SP339

New bioactive angiopoietin-2 ELISA allows the quantification of all three isoforms of angiopoietin-2 in chronic kidney disease

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SUMMARY AND CONCLUSION

This new angiopoietin-2 immunoassay uses high affinity antibodies to quantify free, bioactive human angiopoietin-2. The structural epitope of the recombinant coating antibody lies within the bioactive ANG2 receptor binding site, thus hindering the co-detection of circulating receptor bound ANG2 complexes. Microarray analysis demonstrates the presence of several linear epitopes for the polyclonal detection antibody. The majority of them are N-terminal/central of the ANG2 molecule. This ELISA presumably detects all three angiopoietin-2 isoforms as the binding site of the coating antibody is conserved among all of them and most polyclonal antibody epitopes are present in all three isoforms. Validation data demonstrate assay applicability in nephrological disorders including chronic kidney disease. Sample screening revealed a fivefold increase of ANG2 levels in patients with chronic kidney disease being on hemodialysis. After kidney transplantation circulating ANG2 decreased significantly. In summary, this new ELISA is a well-characterized, high quality tool to detect all human bioactive angiopoietin-2 isoforms in serum and plasma in healthy and diseased patient samples.

INTRODUCTION

Angiopoietin-2 (ANG2) is an important regulator of the angiopoietin-1/Tie-2 receptor signaling system on endothelial cells during angiogenesis. Disruption of this signaling leads to the loss of endothelial integrity and sensitizes the endothelium towards various pro-inflammatory cytokines and growth factors. Thus, ANG2 might cause vascular micro-inflammation in patients with CKD (chronic kidney disease) [1]. ANG2 levels increase with CKD stage [1, 2], are associated with fluid overload [3] and abnormal cardiac structure [4] and correlate with mortality in patients with CKD stages 4–5. Although ANG2 levels recover after successful kidney transplantation, ANG2 continues to be a cardiovascular risk factor in this population [1].

METHODS

An enzyme-linked immunosorbent assay for the detection of all three angiopoietin-2 isoforms in human serum and plasma was developed. Two high quality antibodies are combined in a sandwich test format: A recombinant monoclonal antibody is used as a capture antibody. A biotin-labeled polyclonal affinity-purified antibody serves for detection of the analyte. High resolution epitope mapping of the antibodies via peptide microarray technology allowed the identification of linear antibody epitopes. Technical performance and accuracy of the assay were assessed according to ICH/EMEA guidelines.

HUMAN BIOACTIVE ANG2 ELISA

Epitope mapping of detection antibody

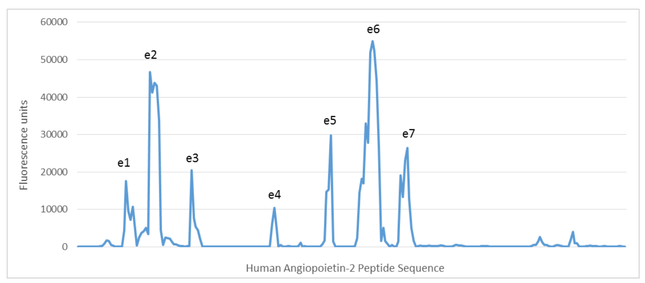

Fig. 1: Microarray analysis illustrates linear epitopes of the polyclonal detection antibody on human ANG2 sequence. Recorded fluorescent signals demonstrate the binding of the antibody to relevant epitopes on the human angiopoietin-2 sequence (O15123) previously printed on the chip. Minor signal intensities below 5000 fluorescence units were not considered. In total, seven linear epitopes were detected, whereas two of them displayed a twofold higher fluorescent signal than others. For the recombinant monoclonal antibody no fluorescence was recorded (not shown). This antibody recognizes a structural epitope within the C-terminus of angiopoietin-2, which covers the bioactive receptor-binding site of the protein.

Antibody epitopes on human ANG2 sequence

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                                                    e1
  1 MWQIVFFTLSCDLVLAAAYNNFRKSMDSIGKKQYQVQHGSCSYTFLLPEMDNCRSSSSPY  60
                e2                          e3
 61 VSNAVQRDAPLEYDDSVQRLQVLENIMENNTQWLMKLENYIQDNMKKEMVEIQQNAVQNQ 120
                                                  166       e4
121 TAVMIEIGTNLLNQTAEQTRKLTDVEAQVLNQTTRLELQLLEHSLSTNKLEKQILDQTSE 180
                                                       hu  e5
181 INKLQDKNSFLEKKVLAMEDKHIIQLQSIKEEKDQLQVLVSKQNSIIEELEKKIVTATVN 240
                                        e6        275
241 NSVLQKQQHDLMETVNNLLTMMSTSNSAKDPTVAKEEQISFRDCAEVFKSGHTTNGIYTL 300
     e7
301 TFPNSTEEIKAYCDMEAGGGGWTIIQRREDGSVDFQRTWKEYKVGFGNPSGEYWLGNEFV 360

361 SQLTNQQRYVLKIHLKDWEGNEAYSLYEHFYLSSEELNYRIHLKGLTGTAGKISSISQPG 420

421 NDFSTKDGDNDKCICKCSQMLTGGWWFDACGPSNLNGMYYPQRQNTNKFNGIKWYYWKGS 480

481 GYSLKATTMMIRPADF                                             496
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polyclonal antibody epitopes (red)

Isoform 2 = 97-148aa missing (yellow box)
Isoform 3 = 268aa missing (blue)
Coiled-Coil Domain: 166-248
Fibronogen-like Domain: 275-496

Fig. 2: Majority of antibody epitopes present in all three human angiopoietin-2 isoforms (O15123-1-3). Epitopes of the detection antibody are located N-terminal/at the centre (depicted in red) of the canonical agiopoietin-2 sequence. The monoclonal antibody recognizes a structural epitope within the C-terminus of angiopoietin-2, which covers the bioactive receptor-binding site of the protein, but does not interfere with any epitope of the polyclonal antibody (not shown). Compared to the canonical sequence (O15123-1) a stretch of 52 amino acids is missing in the N-terminus of isoform 2 (aa 97-148, yellow box). One minor epitope of the detection antibody falls within this region and won't be detected by the latter. Isoform 3 lacks an amino acid at position 268, which is part of another epitope of the detection antibody and might affect its binding to this isoform. Nevertheless, we expect to detect all three angiopoietin-2 isoforms, as the receptor-binding site, which corresponds to the coating antibody binding site, is conserved among all isoforms and, except the epitopes affected, the majority of detection antibody epitopes are present in all isoforms too.

High quality antibodies

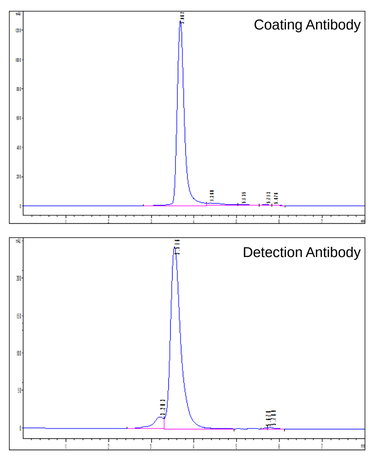
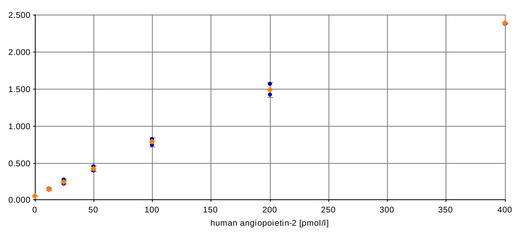


Fig. 3: High Performance Liquid Chromatography (HPLC) analysis demonstrates high quality of employed antibodies. To determine the purification grade of the coating and detection antibody, analytical size exclusion was performed using a Yarra X150 sepharose column from GE Healthcare. Chromatograms of both antibodies show a purity of 96.1 and 92.4% respectively.

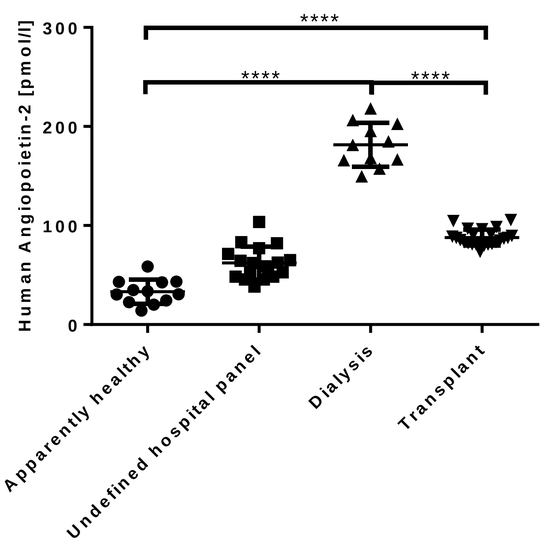
Standard curve



	c [pmol/l]	mean [OD]	CV [%]
STD 1	0	0.048	4%
STD 2	12.5	0.144	7%
STD 3	25	0.246	10%
STD 4	50	0.424	6%
STD 5	100	0.782	5%
STD 6	200	1.495	5%
STD 7	400	2.390	0%

Fig. 4: Typical ELISA standard curve of human angiopoietin-2 protein. It covers a range of 12.5 – 400 pmol/l recombinant angiopoietin-2 spiked into a serum-based matrix. Mean OD values result from a twofold measurement of the calibrator range, whereas the coefficient of variation (<10%) indicates low assay variation.

Increased bioactive ANG2 levels in serum samples with chronic kidney disease



**** p<0.0001

Fig. 5: Circulating Angiopoietin-2 levels of apparently healthy plasma samples measure between standard 3 and 4 of the calibration curve, in median 32.2 pmol/l (n=12). Analysis of an undefined hospital panel (mainly geriatric samples) shows elevated ANG2 levels (median 60.6 pmol/l, n=18). Compared to the healthy population, chronic kidney disease patients on hemodialysis display even a fivefold higher ANG2 concentration in their plasma (median 181.4 pmol/l, n=11). After kidney transplantation ANG2 levels significantly decrease (median 86.9 pmol/l, n=23), but are still higher than in the reference population. Calculated probability is below 0.0001 (Mann-Whitney Test) in all cases.

LITERATURE

1. *Molnar et al. (2014)*: Circulating angiopoietin-2 levels predict mortality in kidney transplant recipients: a 4-year prospective case–cohort study. Transplant International, 27 (6): 541-52
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4. *Tsai et al. (2016)*: Angiopoietin-2, Angiopoietin-1 and subclinical cardiovascular disease in Chronic Kidney Disease. Scientific Reports, 6:39400

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