
<td>Immunoassay</td>
<td>0.86 sensitivity 0.80 specificity</td>
<td>Jackson et al. 2011</td>
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<tr>
<td>CXCL10</td>
<td>Urine</td>
<td>91</td>
<td>Subclinical tubulitis</td>
<td>Immunoassay</td>
<td>0.79</td>
<td>Schaub et al. 2009</td>
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<td>91</td>
<td>IFTA</td>
<td>Immunoassay</td>
<td>0.845</td>
<td>Ho et al. 2011</td>
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<td></td>
<td>Urine</td>
<td>122</td>
<td>Acute and chronic injuries</td>
<td>Screening: Luminex multiplex Validation: Luminex quadruplex</td>
<td>0.93</td>
<td>Hu et al. 2009</td>
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<tr>
<td></td>
<td>Urine</td>
<td>156</td>
<td>AR and BKVI</td>
<td>Immunoassay</td>
<td>0.80 sensitivity 0.76 specificity</td>
<td>Jackson et al. 2011</td>
</tr>
</tbody>
</table>

*Some targeted proteomic results*
An integrated proteomic approach

(Sigdel et al. Mol Cell Proteomics 2014)
Specificity of the candidate biomarkers

(Sigdel et al. Mol Cell Proteomics 2014)
An integrated, European initiative

“Biomarkers of renal graft injuries in kidney allograft recipients”

(FP7-HEALTH.2012)

13 partners (Belgium, France, Germany, Sweden)

Funding: 6 M€
Detect, select and validate:

• Non-invasive, diagnostic and prognostic biomarkers of kidney graft lesions
  ✓ early biomarkers of acute or chronic lesions, as well as of graft loss-of-function
  ✓ by combining transcriptomics, proteomics and metabolomics, in blood and urine

• Mechanism-based tissue biomarkers, to improve the histological analysis and interpretation of graft biopsies
mRNA, micro-RNA, proteins, peptides, lipids, metabolites

**step 1: Discovery "Training set"**
- Blood + biopsy + urine samples
  - 30 normal
  - 30 T-cell mediated reject
  - 30 Ab-mediated reject
  - 30 IFTA

**step 2: Selection "Selection set"**
- Blood + biopsy + urine samples
  - 30 normal
  - 30 T-cell mediated reject
  - 30 Ab-mediated reject
  - 30 IFTA

**step 3: Validation "Trans-sectional study"**
- 450 sets of samples from a representative set of renal transplant patients
- 450 adults + 50 pediatrics. Follow-up from Tx to 5 years. Blood and urine samples at 1, 3, 6, 12 months then annually

**Biomarker candidates**

**step 4: Validation "Biomargin prospective cohort"**

**Consolidation (system biology) + Literature**

**Extended list of biomarker candidates**

**Diagnostic performance of biomarker candidates**

**Validated biomarkers**

**Diagnostic + prognostic performance of the final sets of biomarkers**
In summary

- Already many proteomic biomarker candidates for kidney allograft lesions … at least for AR! The lists are increasing with progress in mass spectrometry.

- However, all non-targeted studies have reported new candidates → have the best been discovered?

- BIOMARGIN:
  - is seeking new candidates, using in parallel CE-TOF, MALDI-TOF/TOF, LC-ESI-Q-TOF and LC-ESI-Orbitrap!
  - will then evaluate and rank as many candidates as possible, using LC-MRM
  - In the context of systems biology and all the other known predictors

- To be adopted clinically, it should be proven that biomarkers improve long-term graft survival and patients’ quality of life (need for randomized, controlled clinical trials)
Ask