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ACE I/D but not *AGT* (-6)A/G polymorphism is a risk factor for mortality in ARDS

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Abstract

The intrapulmonary Renin-Angiotensin-System via tissue concentration of angiotensin II or bradykinin may have multiple effects on pulmonary pathophysiology. Therefore, we investigated, whether the presence of the D allele of the *ACE* I/D polymorphism or the A allele of angiotensinogen (AGT) promoter polymorphism (-6)A/G are independent risk factors for 30 day survival in ARDS patients. In a prospective study adults (white Germans of Caucasian ethnicity) with ARDS (n = 84) were recruited from our intensive care unit, and genotyped for the *ACE* I/D and the *AGT* (-6)A/G polymorphism as were 200 healthy white Caucasian controls. Mortality was increased (p = 0.015) in the *ACE* DD genotype compared to the I allele, and the *ACE* I/D polymorphism was an independent prognostic factor for 30 day survival. Patients with a homozygous DD genotype were at highest risk for death (hazard ratio, HR, 5.7; 95% CI 1.7-19.2; p = 0.005) compared to the II genotype. In contrast, the *AGT* (-6)A/G polymorphism was neither associated with an increased risk for development of ARDS nor with outcome. In patients with ARDS, the *ACE* I/D polymorphism but not the *AGT* (-6)A/G promoter polymorphism is an independent risk factor with a pronounced effect on 30 day survival.

Keywords: Intrapulmonary renin-angiotensin-system, angiotensin converting enzyme insertion/deletion polymorphism, ARDS

Introduction

The acute respiratory distress syndrome (ARDS) is characterized by increased capillary-alveolar permeability, hypoxemia, reduced lung compliance, diffuse bilateral pulmonary infiltrates, and the need for mechanical ventilation. ARDS remains an important cause of death in the intensive care unit (ICU), and few specific therapies are available. Factors predicting the onset or severity of this syndrome are hardly known, but the low incidence of ARDS within the relatively large group of patients at risk suggests the interaction of genetic and environmental factors [1, 2]. The early stages of ARDS are characterized by a high permeability pulmonary edema, alveolar epithelial cell loss, and neutrophil infiltration, which may progress to significant alveolar and interstitial remodelling, and fibrosis. Experimental evidence suggests that activation of the pulmonary renin–angiotensin system (RAS) may influence the pathogenesis of ARDS *via* mechanisms affecting vascular permeability [3], vascular tone [4], fibroblast proliferation [5], and by decreasing alveolar epithelial cell survival [6].

Hence angiotensin II (AT-II) may play a major role in the pathogenesis of ARDS. AT-II is generated through the proteolytic cleavage of angiotensinogen (AGT), which is primarily synthesized in the liver and, to a lesser extent, in the kidney, brain, heart, adrenal, fat, and vascular walls [7, 8]. AGT is first converted by renin to the decapeptide Angiotensin I (AT-I). Thereafter, angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II. However, ACE also degrades bradykinin, which also impacts on vascular tone, vascular permeability, and cardiac function [9]. Accordingly, bradykinin could also contribute to the pathogenesis of ARDS.

Approximately forty-seven percent of the variance in plasma ACE activity is explained by a common insertion/deletion (I/D) polymorphism located in intron 16 of the *ACE* gene, the D allele being associated with increased ACE activity [10]. Circulating ACE is often decreased in patients with ARDS [11]. However, this may reflect attenuated enzyme release from

damaged pulmonary vascular endothelium and may not represent ACE activity in other lung compartments. In fact, an increased ACE activity has been reported in the bronchoalveolar lavage (BAL) despite a decrease in circulating concentrations [11]. In line with this latter observation, the transpulmonary gradient and circulating concentrations of AT-II are increased in patients with ARDS [12], and activation of the circulating RAS, with a preservation of the transpulmonary gradient, was also found in critically ill patients [13]. While ACE is mainly derived from endothelial cells [14] and the lung represents the body's largest endothelial surface, interindividual differences in ACE activity may affect the susceptibility for and survival during ARDS [15]. Since the plasma concentration of AGT is close to the Michaelis constant of the enzymatic reaction between renin and AGT, a rise in plasma AGT levels can lead to a parallel increase in AT-II formation [16].

The human *AGT* gene harbours a (-6)A/G promoter polymorphism [17]. The (-6)A allele displays increased promoter activity compared with (–6)G with concomitantly increased plasma AGT levels [18]. Thus, if AT-II is crucial for the pathogenesis of ARDS, genotypes of the AGT (-6)A/G polymorphism could also affect the susceptibility for and survival during ARDS.

Therefore, we investigated whether genotypes of the *ACE* I/D polymorphism or genotypes of the *AGT* (-6)A/G promoter polymorphism are independent risk factors for 30 day survival in patients with ARDS.

Methods

Study populations

This study was reviewed and approved by the Ethics Committee of the University Hospital Essen. Over a period of two years patients admitted to a specialized ICU of the University Hospital Essen were considered eligible for the study, if they fulfilled the joint American/European Consensus Committee criteria for ARDS [19], had no previous history of ARDS, and written informed consent were obtained. All patients (43 males, 41 females, mean age 43 ± 16 years) were white Germans of Caucasian ethnicity. The specific clinical disorder which evoked acute respiratory distress syndrome (ARDS) was determined over the first 24 to 48 h after the patient met ARDS criteria. As in other studies, some patients had more than one [20] potential predisposing clinical condition associated with the development of ARDS. However, the primary condition responsible was determined by investigators after careful review of the clinical and laboratory data. The primary clinical disorders included pneumonia, sepsis, and other disorders (like trauma, aspiration). Pneumonia was defined as evidence of primary lung infection from bacterial, viral, fugal, or parasitic infection as diagnosed by medical history, Gram's stain, and cultures of tracheal aspirate or bronchoalveolar lavage specimen. The term *sepsis syndrome was used*, as defined by Bone et al.[20]. Patients who met criteria for both sepsis syndrome and pneumonia were classified as pneumonia, assuming that pneumonia evoked the sepsis syndrome. Clinical and demographic data at baseline, including lung injury score, Simplified Acute Physiology Score (SAPS) [21], and the Sequential Organ Failure Assessment (SOFA) score [22] were calculated over the first 24 to 48 h after the patient met ARDS criteria. Patients were categorized as survivors if they were alive thirty days after the diagnosis of ARDS or discharged from the hospital without requirement of mechanical ventilation.

The control sample consisted of 200 healthy white Caucasians individuals of either gender who were recruited at the local Department for Transfusion Medicine, University Hospital Essen. All samples were collected at random from subjects donating blood and the details of this sample have been published previously [23]

Determination of genotypes

DNA was extracted from whole blood samples by modified phenol–chloroform extraction. ACE genotype was determined by three-primer polymerase chain reaction amplification as described previously [24], performed by staff blinded to all subject data. This method yields amplification products of 84 bp for the D allele and 65 bp for the I allele, and products were visualized on polyacrylamide gels. The homozygous insertion (II) genotype was confirmed by repeat polymerase chain reaction in the absence of a primer for the I allele. All samples were randomized, genotyped, and results were replicated by two independent researchers. For genotyping the AGT (-6) A/G polymorphism, PCR was done using primers AGT-Pro-SE 5' CTTCTGGCATCTGTCCTTCTGG 3' biotinylated and primer AGT-Prom-AS 5' CCTAGCCCACAGCTCAGTTACATC 3' resulting in a 200 bp fragment. Genotypes were determined using Pyrosequencing [25] with sequencing primer AGT-Pro-Seq 5'-GGCAGCTTCTTCCCC -3'.

Statistical analysis

The clinical outcome analyzed in this study was survival of the first 30 days dependent on *ACE* and *AGT* genotypes. Kaplan-Meier plots and the log-rank test for trend were used to evaluate the relationship between genotypes and clinical outcome from the date of the primary diagnosis to the end of follow-up. Log-rank tests for *ACE* genotypes were performed using all three genotypes unless stated otherwise. The power (1-ß) of the clinical outcome analysis was 0.87. The impact of gender, SAPS, SOFA, lung injury score (LIS), age, body mass index

(BMI), positive endexpiratory pressure (PEEP), ratio of arterial partial pressure of oxygen and fraction of inspired oxygen (PaO_2/FiO_2), and *ACE* genotypes as prognostic factors for the clinical outcome were analyzed by stepwise multivariate Cox-regression analysis. Non-significant variables ($p>0.05$) were removed stepwise from the model. Hazard ratios and 95% confidence interval (CI) were calculated from the Cox-regression model including all remaining factors for multivariate analysis and for the indicated factor for univariate analysis. Contingency tables and the Pearson´s χ^2-test were used for categorical variables using *ACE* and *AGT* genotypes. Since the *ACE* I/D polymorphism appeared to show a gene-dose effect, linear analysis of variance (ANOVA) was used for comparison of continuous parametric variables, where appropriate. The Kruskal-Wallis-test was used for continuous nonparametric variables. The Hardy-Weinberg equilibrium was tested by a goodness-of-fit χ^2 test. Differences were regarded significant at a p-value of less than 0.05. All statistical analysis was done using SPSS 11.0 (SPSS, Chicago, USA). Continuous variables are given as means ± SD , as indicated.

Results

Using Kaplan-Meier estimates to compare outcome, 30 day survival was significantly associated with *ACE* genotypes (p=0.011, fig. 1) with an apparent gene-dose effect. Patients homozygous for the *ACE* DD genotype displayed a significantly higher risk for death than patients with II genotypes, with heterozygous patients being at intermediate risk (hazard ratios: DD versus II: 3.6, 95% CI 1.3-8.7, p = 0.011; ID versus II: 2.2, 95% CI 0.7-6.1, p = 0.162). Thirty day survival rates were 73.2 % for II, 64.0 % for ID, and 50.0 % for DD genotypes, respectively (fig. 1). In contrast, no such association was found for AGT genotypes (fig. 2).

Multivariate proportional hazard analysis including age, gender, LIS, BMI, PEEP, SAPS, and PaO_2/FiO_2 as covariates revealed the *ACE* I/D polymorphism to be the most important and independent prognostic factor for 30 day survival. Homozygous DD subjects were at highest risk for death (hazard ratio, HR, 5.7, 95% CI, 1.7-19.2; p = 0.005) compared to II genotypes (table 1).

Clinical characterization of the ARDS patients by *ACE* I/D genotypes is displayed in table 2. We found no significant associations of *ACE* genotypes with gender, age, LIS, BMI, PaO_2/FiO_2, PEEP, SAPS, SOFA, or duration of ICU stay. Likewise, none of these variables was associated with *AGT* genotypes (table 3).

The most common cause evoking ARDS were pneumonia (67%), sepsis (24%), trauma (4%), and aspiration (5%), and overall survival was 68%. Demographic characteristics of the ARDS patients and the sample of healthy individuals are displayed in table 4. We found no statistically significant differences regarding demographic characteristics between ARDS and the control group except a slight difference for age. Mean age in the ARDS group was 43 ± 16 years and 37 ± 2 in the control group.

For both samples, genotype distributions were compatible with the Hardy-Weinberg equilibrium. *ACE* and *AGT* genotypes, and allele frequencies were similar between ARDS patients and the blood donor sample (table 4). ACE and AGT genotypes were not in linkage disequilibrium and thus, no interaction between ACE and AGT genotypes and an association with ARDS was observed.

Discussion

This study shows for the first time in a multivariate analysis that the D allele of the *ACE* I/D polymorphism is significantly associated with an increased thirty day mortality in patients with ARDS. The hazard ratios of nearly 6 for the homozygous DD genotype and approximately 5 for the heterozygous ID genotypes compared with the II genotype not only indicate that the D allele may have an important effect on the activity of the intrapulmonary ACE system, but also underscore the potential relevance of the ACE system for pulmonary pathology. Associations between the D allele and the development or progression of sarcoidosis [26], asthma [27], and berylliosis [28] have been described. Due to the influence of the pulmonary RAS on vascular permeability [3], vascular tone [4], fibroblast activity [5], and alveolar epithelial cell survival [6], its strong effect upon the natural course of ARDS is comprehensible. Within the lung, the pulmonary circulation is a potentially important site of RAS activation. ACE inhibitors attenuate pulmonary vasoconstriction in healthy humans and in patients with cor pulmonale [4], as do type-1 angiotensin receptor antagonists [29]. Infusion of angiotensin-I [30] or angiotensin-II [3] can evoke pulmonary edema independently of catecholamine release. Angiotensin-II may, therefore, also affect microvascular permeability. Finally, angiotensin-II is a pro-apoptotic factor for alveolar epithelial cells *in vitro* [31]. The loss of an intact epithelial barrier - with implications for the movement of fluid and cells between the vascular, interstitial, and alveolar spaces - is another early event in ARDS that might be influenced by the RAS. Any single mechanism or all mechanisms combined could thus impact on ARDS and its outcome.

A significant association was observed between the D allele and mortality in our patients. Several pathways influencing the course of ARDS and/or additional mechanisms may be involved. For example, fibroproliferation could have a significant effect on outcome in ARDS

[5], [32] and it was previously shown that Angiotensin -II is a mitogen for lung fibroblasts [5]. Furthermore, both ACE inhibitors and type-1 angiotensin receptor antagonists attenuate collagen deposition and interstitial fibrosis in experimental models of lung injury [33]. In patients with left ventricular dysfunction, ACE inhibition improved gas transfer and ventilation-perfusion-coupling by a yet unknown mechanism [34]. It would, therefore, not be unexpected if increased ACE activity, associated with the *ACE* D allele, evoked opposite effects in patients with ARDS. Finally, ACE is expressed in activated alveolar macrophages [35] and lymphocytes [10]. In activated pulmonary macrophages, ACE inhibition decreased free radical expression; however, its role in modulating the inflammatory response has not been clearly defined [35].

It is well known that the I/D polymorphism accounts for 47% of the variance in plasma ACE activity in healthy white individuals, ACE activity being highest in those with the DD genotype [10]. However, ACE concentration and activity is of course not only influenced by genotype, but also by a myriad of mechanisms including pharmacological therapy [36] and fluid management [37]. We, therefore, made no attempt to measure concentration or activity of ACE in bronchoalveolar lavage or serum. Linkage and segregation analysis has shown that circulating angiotensin-I converting enzyme (ACE) levels are influenced by a major quantitative trait locus that maps within or close to the ACE gene. Multiple variants which are in linkage disequilibrium with the I/D polymorphism have been described, but none of these variants has been associated with ACE levels. In addition, it cannot be excluded that the ACE I/D polymorphism is in linkage disequilibrium with one or more variants in genes other than ACE and that these yet unidentified SNPs explain the associations described here [38, 39]. Even if many hints point to angiotensin II as a main factor in ARDS, it should be kept in mind that ACE also degrades bradykinin, which also has an impact on vascular tone, vascular permeability, and cardiac function [9].

Unexpectedly, the *AGT* (-6)A/G polymorphism did not affect survival during ARDS, although genotypes of this polymorphism could be associated with an increase in plasma AGT concentrations, that subsequently increased AT-II generation [16, 18]. Therefore, our results could be explained by the hypothesis, that the association seen with the *ACE* I/D polymorphism relates to bradykinin degradation rather than to increased AT-II formation.

We could not confirm in our Caucasian sample the association between genotypes of the *ACE* I/D polymorphism and susceptibility for ARDS, as suggested by Marshall et al. [15], although patient population differ only slightly with regard to clinical characteristics such as SAPS, LIS, PaO_2/FiO_2, duration of ICU stay, and overall mortality. However, one important difference may consist in etiology of respective ARDS patients. In our sample, pneumonia was the underlying cause in 60 % of patients, while pneumonia was diagnosed in only 30 % of cases in the sample of Marshall et al.. Thus, it could be speculated that the association between the D allele and susceptibility for ARDS exists only in secondary (non pneumonia) ARDS. This hypothesis appears to be supported by data generated with regard to the Asian epidemic of Severe Acute Respiratory Syndrom (SARS), as the susceptibility for ARDS was not associated with the D allele of the ACE polymorphism [40].

Despite 30 years of research into the mechanisms and consequences of the acute respiratory distress syndrome, efforts to identify a reliable, pulmonary-specific risk factor for death have been disappointing. Variables that are independently associated with mortality are not specific for abnormalities of pulmonary pathophysiology, such as sepsis, nonpulmonary organ system dysfunction, age, or cirrhosis [41, 42]. Although indexes of hypoxemia, such as the partial pressure of arterial oxygen (PaO_2), the fraction of inspired oxygen (FiO_2), or the ratio of PaO_2 to FiO_2, were initially thought to have prognostic value [42, 43], subsequent studies established that these variables were not independently associated with the risk of death when

they were measured early in the course of the acute respiratory distress syndrome [41, 42]. Furthermore, the majority of ARDS patients eventually die of multiple organ failure, and this study cannot explain why the ACE gene polymorphism affects outcome. Also, we do not know whether another pathway is triggered dependent on genotypes of the ACE polymorphism. For instance, there is laboratory evidence of involvement of angiotensin in the activation of nuclear factor-[kappa]B. This may suggest a proinflammatory effect for the development or aggravation of the systemic inflammatory response syndrome [44]. However, these are only speculations. Furthermore, ARDS by definition, encompasses a relatively small range of the phenotype pulmonary dysfunction with almost all patients showing severe dysfunction, pulmonary hypertension, and high shunt. Thus, we would not expect to uncover a correlation of phenotype (shunt, and the like) and genotype.

Although all ARDS patients were treated with a standardized multimodal concept which included analgosedation, fluid, ventilation, hemodynamic, antibiotic and diagnostic management, we cannot exclude due to the multifactorial nature of the disorder that unknown potentially confounding factors still exist. Thus, the results of this study should not be over-interpreted. However, this investigation shows that the ACE I/D polymorphism affects pulmonary disorders and also underscores the potential relevance of the ACE system for ARDS.

Our findings could have clinical impact in the future. This impact could include identification of novel candidate genes in the bradykinin pathway, tailoring of drug therapy related to the RAS system, and stratification patients for clinical trials.

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Table 1: Multivariate stepwise cox regression analysis

		p-value	HR	95% CI
ACE				
	II		1*	
	ID	0.008	5.2	1.5-17.6
	DD	0.005	5.7	1.7-19.2
SAPS		0.001	1.04	1.02-1.07
PaO_2/FiO_2		0.013	0.99	0.98-0.99
BMI		0.022	0.86	0.75-0.98
PEEP		0.010	0.87	0.78-0.97

HR. hazard ratio; 95 % CI; 95 % confidence interval; *, reference

Table 2: *ACE* genotype distribution and clinical characteristics in patients with ARDS

	All	II	ID	DD	p-value
n (%)	84	23 (27%)	36 (43%)	25 (30%)	
Gender (m/f) [%]	43/41	11/12 (30%/24%)	21/15 (35%/46%)	11/14 (35%/29%)	0.56
Mean age at diagnosis (years ± SD)	43 ±16	45 ± 17	43 ± 15	42 ± 17	0.74
LIS	3.2±0.4	3.2 ± 0.6	3.3 ± 0.6	3.15 ± 0.5	0.75
BMI (kg/m²)	26 ± 6	25 ± 4	27 ± 6	24 ± 4	0.17
PaO_2/FiO_2 (mmHg)	116 ± 65	98 ± 58	126 ± 74	119 ± 56	0.27
PEEP (mbar)	16 ± 4	16 ± 3	17 ± 4	16 ± 5	0.71
SAPS	50 ± 17	49 ± 19	49 ± 16	51 ± 19	0.88
SOFA	12 ± 4	12 ± 3	13 ± 4	12 ± 4	0.30
Median duration of stay (days) [range]	18 [2-174]	22 [6-48]	17 [3-73]	16 [2-174]	0.11
Pneumonia	56	19	22	15	
Sepsis	20	5	10	6	
Trauma or aspiration	8	2	4	2	0.9

P values were calculated using the chi-square test for categorical variables, ANOVA for continuous parametric variables, and the Kruskal-Wallis-test for continuous nonparametric variables.

Table 3: *AGT* genotype distribution and clinical characteristics in patients with ARDS

	All	GG	GA	AA	p-value
n (%)	84	24 (29%)	49 (58%)	11 (13%)	
Gender (m/f) [%]	43/41	18/6 (21%/7%)	21/28 (25%/33%)	4/7 (5%/8%)	0.028
Mean age at diagnosis (years ± SD)	43 ±16	42 ± 15	44 ± 17	44 ± 14	0.77
LIS	3.2±0.4	3.2 ± 0.5	3.3 ± 0.5	3.3 ± 0.5	0.53
BMI (kg/m²)	26 ± 6	25 ± 3	26 ± 5	27 ± 6	0.10
PaO_2/FiO_2 (mmHg)	116 ± 65	119 ± 66	113 ± 65	125 ± 76	0.27
PEEP (mbar)	16 ± 4	15 ± 5	17 ± 4	15 ± 6	0.75
SAPS	50 ± 17	51 ± 19	47 ± 16	51 ± 19	0.55
SOFA	12 ± 4	12 ± 4	12 ± 3	14 ± 3	0.19
Median duration of stay (days) [range]	18 [2-174]	26 [6-48]	23 [3-73]	34 [2-174]	0.11
Pneumonia	56	14	33	9	
Sepsis	20	6	12	2	
Trauma or aspiration	8	4	4	0	0.52

P values were calculated using the chi-square test for categorical variables, ANOVA for continuous parametric variables, and the Kruskal-Wallis-test for continuous nonparametric variables.

Table 4: Genotype frequencies of the *ACE* I/D and the *AGT* (-6)A/G promoter polymorphism and demographic characteristics in ARDS patients and in healthy blood donors

		Patients	Controls	p-value
	∑	84	200	
ACE	II	23 (27%)	39 (20%)	
	ID	36 (43%)	106 (53%)	
	DD	25 (30%)	55 (27%)	0.22
Angiotensinogen	GG	24 (29%)	72 (36%)	
	AG	49 (58%)	92 (46%)	
	AA	11 (13%)	36 (18%)	0.28
Age		43 ± 16	37 ± 2	0.001
BMI (kg/m²)		26 ± 6	25 ± 4	0.578
Gender (m/f)		43/41	123/72	0.06

BMI, body mass index. P-values were calculated using χ^2 test for categorical variables and Student's t-test for continuous parametric variables.

Figure 1

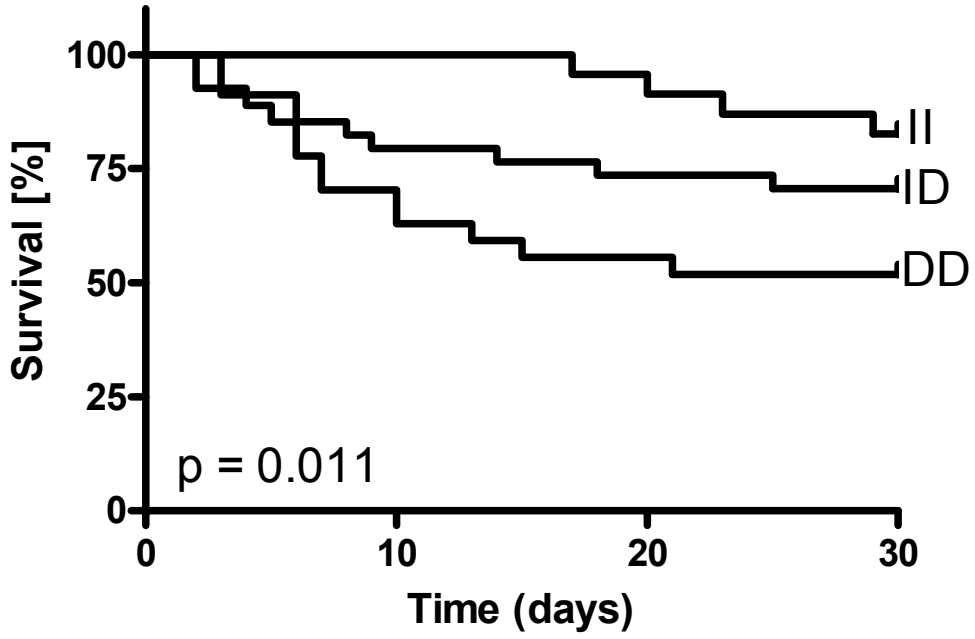
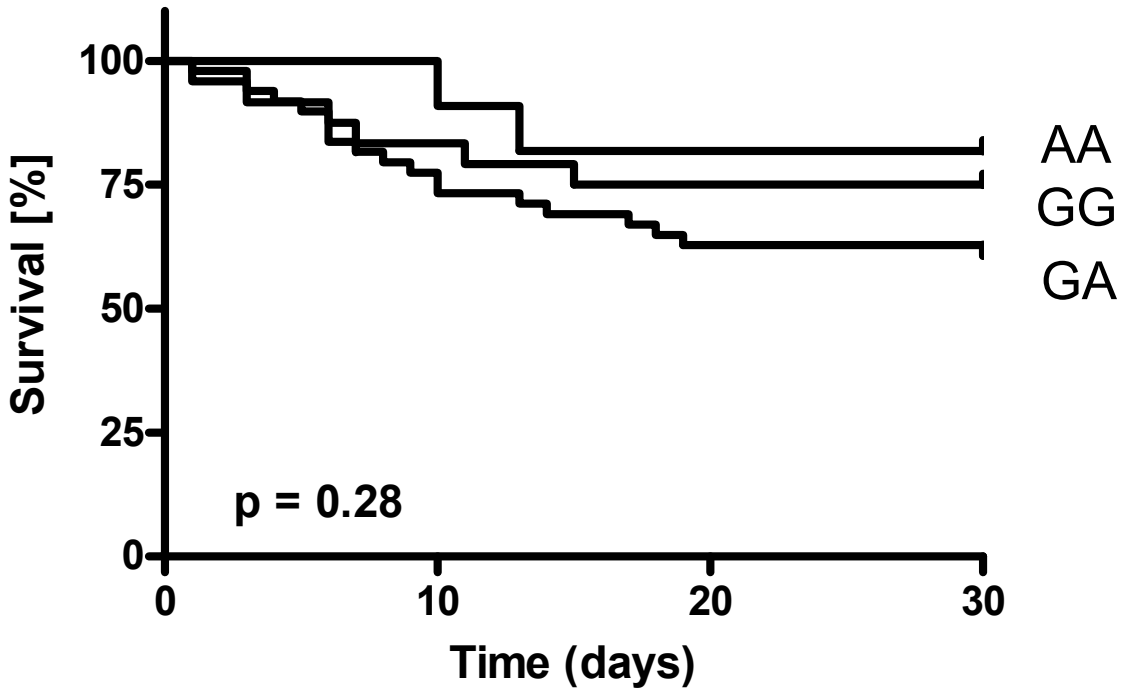


Figure 2



Legends

Figure 1

Thirty day survival in patients with ARDS dependent on genotypes of the *ACE* I/D polymorphism. Kaplan-Meier estimates were used to calculate probabilities of 30 day survival. Thirty day survival was significantly increased in the II genotypes compared to carriers of a D allele.

Figure 2

Thirty day survival in patients with ARDS dependent on genotypes of the Angiotensinogen promoter polymorphism. Kaplan-Meier estimates were used to calculate probabilities of 30 day survival. Thirty day survival was not significantly different between Angiotensin promoter polymorphism genotypes.