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High levels of Serum Angiogenic growth factors in patients with AL amyloidosis: comparisons with normal individuals and multiple myeloma patients

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Key Words:	AMYLOIDOSIS, ANGIOGENESIS, MYELOMA, VEGF, Angiopoietin

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For Peer Review

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7 **High levels of Serum Angiogenic growth factors in patients with AL**
8 **amyloidosis: comparisons with normal individuals and multiple myeloma**
9 **patients**
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Summary

We evaluated the serum levels of five angiogenic cytokines in 82 patients with primary systemic amyloidosis (AL). Angiopoietin-1, VEGF bFGF and angiogenin were higher in AL patients than in controls (n=35) and newly-diagnosed, symptomatic, myeloma patients (n=35). The ratio of Angiopoietin-1/Angiopoietin-2 was lower in AL compared to controls but higher compared to myeloma patients. Angiopoietin-2 correlated with indices of cardiac dysfunction; however, none of the angiogenic growth factors was of prognostic significance. The increased levels of angiogenic cytokines observed in AL seem to represent either a toxic effect of amyloid fibrils or light chains, or a compensatory response to organ dysfunction.

Keywords: Amyloidosis, Angiogenesis, Myeloma, VEGF, Angiopoietin

Introduction

In primary systemic light chain (AL) amyloidosis a monoclonal light chain produced by a plasma cell clone forms amyloid fibrils which are deposited in the microcirculation causing tissue damage and organ dysfunction. Kidneys, heart, liver, nerves, GI tract and soft tissue are most commonly involved. AL amyloidosis is fatal due to complications arising from the amyloid infiltrated organs, mainly due to cardiac amyloidosis (Merlini and Stone 2006). The extent of plasma cell clone in AL amyloidosis is usually modest, with a low proliferative activity, in contrast to multiple myeloma (MM). The intrinsic characteristics of the plasma cell clone, the effects of the amyloidogenic light chain to the plasma cells but also the plasma cell-bone marrow microenvironment interaction may explain the less “aggressive” clonal course in AL amyloidosis. Angiogenesis, a critical step for MM progression, is part of the complex clone-microenvironment interactions and is regulated by several molecules.. Vascular endothelial growth factor (VEGF) induces the formation of new blood vessels, at least during the initial steps of angiogenesis (Podar and Anderson 2005). Angiogenin and basic fibroblast growth factor (bFGF) are also potent stimulators of both physiological and pathological angiogenesis. Angiopoietin-1 (Ang-1) and -2 (Ang-2) play a significant role in vessel formation, sprouting, stabilization and maturation. The levels of these growth factors have been found to be elevated in the serum of patients with multiple myeloma (MM) and have been correlated to disease stage (Anargyrou, *et al* 2008, Rajkumar, *et al* 2002, Sezer, *et al* 2001). Furthermore, they play a role in the functional integrity of several organs such as renal glomeruli, endothelium, peripheral nerve (Carmeliet 2005) and may have a prognostic significance for patients with AL. However, such data are not available. Thus, we measured the levels of the aforementioned angiogenic cytokines in the serum of patients with AL amyloidosis and analyzed for possible correlations with disease features, organ involvement and outcome.

Patients & Methods

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3 **This study included patients who had been diagnosed with AL**
4 **amyloidosis from January 2000 until June 2008 and who had available serum**
5 **samples prior to treatment initiation.** Definition of organ involvement, hematologic
6 and organ response and progression were based on consensus criteria (Gertz, *et al*
7 2005). Serum was collected and stored at -80°C until measurement. Serum samples
8 were also collected from 35 age-matched healthy controls and 35 newly-diagnosed,
9 untreated, symptomatic myeloma patients. **An approval for the collection of the**
10 **serum samples and publication of patient data was obtained from the**
11 **institutional review board of Alexandra Hospital. All patients and healthy**
12 **controls gave an informed consent for the collection and study of serum**
13 **samples.**

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27 Serum levels of Ang-1, Ang-2, VEGF, angiogenin, and bFGF were evaluated
28 using ELISA methodology, as previously described (Anargyrou, *et al* 2008). Free light
29 chains were assessed with the Freelite assay (Binding Site, Birmingham, UK) and
30 the N-terminal fragment of pro B-type natriuretic peptide (NT-proBNP) using a
31 standard assay (Roche Diagnostics GmbH, Mannheim, Germany).

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38 **For the comparisons between groups we used the unequal variance t-**
39 **test, since there were significant differences in variances. Spearman's**
40 **nonparametric correlation coefficient and linear regression were used for the**
41 **identification of correlations between continuous variables. Due to the skewed**
42 **distribution of the cytokine levels we also used logarithmic transformation.**
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49 Survival was calculated from the day of treatment initiation until the date of death or
50 last follow-up. Time to events comparisons were made with the use of log rank test.
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53 Cox proportional hazards models were used for time to event multivariate analysis.
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56 All analyses were performed using SPSS 15 software.

57 **Results & Discussion**

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60 Eighty-two patients, (median age 63, range 39-86), were included in this
analysis. The median number of involved organs was 2 (range 1-4); most had kidney

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3 (76%) and/or heart involvement (56%) and 25% had advanced symptomatic
4 congestive heart failure (CHF) with a New York Heart Association (NYHA) class of
5 ≥ 2 (supplemental Table S1).
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10 Serum levels of VEGF ($p < 0.001$), bFGF ($p < 0.001$) and angiogenin ($p < 0.001$)
11 were significantly higher in AL patients compared to normal controls. Serum levels of
12 Ang-1 ($p < 0.001$) and Ang-2 ($p < 0.001$) were both elevated in AL patients but their
13 respective Ang-1/Ang-2 ratio was lower than in controls ($p = 0.005$). Patients with AL
14 had significantly higher levels of VEGF ($p < 0.001$), angiogenin ($p < 0.001$) and Ang-1
15 ($p = 0.001$), and Ang-1/Ang-2 ratio than in patients with myeloma ($p < 0.001$) (Table 1
16 and figure 1A-D).
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25 There were no significant correlations of the bone marrow plasma cell
26 infiltration with the levels of the angiogenic growth factors. There was a significant
27 correlation among levels of Ang-2 and Ang-1/Ang-2 ratio with cardiac involvement
28 ($p = 0.001$ and 0.022 , respectively), with more severe, symptomatic CHF (NYHA ≥ 2)
29 ($p = 0.011$ and $p = 0.006$, respectively) and NT-proBNP ($p = 0.017$ and $p < 0.001$,
30 respectively) (Supplemental table S2). In multiple linear regression a strong
31 correlation was found for Ln of NT-proBNP and Ln of Ang-1 and of Ang-2 ($p < 0.001$
32 for both, $R^2 = 50\%$); however, Ang-1 was negatively and Ang-2 positively correlated to
33 NT-proBNP (Supplemental Figures S1 & S2).
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44 Angiogenin was strongly correlated with indices of renal function: Glomerular
45 filtration rate, as assessed by the MDRD equation (eGFR), was inversely correlated
46 ($p = 0.006$) and amount of proteinuria was positively correlated to angiogenin
47 ($p = 0.009$).
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53 Patients were not treated uniformly, while 31% received upfront novel agents
54 such as bortezomib or lenalidomide. According to consensus criteria, 64% achieved
55 a hematologic response, including 20% who achieved a hematologic CR and 30%
56 achieved an organ response (17 renal, 10 cardiac, 1 liver response). None of the
57 angiogenic molecules correlated with hematologic or organ response. The median
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3 survival of the cohort is 58 months. **None of the angiogenesis related factors was**
4 **associated with survival in univariate or multivariate analysis. NT-proBNP**
5 **($p < 0.001$) was the most significant factor associated with survival in this cohort**
6 **addition of any of the angiogenic cytokines did not improve the prognostic**
7 **model.**
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12 This is the first study of the serum levels of several angiogenesis-related
13 cytokines in patients with AL amyloidosis. We found that all studied cytokines were
14 significantly elevated (at least 2-fold or higher) than in controls but also than in
15 myeloma patients (with the exception of Ang-2). Furthermore, patterns of
16 antagonistic ligands (Ang-1 and Ang-2) are different between patients with AL and
17 tsymptomatic myeloma. Although some of these cytokines correlated with disease
18 features, they were not associated with the outcome of our AL patients.
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30 The increased levels of these cytokines, especially of VEGF, could be the
31 result of a “stress” response to the global deposition of amyloid fibrils and to the
32 direct or indirect “toxic” effects of the amyloid fibrils or of the “amyloidogenic” light
33 chain. Amyloidogenic light chains may have a direct toxic effect in cells and may
34 initiate a stress response *in vitro* (Brenner, *et al* 2004). Abnormal vascular
35 morphology and global endothelial dysfunction have also been described in
36 amyloidosis (Garcia-Garcia, *et al* 2002, Modesto, *et al* 2007). The lack of correlation
37 of BM plasma cell infiltration with VEGF serum levels in our study and the results of a
38 previous study indicating that BM angiogenesis in patients with AL is similar to that of
39 healthy controls (Rajkumar, *et al* 2002) indicates that excess VEGF is probably not
40 produced by plasma cells, and may not reflect increased BM angiogenesis.
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54 Nevertheless, angiogenic cytokines have pleotropic effects, beyond the
55 induction and regulation of angiogenesis. Abnormal levels of VEGF have been found
56 in macular degeneration, diabetic retinopathy, acute and chronic lung injury,
57 neurodegenerative diseases (Carmeliet 2005). Serum levels of VEGF are
58 significantly elevated in plasma cell dyscrasias associated with systemic symptoms,
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3 such as POEMS (Kastritis, *et al* 2006) and Schnitzler's syndrome (Kastritis, *et al*
4 2008), and decline significantly after successful treatment. VEGF induces vascular
5 leak and promotes peripheral edema and effusions. Anti-VEGF therapy was of some
6 benefit in some AL patients with refractory pleural effusions, perhaps by blocking the
7 effects of increased VEGF on vascular permeability (Hoyer, *et al* 2007).

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14 **The correlation of angiogenin levels with proteinuria and eGFR,**
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16 **indicates that it may play a role in the pathophysiology of glomerular leak in AL**
17 **amyloidosis-related nephrotic syndrome. Angiogenin is a potent angiogenesis**
18 **factor and an acute phase protein that activates endothelial cells, while it is**
19 **(along with VEGF) secreted by proximal tubular epithelial cells under hypoxic**
20 **conditions and may modulate angiogenesis and vascular remodeling in the**
21 **renal interstitium (Nakamura, *et al* 2006).**
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30 Ang-1 and Ang-2 are antagonistic ligands for the receptor Tie-2 and are
31 crucial for maturation and stabilization of the vascular wall: Ang-1 stabilizes the
32 vascular wall, while Ang2- induces vessel destabilization, which leads to the
33 angiogenic sprouting. The balance of the serum levels of these two cytokines was
34 significantly different for AL and myeloma patients, being towards Ang-1 for AL and
35 towards Ang-2 (and thus, towards vascular destabilization) for myeloma patients.
36 Ang-1 and Ang-2 correlated with cardiac involvement and indices of the severity of
37 cardiac dysfunction. Both angiopoietins also correlated with NT-proBNP, but in
38 opposite directions, indicating a compensatory response to cardiac dysfunction. Ang-
39 2 levels have been correlated with acute onset and more severe ischemic CHF, but
40 with normal levels of Ang-1 (Chong, *et al* 2004). However, in our study, NT-proBNP
41 was the most important prognostic factor. This indicates that NT-proBNP, as a
42 biomarker, captures more of the diversity of factors that influence outcome in AL. **The**
43 **fact that our patients were not treated homogeneously, and that several of**
44 **them were treated upfront or at a later stage with novel agents may mitigate**
45 **possible correlations of angiogenesis-related molecules with disease outcome.**
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3 Serial assessments could help identify if any of these factors may have a value as a
4 prognostic biomarker. **The use of these markers as a diagnostic tool was not**
5 **assessed. A study designed to address this issue could validate whether some**
6 **of these factors could be used to identify myeloma patients with evidence of**
7 **amyloidosis.**

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14 In conclusion, **the serum levels of angiogenic cytokines are significantly**
15 **elevated in patients with AL amyloidosis compared to both normal individuals**
16 **and myeloma patients and this is probably the result of direct or indirect**
17 **activity of amyloid fibrils or light chains, or a compensatory response to organ**
18 **dysfunction. The major differences from myeloma patients are indicative of the**
19 **distinct pathophysiology of the diseases.**
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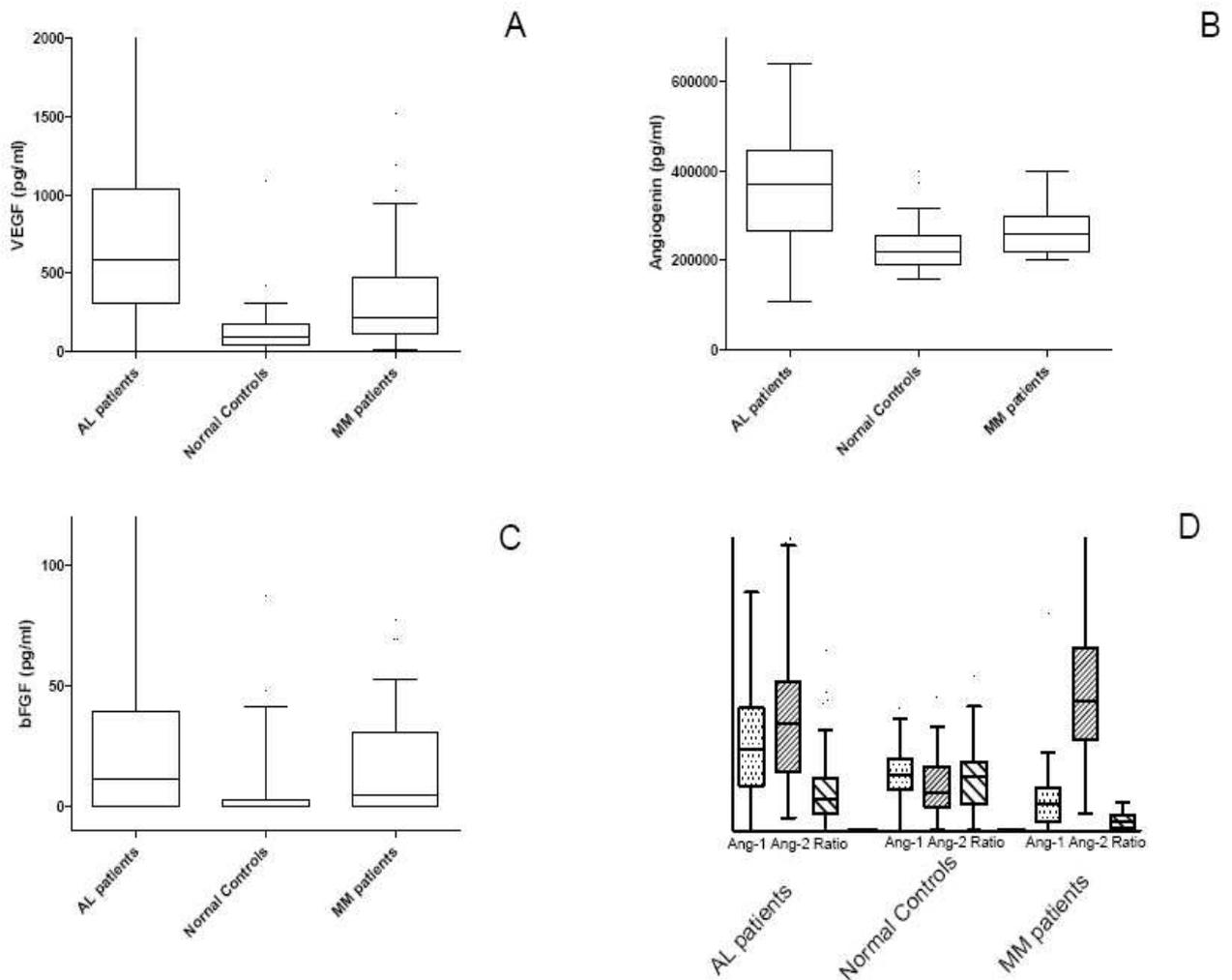
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Table 1. Mean values (\pm SD) of angiogenesis related factors (values have been rounded)

	VEGF (pg/mL)	Angiogenin (pg/mL)	Angiopoietin-1 (pg/mL)	Angiopoietin-2 (pg/mL)	ratio Angiop1/2	bFGF (pg/mL)
AL patients	719 (\pm 555)	400590 (\pm 207726)	48068 (\pm 26623)	5998 (\pm 3602)	11,67 (\pm 11)	31.15 (\pm 45)
Controls	143 (\pm 187)	232005 (\pm 56021)	28805 (\pm 15659)	2452 (\pm 1534)	26,15 (\pm 74)	10.45 (\pm 29)
Myeloma patients	355 (\pm 372)	268631 (\pm 53670)	17443 (\pm 19700)	7140 (\pm 3529)	5.94 (\pm 20)	16.37 (\pm 22)
p-value AL vs Controls	<0.001	<0.001	<0.001	<0.001	0.005	<0.001
p-value AL vs Myeloma	<0.001	<0.001	<0.001	0.117	<0.001	0.021

Figures

Figure 1 : serum levels of VEGF (1A), Angiogenin (1B), bFGF(1C) in patients with AL amyloidosis, normal controls and myeloma patients .Figure 1D show Ang-1 (:::), Ang-2 (///) and Ang-1 to Ang-2 ratio (\\\) in patients with AL amyloidosis, normal controls and myeloma patients. The equilibrium of Ang-1/Ang-2 in AL patients is not significantly different than in controls, due to increase of both Ang1 and Ang2, whereas in myeloma patients a significant shift of the pattern compared to controls is evident, due to low Ang-1 and high Ang-2 levels.



Supplemental tables and figures

Table S1: Characteristics of patients with AL amyloidosis

Male /.Female	39 (48%) / 43 (52%)
Age (median /range)	63 (39-86)
Number of involved organs (median / range)	
Organ involvement	
Heart	45 (56%)
Renal	61 (76%)
Liver	11 (14%)
Nerve	22 (29%)
NYHA ≥ 2	20 (25%)
Creatinine mg/dl (median / range)	1.1 (0.4-10.6)
Bone Marrow PCs (median / range)	12 % (0-90)
Serum Albumin (gr/dl)	3.3 (1.7-5.0)
IVS (mm)	12 (7-23)
NT-pro-BNP (ng/L)	1782 (33-74981)
Troponin-T ($\mu\text{g/L}$)	0.01 (0-4.19)
Urine Albumin (mg/24h)	3300 (0-20000)
Involved FLC (mg/L)	152 (11-15100)
B2-microglobulin (mg/L)	2.16 (0.03-28.30)
Alkaline Phosphatase (IU/L)	88 (33-4701)

Table S2: p-values for involvement of specific organs and levels of angiogenic growth factors

	Heart	Renal	Liver	Peripheral nerve
VEGF	0.564	0.655	0.033	0.146
Angiogenin	0.195	0.049	0.977	0.325
Angiopoietin-1	0.595	0.515	0.471	0.955
Angiopoietin-2	0.008	0.968	0.472	0.282
ratio Angiopoietins 1/2	0.022	0.295	0.170	0.078
bFGF	0.976	0.485	0.983	0.558

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Figure S1 : Correlation of Ang2 with NTproBNP (natural logarithm of NTproBNP)

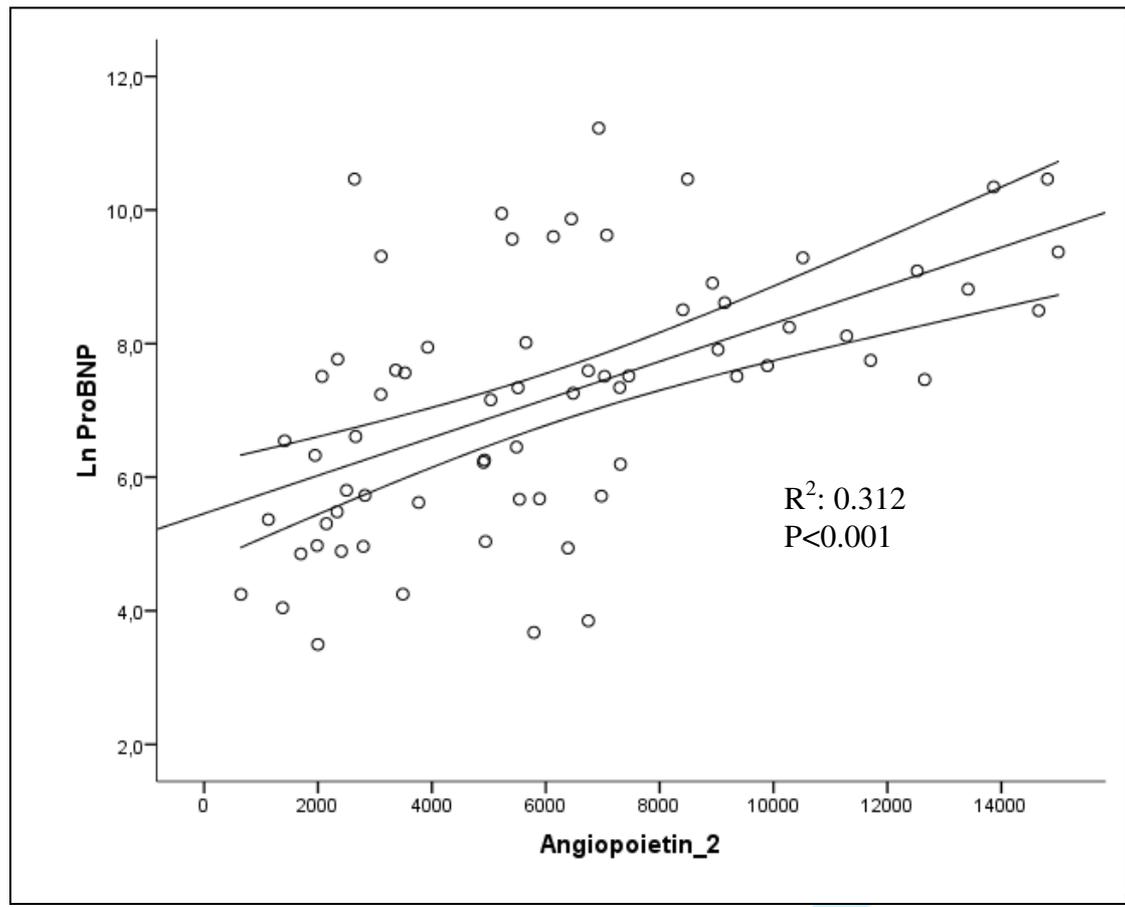


Figure S2: 3-dimensional display of the correlation of Ang-1 and Ang-2 to NTproBNP (natural logarithms)

