Supplementary Materials and Methods

LC-MRM analysis

Targeted analysis was performed on an Agilent 6490 triple quadrupole mass spectrometer coupled with a nano-Chip-LC Agilent Infinity Series HPLC1290 system, as described recently (1). Solvent compositions were 97.8% H_2O, 2% ACN and 0.2% FA for solvent A, and 97.8% ACN, 2% H_2O and 0.2% FA for solvent B.

MRM Assay Development

The selection of targeted peptides was based on the same criteria as recently published (1). Briefly, only prototypic peptides with a length of 8 to 25 amino acids and without any methionine residue or missed cleavages were accepted. Additionally, peptide extracted ion chromatograms (XICs) on MS1 and MS2 level were manually checked. At the MS1 level, this involved selection of the right peak, considering co-elution of all precursors. Identification triggers over all samples and technical replicates had to align within a certain time window. Peptides had to be identified only once over the whole chromatographic run (multiple identifications were only accepted for very broad peaks) and had to be identified in at least two of the three different sample types (low TL, high TL, control). Thereby, 940 transitions for 188 peptides (93 proteins) were used for unscheduled MRM measurements, applying different dwell times depending on the intensities achieved for peptide precursors in shotgun MS: 100ms for those with the lowest intensities ($<10^6$), 50ms for those with moderate intensities (10^6-10^7), and 20ms for those with highest signal intensities ($>10^7$). One biological sample, each of the low TL, high TL and control group, previously used for shotgun MS, was injected multiple times for measuring the 940 selected transitions in unscheduled MRM mode.

The peak picking for the scheduled MRM assay was done based on the same criteria as recently published (1), which were: selection of only co-eluting peaks with consistent peak shape for at least three out of the five investigated product ions and a dot-product (dotp) value of at least 0.8. Additionally, measured and calculated (based on the Skyline SSRcalc 3.0) retention times had to match with a correlation greater R= 0.9. Finally, three most intense and interference-free transitions were selected per peptide for the scheduled MRM method, resulting in 372 transitions for 126 peptides interfered from 88 proteins. The interference free properties of transitions corresponding to peptides derived from proteins,

which were found significantly regulated in the targeted proteomics analysis (Figure 2), are shown in the Figure S1. The unchanged peak area percentages over 90 injections indicate the interference-free character of selected transitions. Although trem-like transcript 1 protein (TREML1) fulfil the threshold criteria for significant regulation, it was excluded from the panel of biomarker candidates since selected peptides show strong variation of the transition peak area percentage (Figure S1). In order to maintain a minimum dwell time of 20ms, an additional and final revision was performed, limiting the number of peptides per protein to two, removing peptides with insufficient signal intensities in all biological groups and removing proteins with low biological significance (based on literature and shotgun data). In the end, a final scheduled MRM method for 92 peptides (276 transitions) derived from 58 target proteins and using a 3min time window was established (Table S3). Transitions of standard peptides spiked in each samples were included in the final MRM method. All clinical samples were measured as technical triplicates. Skyline software (2) was used to implement this MRM method, allowing data analysis and data preparation for statistical analysis.

Statistical analysis of MRM data

The statistical analysis of the data was performed with MSStats (v. 2.3.5) (3) employing the linear mixed effects model. The manual inspection regarding correct peak selection, interferences, and integration boundaries of the data was done with Skyline. All other steps were conducted in R using MSStats package. Data pre-processing and quality control steps consisted on $\log_2$ transformation and normalization to the four spiked standard peptides. The missing peaks were replaced with NA value. Furthermore, in order to remove the very low abundant transitions and to consider only the most informative features for further analysis, the Feature Selection was set to TRUE by applying the default setting. Both, for comparison of the abundances between sample groups and for abundance calculation of each protein sample, missed values were subjected to imputation using the average minimum intensity across all runs. Additionally, p-values were adjusted according to Benjamini and Hochberg and a significant threshold of 0.05 was applied. The statistical analysis for the screening study was restricted to the small number of samples, three samples per group (low TL, high TL, control). However, due to higher number of patient samples included in the validation study - fifteen per group (low TL, high TL) - the statistical analysis was performed with expanded scope of conclusions for biological replication.

References

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