AGE (2013) 35:993–1005
DOI 10.1007/s11357-012-9408-8

Variations of the angiotensin II type 1 receptor gene are associated with extreme human longevity

Ariela Benigni · Silvia Orisio · Marina Noris · Paraskevas Iatropoulos · Davide Castaldi · Kei Kamide · Hiromi Rakugi · Yasumichi Arai · Marta Todeschini · Giulia Ogliari · Enyu Imai · Yasuyuki Gondo · Nobuyoshi Hirose · Daniela Mari · Giuseppe Remuzzi

Received: 24 November 2011 / Accepted: 4 April 2012 / Published online: 9 May 2012

Abstract Longevity phenotype in humans results from the influence of environmental and genetic factors. Few gene polymorphisms have been identified so far with a modest effect on lifespan leaving room for the search of other players in the longevity game. It has been recently demonstrated that targeted disruption of the mouse homolog of the human angiotensin II type 1 receptor (AT_1R) gene (*AGTR1*) translates into marked prolongation of animal lifespan (Benigni et al., J Clin Invest 119 (3):524–530, 2009). Based on the above study in mice, here we sought to search for *AGTR1* variations associated to reduced AT_1 receptor protein levels and to prolonged lifespan in humans. *AGTR1* was sequenced in 173 Italian centenarians and 376 younger controls. A novel non-synonymous mutation was detected in a centenarian. Two polymorphisms in *AGTR1* promoter, rs422858 and rs275653, in complete linkage disequilibrium, were significantly associated with the ability to

Ariela Benigni and Silvia Orisio contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11357-012-9408-8) contains supplementary material, which is available to authorized users.

A. Benigni · S. Orisio (✉)
Department of Molecular Medicine,
Mario Negri Institute for Pharmacological Research,
Centro Anna Maria Astori,
Science and Technology Park Kilometro Rosso,
Via Stezzano 87, 24126 Bergamo, Italy
e-mail: silvia.orisio@marionegri.it

M. Noris · M. Todeschini
Transplant Research Center,
"Chiara Cucchi De Alessandri & Gilberto Crespi",
Mario Negri Institute for Pharmacological Research,
24020 Ranica, Italy

P. Iatropoulos
Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Mario Negri Institute for Pharmacological Research,
24020 Ranica, Italy

D. Castaldi
Ph.D. School of Informatics, DISCo,
University of Milan—Bicocca,
20126 Milan, Italy

K. Kamide · H. Rakugi
Department of Geriatric Medicine and Nephrology,
Osaka University Graduate School of Medicine,
565-0871 Osaka, Japan

Y. Arai · N. Hirose
Division of Geriatric Medicine,
Department of Internal Medicine,
Keio University School of Medicine,
160-8582 Tokyo, Japan

attain extreme old age. We then replicated the study of rs275653 in a large independent cohort of Japanese origin (598 centenarians and semi-supercentenarians, 422 younger controls) and indeed confirmed its association with exceptional old age. In combined analyses, rs275653 was associated to extreme longevity either at recessive model (P=0.007, odds ratio (OR) 3.57) or at genotype level (P=0.015). Significance was maintained after correcting for confounding factors. Fluorescence activated cell sorting analysis revealed that subjects homozygous for the minor allele of rs275653 had less AT_1R-positive peripheral blood polymorphonuclear cells. Moreover, rs275653 was associated to lower blood pressure in centenarians. These findings highlight the role of *AGTR1* as a possible candidate among longevity-enabling genes.

Keywords Angiotensin II type I receptor · Genetic polymorphism · Centenarians · Human longevity

Abbreviations

AT_1R	Angiotensin II type 1 receptor
$AT_{1A}R$	Angiotensin II type 1A receptor
$AT_{1B}R$	Angiotensin II type 1B receptor
$AT_{1A}R^{-/-}$	Mouse deficient for angiotensin II type 1A receptor
AGTR1	Human angiotensin II type 1 receptor gene
FOXO3A	Forkhead box O3A
ROS	Reactive oxygen species
AngII	Angiotensin II
NCBI	National Center of Biotechnology Information
PBMC	Peripheral blood mononuclear cells
PMN	Polymorphonuclear cells
OR	Odds ratio
FACS	Fluorescence activated cell sorting
HW	Hardy-Weinberg

G. Ogliari · D. Mari
Department of Medical Sciences,
Geriatric Unit, IRCCS Ca' Granda Foundation Maggiore Policlinico Hospital, University of Milan, 20122 Milan, Italy

E. Imai
Department of Nephrology,
Nagoya University Graduate School of Medicine,
466-8550 Nagoya, Japan

Y. Gondo
Department of Clinical Thanatology and Geriatric Behavioral Science,
Osaka University Graduate School of Medicine,
565-0871 Osaka, Japan

G. Remuzzi
Mario Negri Institute for Pharmacological Research,
Centro Anna Maria Astori,
Science and Technology Park Kilometro Rosso,
Via Stezzano 87, 24126 Bergamo, Italy

G. Remuzzi
Unit of Nephrology and Dialysis,
Azienda Ospedaliera Ospedali Riuniti di Bergamo,
24128 Bergamo, Italy

Introduction

The phenotype of human longevity has many diverse determinants. Non-genetic factors such as health habits, socioeconomic conditions, diet and physical activity account for an important part; however, emerging evidence suggests that genetic factors contribute in a major way to variation in human lifespan particularly in populations known for exceptional survival (Sirtori et al. 2001; Bilal et al. 2008). Genetic determinants of longevity were initially reported in small, short-lived organisms including yeast, worm and flies, but a considerable number of these genes turned out to extend lifespan in mammals as well. Among plausible candidates for human longevity, polymorphisms in apolipoprotein E gene have been found to influence lifespan in multiple populations, probably through their association with lower risk of cardiovascular disease and dementia (Corder et al. 1996). More recently, findings in two independent populations documented that polymorphisms in the human forkhead box O3A (FOXO3A) gene, which encodes a key regulator of the insulin/insulin growth factor 1 signalling pathway, were associated with the ability to attain exceptional old age (Flachsbart et al. 2009; Willcox et al. 2008). However, as can be expected for a polygenic trait like longevity, variations of FOXO3A and of other genes in the insulin/insulin growth factor 1 signalling pathway found no or only modest effect on human lifespan (Flachsbart et al. 2009; Willcox et al. 2008; Pawlikowska et al. 2009). Thus, investigations on further candidates with substantial beneficial effects on human lifespan are needed to fully disclose the determinants of human ageing.

A recent study demonstrated that mice deficient for angiotensin II type 1A receptor ($AT_{1A}R$) ($AT_{1A}R^{-/-}$)

developed less atherosclerosis and cardiac injury while ageing and had a significant prolongation of lifespan than wild-type littermates (Benigni et al. 2009). In rodents, two isoforms of AT_1R have been identified, $AT_{1A}R$ and $AT_{1B}R$, while human cells express a single AT_1 receptor (AT_1R). Angiotensin II (AngII) has been involved in organ senescence, given its capacity to robustly stimulate mitochondrial production of reactive oxygen species (ROS) that are crucially implicated in age-related organ damage including heart, kidney and brain (Wilson 1990; de Cavanagh et al. 2007) and contribute to the plethora of clinical phenotypes associating with ageing (Min et al. 2009). Over time, exuberant ROS production exceeds the antioxidant defence of the mitochondrial and the nuclear enzymes, so that accumulating damage untimely destroys cells by apoptosis or necrosis (Benigni et al. 2010). The protection observed in $AT_{1A}R^{-/-}$ mice from vascular damage and the associated extension of lifespan were interpreted as a consequence of mitochondrial integrity provided by the expression of survival genes that limited oxidative stress (Benigni et al. 2009). These findings prompted us to investigate the possible role of variations of *AGTR1* in influencing human longevity, with the hypothesis that mutations causing reduced AT_1R expression levels might associate to prolonged lifespan in humans as observed in $AT_{1A}R$-deficient mice. Our studies identified two allelic variants in *AGTR1* promoter which are significantly overrepresented in Italian centenarians. This association was subsequently replicated in an independent population of Japanese centenarians and controls.

Also, we demonstrate that the allelic variants in the promoter of *AGTR1* are associated with reduced AT_1R receptor expression levels in peripheral blood neutrophils. In addition, screening of the whole coding region of *AGTR1* in Italian cohort disclosed in one centenarian a novel mutation leading to amino acid substitution. Finally, we disclose an association between variants in *AGTR1* promoter and diastolic blood pressure in centenarians.

Methods

Study population

A cohort of 173 Italian centenarians (between 99 and 106 years of age, mean 100.9±1.7 years) living in Lombardy region, northern Italy, was recruited through years 2004–2009. The gender ratio was 83 % females to 17 % males. Subjects aged more than 99 years and 6 months were considered centenarians as they were in their 100th year of life. Centenarians were recruited through PRIN 2006 study. The younger control subjects (between 49 and 78 years of age, mean 65.9±9.1 years, 43 % females and 57 % males) were Italians unrelated to centenarians and recruited in the same geographical area through COFIN 2000 study.

The Japanese replication sample consisted of 589 centenarians/semi-supercentenarians ageing 99–115 years (mean 104.5±3 years). The gender ratio was 84 % females to 16 % males. Centenarians and semi-supercentenarians were recruited through year 2000 until now through four series of screening involving the Tokyo metropolitan area; the centenarians address books published by Ministry of Health, Labor and Welfare from 1997 to 2002; all the Japanese nursing homes; the centenarian address lists available at local prefectures; and finally through articles published by local newspapers. Younger controls (between 69 and 72 years of age, mean 70.1±0.9 years, 52 % females and 48 % males) were 422 volunteers from Itami City and Asago City, Hyogo prefecture, Japan, who participated in SONIC Study for elderly subjects to investigate factors related to successful ageing in Japan. Written informed consent was obtained from all study participants.

DNA analysis of *AGTR1*

In the Italian group *AGTR1* exons, their flanking regions, the 5′- and 3′-UTR regions (including 250 bp of the promoter upstream 5′-UTR) were amplified from genomic DNA and sequenced in the two directions on an ABI 3730 DNA analyser (Applied Biosystems). Primers (Online Resource 1) were designed according to *AGTR1* genomic reference NC_000003, built 37.1, at National Center for Biotechnology Information. Variants are referred to transcript variant 4 (NM_031850) RNA reference sequence and numbering begins with the ATG start codon. For quality control purposes, 5 % random sample was sequenced twice. Moreover, subjects carrying very rare genotypes (<5 %) were confirmed by repeating PCR reactions, followed by resequencing of both DNA strands.

For the replication study in the Japanese group, SNP genotyping was performed by TaqMan SNP assay (Applied Biosystems). The genotyping conditions for

TaqMan Assay were established by repeated genotyping of 48 controls.

Analysis of AT_1R presence in peripheral leukocytes

Fresh blood samples were processed for peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMNs) purification as described (Noris et al. 1999; Noris et al. 1996), labelled with an anti-human AT_1R antibody (Anti AT-1, N-10, Santa Cruz) followed by incubation with fluorescein isothiocyanate goat F(ab')2 fragment anti-rabbit IgG (eBioscience). To identify the various leukocyte subsets, cells were stained with fluorochrome-conjugated mAb antibodies against CD45, CD16, CD14, CD3, CD4 (BD Bioscience and Biolegend). The samples were analysed by fluorescence activated cell sorting (FACS) analysis using FACSAria cytometer (BD Bioscience). Results were expressed as percentage of AT_1R positive cells (percent).

Statistical analysis

Quantitative variables were expressed as mean±SEM. ANOVA and *T* test were used to compare continuous variables. Categorical variables were analysed by chi-square test. If $n<5$, exact Fisher test statistics was used. In the presence of a category with "0" frequency, the statistical analyses were performed using Elrm software for R (Zamar et al. 2007). Markers deviating from the Hardy–Weinberg (HW) equilibrium in the control population ($P<0.05$) were excluded from the analysis. Odds ratios (OR) and 95 % confidence intervals were analysed using SISA statistics.

Haploview software v4.1 (Barrett et al. 2005) was used for blocks definition, haplotypes estimation and allelic association tests. Nominal *P* values were reported and were corrected for multiple testing by permutation 100,000 times. The Bonferroni correction was applied to adjust for multiple testing when appropriate. Logistic regression analysis was performed using MedCalc software. Level of significance was set at a *P* value <0.05 assuming a two-tailed model.

Bioinformatic analysis

The sequence variants found in *AGTR1* promoter were analysed for potential transcription factors binding sites using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). The effect of base substitution on protein stability was examined "in silico" using SIFT (Ng and Henikoff 2002; Kumar et al. 2009) and PolyPhen-2 (Sunyaev et al. 2001).

Results

Variations in *AGTR1* and longevity in Italian group

In the Italian cohort, we sequenced exons and putative functional regions of *AGTR1* to determine if any gene variant, which may alter the amino acidic sequence or transcriptional regulation, is differently represented in centenarians vs. younger controls. We identified eight not previously reported variants (Fig. 1 and Online Resource 2), including a non-synonymous variant (c.893A>G) leading to amino acid substitution at position 298 (p.Asn298Ser), which was found in a centenarian and not in controls. This change occurs in the seventh transmembrane domain of AT_1R and is predicted to be damaging in silico. In addition to the rare variants, we have also observed ten known SNPs during the sequencing of *AGTR1*, and therefore, we have evaluated their distribution in centenarians and in controls (Fig. 1; Table 1). Of the ten SNPs observed, two were not in HW equilibrium and were removed from further analyses. The rs422858 is a dinucleotide change (AG/CC), but for practical reasons here, the major allele AG was defined as "A" and the CC allele as "C" (Erdmann et al. 1999). Single marker analysis in the eight SNPs which passed the HW equilibrium test revealed significantly different rs422858 and rs275653 genotype distributions between centenarians and controls ($P=0.004$ for both markers, Table 1). Rs422858 and rs275653, located in the promoter region of *AGTR1* at −174/173 bp and at −113 bp, respectively, are in complete linkage disequilibrium. Under the recessive model, the minor alleles of rs422858 and rs275653 were significantly associated to extreme old age (unadjusted $P=0.003$; Bonferroni-corrected $P=0.021$; OR=4.55) (Table 1). A significant difference was observed for rs422858 and rs275653 also under the dominant and the additive models, but this difference did not pass the Bonferroni correction for the dominant model.

Rs2307085, located on exon 1, was nominally significantly associated to longevity at both genotypic ($P=0.022$) and allele levels ($P=0.023$) (Table 1). Rs422858 and rs275653 together with rs2307085 were found to be in a single Haplotype block (Fig. 1, Online Resource 3).

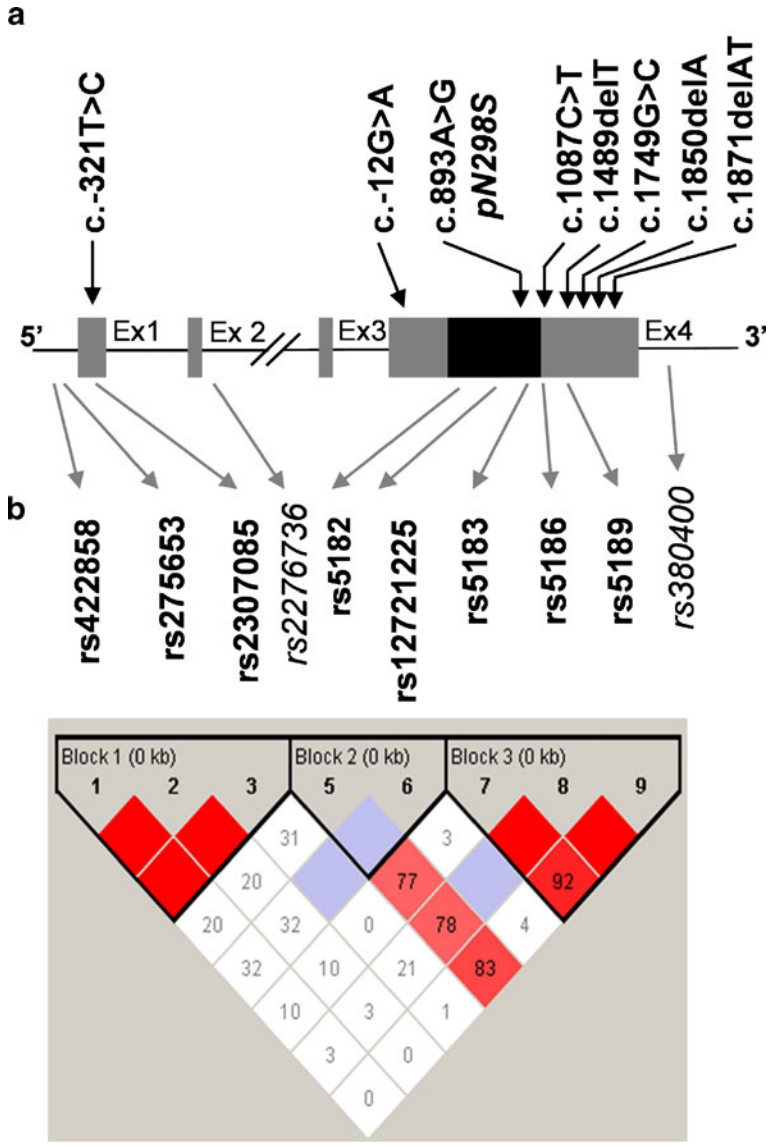

Fig. 1 Genomic map and LD plot of *AGTR1* (not in scale). The *filled boxes* are exons, numbered *1* to *4*; *shaded boxes* non-coding regions, *black box* coding region. **a** Over the genomic *AGTR1* map the position of new rare substitutions found in the Italian population is indicated. Below are the known variations, SNP numbers and their location relative to each other. **b** The LD block structure was calculated using the solid spine method in Haploview. The LD plot on the locus is based on the measure of D'/LOD. SNPs numbered in *italic* were excluded from analyses because they did not respect the HW distribution

In this block, the haplotype A-A-C was associated with a reduced lifespan (nominal $P=0.007$, permuted $P=0.024$); consistently, the minor C-G-C haplotype was nominally associated with longevity ($P=0.028$) (Online Resource 3). Stepwise logistic regression analysis showed a significantly different distribution of the rs275653 (and the linked rs422858) genotypes among centenarians and controls ($P=0.0066$), while no significance was observed for all the other SNPs. After adjusting for gender, rs275653 exhibited a stronger effect than without adjustment ($P=0.00095$).

Logistic regression of the allele distributions of all markers confirmed the previous findings, with only the minor allele of rs275653 being significantly associated to longer lifespan ($P=0.0092$; OR=1.56) even after correction for gender ($P=0.00083$). For each SNP examined, there was no significant difference in the allelic and genotypic distribution between males and females.

AGTR1 association replicated in Japanese centenarians and combined analyses

These results prompted us to validate the association data between variants of rs275653 and longevity in a Japanese population of 589 centenarians/semi-supercentenarians and in younger individuals whose age was comparable to that of Italian controls. We chose to study a population of different ethnic origin because the Japanese population is

Table 1 Association analysis of genotypes and alleles of *AGTR1* SNPs with longevity in Italian cohort

			Genotype frequencies				Recessive model (mm vs. Mm+MM)			Dominant model (mm+Mm vs. MM)			Additive model MM vs. mm			Allele analysis					
SNP	M/m		MM	Mm	mm	HWE *P*	OR	95% CI	P_{nom}	OR	95% CI	P_{nom}	OR	95% CI	P_{geno}[b]	MAF	OR	95% CI	P_{nom}	P_{perm}	MAF
rs422858	A/C	Ce	0.607	0.335	0.058	0.551	4.55	1.5–12.5	**0.003**[a]	1.49	1.0–2.2	**0.038**	4.99	1.7–14.9	**0.004**[a]	0.225	1.55	1.1–2.1	**0.007**	**0.044**	0.225
		Co	0.697	0.290	0.013											0.158					0.158
rs275653	A/G	Ce	0.607	0.335	0.058	0.551	4.55	1.5–12.5	**0.003**[a]	1.49	1.0–2.2	**0.038**	4.99	1.7–14.9	**0.004**[a]	0.225	1.55	1.1 2.1	**0.007**	**0.044**	0.225
		Co	0.697	0.290	0.013											0.158					0.158
rs2307085	C/A	Ce	0.965	0.035	0.000	1.000	nc			4.45	1.1–18	**0.031**	nc		**0.022**	0.017	4.39	1.1–17.7	**0.023**	0.149	0.017
		Co	0.992	0.008	0.000											0.004					0.004
rs2272656	A/G	Ce	0.434	0.497	0.069	0.010															
		Co	0.389	0.507	0.105																
rs5182	C/T	Ce	0.365	0.462	0.173	0.156	0.89	0.5–1.5	0.669	1.00	0.7–1.5	0.991	0.94	0.57–1.6	0.798	0.404	0.97	0.7–1.3	0.818	1.000	0.404
		Co	0.366	0.445	0.189											0.412					0.412
rs12721225	G/T	Ce	0.994	0.006	0.000	1.000	nc			2.75	0.3–23	0.331	nc		0.331	0.003	0.37	0.0–3.0	0.332	0.929	0.003
		Co	0.984	0.016	0.000											0.008					0.008
rs5183	A/G	Ce	0.974	0.026	0.000	0.930	nc		0.340	0.99	0.6–1.8	0.984	nc		0.873	0.050	0.27	0.5–1.7	0.716	1.000	0.050
		Co	0.974	0.025	0.001											0.056					0.056
rs5186	A/C	Ce	0.491	0.396	0.112	0.934	1.72	0.9–3.2	0.081	1.07	0.7–1.5	0.714	1.7	0.9–3.3	0.599	0.311	1.15	0.9–1.5	0.302	0.875	0.311
		Co	0.508	0.424	0.068											0.280					0.280
rs5189	G/T	Ce	0.918	0.082	0.000	0.761	nc		0.490	0.78	0.7–2.5	0.467	nc		0.571	0.041	0.75	0.4–1.4	0.379	0.945	0.041
		Co	0.897	0.097	0.005											0.054					0.054
rs380400	G/A	Ce	0.717	0.252	0.031	0.001															
		Co	0.705	0.235	0.059																

M major allele, *m* minor allele, *Ce* centenarians, *Co* controls, *HWE* Hardy–Weinberg equilibrium, P_{nom} nominal *P*, P_{geno} genotype *P*, P_{perm} *P* values after 100,000 permutation tests. Statistically significant *P* values are shown in bold

OR odds ratio, *CI* confidence interval

[a] Statistically significant after Bonferroni correction (seven tests)

[b] *P* value of chi-square test for genotype frequencies (2DF)

Table 2 Association of rs275653 with longevity in single populations and in the combined group

		Genotype frequency				Recessive model (GG vs. AA+AG)			Dominant model (GG+AG vs. AA)			Additive model (AA vs. GG)			Allele			
Population		AA	AG	GG	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	MAF	OR	95% CI	*P*
Italians	Ce	0.607	0.335	0.058	**0.004**	4.55	1.5–15.5	**0.003**	1.49	1.0–2.2	0.038	4.99	1.7–14.9	**0.001**	0.225	1.55	1.1–2.1	**0.007**
	Co	0.697	0.290	0.013											0.158			
Japanese	Ce	0.829	0.160	0.012	0.077	6.82	1.02–∞	**0.046**[a]	1.0	0.7–1.4	1.000	6.63	1.0–∞	**0.047**[a]	0.092	1.01	0.8–1.5	0.555
	Co	0.832	0.168	0.000											0.084			
Combined	Ce	0.778	0.199	0.022	**0.015**	3.57	1.33–10.0	**0.007**	0.94	0.7–1.2	0.639	3.51	1.3–9.6	**0.009**	0.122	1.03	0.8–1.3	0.797
	Co	0.768	0.226	0.006											0.119			

Combined group consisted of 762 centenarians and 798 controls. Statistically significant *P* values are shown in bold

Ce centenarians, *Co* controls, *MAF* minor allele frequency, *OR* odds ratio, *CI* confidence interval

[a] Elrm test

one of the most long-lived and includes a high number of centenarians. Data of 1000 Genomes project in Japanese population indicate that rs275653 and rs422858 are in complete LD as in Italian subjects, so we chose to genotype rs275653 because of the availability of a commercial TaqMan assay. In accordance to 1000 Genomes and HapMap data, in Japanese population the minor allele of rs275653 had a lower frequency (0.084) than in Italian controls (0.158). Case–control analysis of rs275653 in Japanese cohort revealed that the GG genotype was significantly associated to longevity under the recessive and additive models (P=0.046 and P=0.047, respectively, Table 2). Genotype distribution evidenced a trend (P=0.077) while no difference was observed with the dominant model and at allele level (Table 2).

Considering that, despite the different frequencies of the minor rs275653 G allele between Italian and Japanese cohorts, an enrichment of the GG genotype was evident in both groups of centenarians as compared to controls, we performed further analyses merging the two populations. In the combined analysis, association of rs275653 with longevity was observed using the recessive (P=0.007) and the additive models (P=0.009) (Table 2). Also, genotypic frequencies were significantly different between centenarians and controls in the overall population (P=0.015). After adjusting for confounding factors, namely gender, antihypertensive treatment and population of origin, logistic regression gave P=0.00034 and P=0.00151 for the association between longevity and rs275653 considering either genotype distribution or the recessive model, respectively (Table 3).

FACS analysis of AT_1R on leukocyte populations

Given that the rs275653 SNP and the linked rs422858 are localized in the promoter region, we hypothesized that they might affect *AGTR1* expression. We conducted in silico analyses to evaluate whether the allelic variants of rs275653 and rs422858 were associated with the presence/absence of DNA binding factors in *AGTR1* promoter. Using *Tess*, we found that the region including the major allele variant A of rs422858 contains an E-box element (CANNTG), which can be the target of helix-loop-helix transcription factors, including Tal1 and NF-uEA. By contrast, the C allele, which is more frequent in our centenarians, causes the loss of the E-box site. No putative binding sites were predicted for allele variants of SNP rs275653 which, however, is in

Table 3 Logistic regression analysis of rs275653 and longevity in the combined group

Variables included in the analysis	Overall			
	OR	95% CI	*P*	Model fit
rs275653—genotype	2.04	1.38–3.02	0.00034	<0.00001
rs275653—recessive model	1.96	1.29–2.96	0.00151	
rs275653—dominant model	–		nr	
Gender (female vs. male)	2.76	1.94–3.91	<0.00001	
Antihypertensive treatment	3.62	2.59–5.06	<0.00001	
Population	–		nr	

OR odds ratio, *CI* confidence interval, *nr* not retained in the model

complete linkage disequilibrium with rs422858. Thus, we sought to investigate whether genetic variants in *AGTR1* promoter could affect AT_1R receptor presence on peripheral blood leukocytes by FACS. Due to the low frequency of subjects homozygous for the minor alleles of rs422858 and rs275653, we could identify one Italian centenarian with this genotype alive at the time of laboratory determinations, so we decided to extend the observation of AT_1R presence to younger controls. Three groups of subjects, each including one centenarian and four younger controls and differing for their genotype at rs275653 (GG, AG and AA), were evaluated. The three groups of subjects were comparable for total cholesterol (GG 227±48 mg/dL; AG 198±30 mg/dL; AA 185±24 mg/dL) and lipids. By FACS analysis, we found specific expression of AT_1R both on PMNs (57.5±14 %) and on PBMC (17.3±8.9 %), which include monocytes, B and T cells. A representative flow cytometric analysis of AT_1R on PMNs and on their $CD16^+$ subset is shown in Fig. 2a, b. In PMNs of subjects homozygous for the minor rs275653 variant, significantly less cells positive for AT_1R (GG: mean 42.8±8.8 %, median 43.5 %) were found as compared to heterozygous (AG: mean 68.9±10.9 %, median 66.8 %, P=0.0024) or homozygous individuals for the common allele (AA: mean 60.7±10.4 %, median 61.0 %, P=0.0185) (Fig. 2c). Analysis of AT_1R cell surface presence in the PMN $CD16^+$ fraction showed significantly lower percentage of AT_1R-positive cells in subjects homozygous for the minor rs275653 allele (GG: mean 68.0±8.1 %, median 64.4 %) in respect to heterozygous (AG: mean 87.0±6.1 %, median 87.4 %, P=0.0030) or homozygous subjects for the common allele (AA: mean 81.1±4.5 %, median 80.5 %, P=0.0134) (Fig. 2d). AT_1R protein levels in PBMCs showed a similar trend as in PMNs; however, differences among rs275653 genotype subgroups did not reach statistical significance (AT_1R-positive cells in PBMC according to genotype GG: mean 16.9±5.7 %; AG: mean 20.5±7.7 %; AA: mean 19.3±7.1 %). Failure to find a statistical difference in the percentage of AT_1R-positive cells among genotypes is likely attributable to the high individual variability along with the small number of subjects analysed.

Association of rs275653 and blood pressure

High blood pressure is associated with severe complications, including heart, kidney disease and stroke, which reduce life expectancy in humans. The renin–angiotensin system is involved in blood pressure regulation and vasoconstriction to the extent that knocking out the AT_1R gene causes hypotension in mice (Oliverio et al. 1998). Based on this observation, we evaluated whether rs275653 was associated with changes in blood pressure.

Centenarians with GG genotype of rs275653 had lower blood pressure, either systolic (SBP), diastolic (DBP) or mean arterial pressure (MAP) with respect to centenarians with AA genotype. This difference was statistically significant for DBP under recessive (P=0.040) and additive model (P=0.031) as well as at allele analysis (P=0.037) (Fig. 3). A significant difference was observed for SBP and MAP at allele analysis. In control subjects, systolic blood pressure levels were slightly reduced in subjects with rs275653 GG genotype; however, the difference did not reach a statistical significance (not shown).

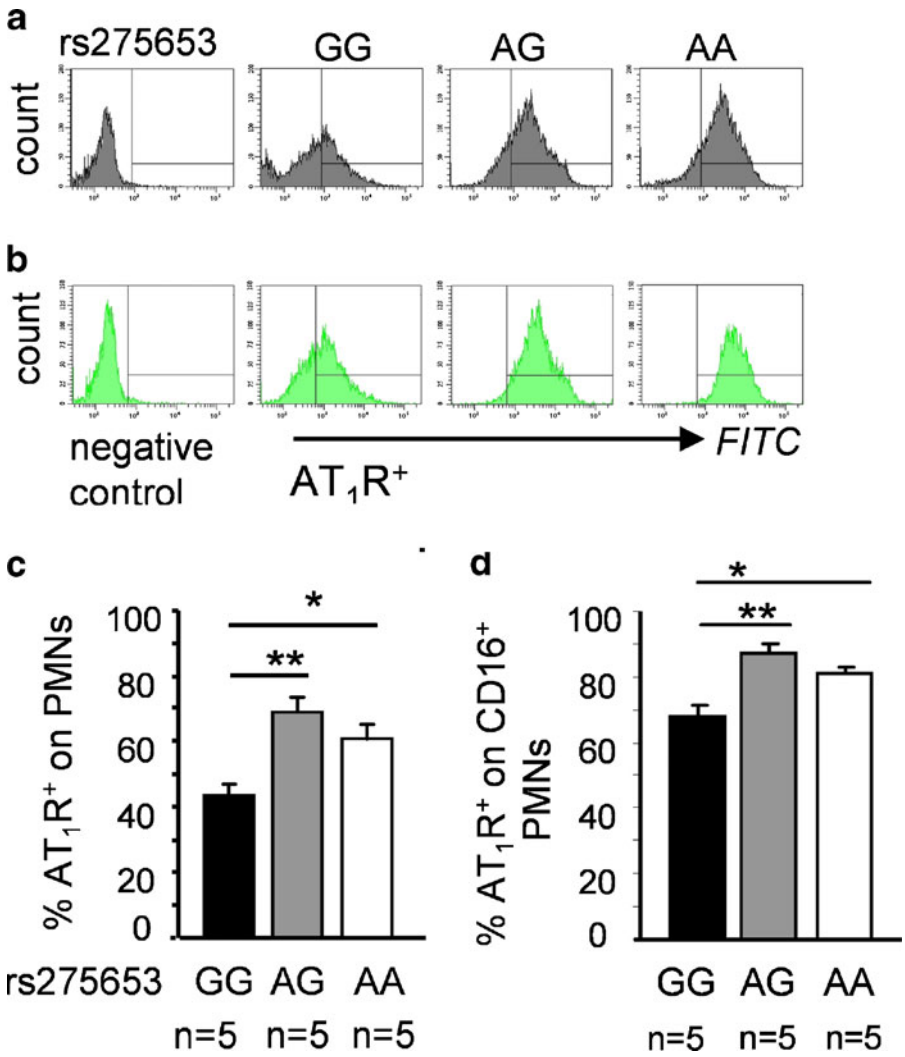

Fig. 2 AT_1R expression in PMNs and in the $CD16^+$ fraction of PMNs of subjects according to rs275653 genotype. Evaluation of AT_1R expression in controls was done twice to correct for inter-assay variability, and the means of the two evaluations were considered in the statistical analysis. Centenarians were analysed once. Representative flow cytometry analysis of AT1R expression on total PMNs (**a**) and $CD16^+$ fraction of PMNs (**b**) in subjects with different genotypes for rs275653 locus: homozygous for minor (GG) and major (AA) alleles and heterozygous (AG). On the *left graphs*, the fluorescence of negative controls is shown. **c** Percentage of cells positive for AT_1R surface expression in PMNs. Overall differences were evaluated by ANOVA (P=0.0036); individuals with GG genotype had significantly lower expression of AT_1R as compared to those with AG (P=0.0024) and AA (P=0.0185) genotypes. No statistically significantly difference was observed between AG and AA genotypes. **d** AT_1R surface expression (percentage of positive cells) in $CD16^+$ fraction of PMNs. Overall there was a statistically significant difference among groups (P_{ANOVA}=0.0016). AT_1R expression in the group of subjects homozygous for the minor variant of rs275653 was lower in respect to AG (P=0.0030) and AA (P=0.0134) groups. Each *column* shows the mean±SEM

Discussion

Here we have identified a region in the promoter of human *AGTR1* associated to extreme longevity. Studies on a test group of Italian centenarians and controls provided evidence of a significant association between SNP variants of *AGTR1* and human lifespan. These findings are sustained by data obtained in the cohort of centenarians and semi-supercentenarians of Asian descent, thus ensuring a validation on a genetically diverse population.

Although studies in short-lived organisms and in human isolated populations with exceptional lifespan have clearly shown that genes play a role in longevity,

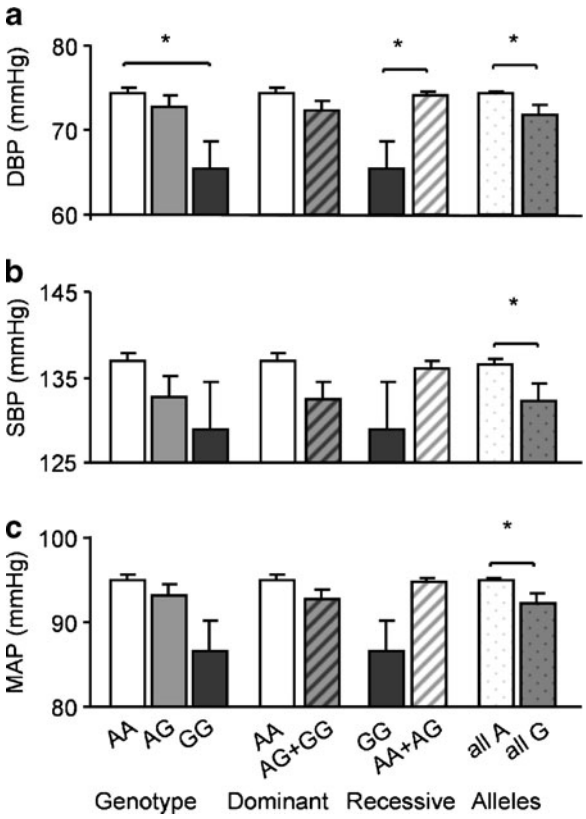

Fig. 3 Blood pressure in Italian and Japanese centenarians according to rs275653 genotype. Data are represented as mean± SEM. **a** Diastolic blood pressure was significantly lower in centenarians with GG genotype as compared to those AA genotype (additive model, P=0.031) or to centenarians with AA and AG genotypes (recessive model, P=0.040). G allele was also associated to reduced DBP (P=0.037). SBP and MAP (**b** and **c**, respectively) were statistically different in centenarians carrying the rs275653 G allele (P=0.046 and P=0.042, respectively)

the specific genetic variants involved are still largely unknown. Evidences in mammals point to a role of genes involved in the major causes of death, i.e. cardiovascular disease, cancer and brain disease. The vasoactive hormone AngII has been involved in organ senescence, and in previous studies, we demonstrated that disruption of the mouse gene encoding for $AT_{1A}R$ markedly prolonged animal lifespan (Benigni et al. 2009) and protected mice from vascular and cardiac injury.

Here we demonstrate that in an Italian cohort, the C variant of rs422858 and the linked G-variant of rs275653 are overrepresented in centenarians with an OR of 4.55 according to the recessive model, in line with what should be expected for a polygenic trait like longevity. Replication of rs275653 genotyping in the Japanese cohort, characterized by a large number of centenarians and semi-supercentenarians of homogeneous ethnic origin, confirmed an enrichment of the G allele in centenarians and its association to longevity according to the recessive model. Combined analyses sustained the association at genotype level. Data of European and Japanese populations from 1000 Genomes and from HapMap databases as well as our results on the Italian group demonstrate that rs275653 is in complete linkage disequilibrium with rs422858. The absence of the rs275653 GG genotype in our Japanese controls diverges from the data of the small cohort of Japanese subjects in 1000 Genomes database (rs275653 GG genotype: one over 90 subjects). However, comparison of the genotype prevalence in Japanese population of 1000 Genomes and in our Japanese controls failed to evidence any statistical difference both at genotype and at allele level.

In silico analyses indicate that the major allele variant of rs422858 belongs to an E-box element which is lost in the presence of the minor allele. Supportive evidence to our genetic data comes from ex vivo findings indicating that the longevity-associated variants have functional consequences on the expression of the corresponding receptor protein on the surface of circulating cells. Indeed, by FACS experiments, we demonstrated that PMNs of subjects homozygous for the minor rs275653 allele (and consequently for the C variant of rs422858) had a significantly lower percentage of AT_1R-positive cells with respect to subjects heterozygous and homozygous for the major allele. A similar difference was observed when the $CD16^+$ PMN fraction was analysed. Consistently, previously reported transfection experiments in adrenal cortical and in vascular smooth muscle cells (Kumar et al. 2005) documented that reporter constructs containing the A rs422858 variant had higher promoter activity than constructs with the minor C variant, suggesting that this SNP could affect the levels of AT_1R gene transcription.

Our findings are in line with the hypothesis that SNP variations in the *AGTR1* promoter can influence protein levels of the corresponding receptor and that a region including or linked to the *AGTR1* promoter could play a role in modulating local RAS system. Since our sequencing-based screening study was confined to the proximal portion of *AGTR1* promoter and to coding regions and intron/exon boundaries, the possibility that rs275653 and the linked rs422858

and rs2307085 are in linkage disequilibrium to variants in other regulatory elements in the promoter or in introns cannot be excluded.

It is likely that the rs275653 and rs422858 minor allele variants or other linked elements in the *AGTR1* promoter dampen cell surface levels of AT_1R protein in PMNs as well as in their $CD16^+$ subset. AT_1R levels in several cell types including neutrophils have been shown to be affected by hypercholesterolemia or dyslipidemia (Marino et al. 2007, 2009; Guasti et al. 2008). In the present study, blood cholesterol and lipids were not different among subjects with GG, GA or the AA genotypes of rs275653, thus ruling out that changes in blood lipid profile accounts for the differences in AT_1R expression.

Local AngII can be produced by tissues and cells, including circulating cells, due to the presence of the complete enzymatic machinery required to synthesize and transform angiotensinogen (Jurewicz et al. 2007; Rasini et al. 2006). Moreover, AngII and its precursors can increase, in a dosage-dependent manner, the mitogen-stimulated T and NK cell proliferation thus playing a role in the induction of inflammatory response and oxidative stress (Jurewicz et al. 2007). In PMNs of patients with peripheral artery disease, increased levels of AT_1R have been demonstrated, suggesting a role for AT_1R in the inflammatory processes which occur in atherosclerosis (Marino et al. 2009). AT_1R stimulation of human neutrophils by AngII increases O_2^- and ROS production (El Bekay et al. 2003; Paragh et al. 2002) through increased cytosolic Ca^{++} levels, and such response is exacerbated in diabetes (Inukai et al. 2005) and hypertension (Alba et al. 2008). Based on the above evidence, the results of the present paper might be taken to suggest that a reduced number of AT_1R on PMNs of subjects carrying the minor rs275653 and rs422858 alleles translates into a reduced capability of such cells to generate ROS, thus creating anti-inflammatory state as to slow cell senescence. Further studies are needed to confirm this assumption and assess whether the variation observed in receptor expression only takes place in PMNs or is shared by other cell types.

Allelic variations in *AGTR1* promoter, including the rs422858 major allele, have been associated with hypertension in Caucasian women (Kumar et al. 2005). Very recently, SNPs in *AGTR1* promoter have been associated to the risk of hypertension also in Mexicans (Martinez-Rodriguez et al. 2011) and to salt sensitivity of blood pressure in a Chinese population (Gu et al. 2010). Other studies have found an association of another SNP, the rs5186 (A1166C) in the 3′-UTR region of *AGTR1*, with hypertension and the metabolic syndrome (Mottl et al. 2008; Palatini et al. 2009). The A1166C SNP affects AT_1R expression (Sethupathy et al. 2007) since the A variant is the target of the microRNA hsa-miR155. In untreated hypertensive young subjects, a correlation among protein expression levels and rs5186 genotype has been recently demonstrated (Ceolotto et al. 2011). In the Italian cohort, we failed to see any association of rs5186 with human longevity, suggesting that the mechanisms by which rs422858/rs275653 or linked factors affect lifespan are not limited to its effects on blood pressure. In both Italian and Japanese centenarians, however, we observed a statistically significant decreased systolic blood pressure in homozygous for GG rs275653 genotype suggesting that this polymorphism might influence blood pressure. In control subjects, we observed a reduction of SBP in subject homozygous for GG genotype; however, this difference was not statistically significant. It is likely that the wide range of age in the control population may have masked the association between the G allele of rs275653 and lower blood pressure.

Through *AGTR1* exon sequencing of the Italian centenarians and younger controls, we discovered few novel variants, all but one in the non-coding regions. Interestingly, the only novel variant leading to amino acid substitution (c.893A>G, p.Asn298Ser) was found in a centenarian. This mutation is not in LD with promoter SNPs, and it is likely a private mutation. It occurs in the highly conserved NPxxY motif of most G-protein-coupled receptors, which is involved in the initiation of signal transduction following ligand binding (Rosenbaum et al. 2009). Site-directed mutagenesis studies have previously shown that Asn298Ser replacement reduced the number of binding sites and impaired the receptor signalling in respect to the wild-type receptor, suggesting that the change found in our centenarian could affect AT_1R activity (Nikiforovich et al. 2006). Further studies are needed to confirm this finding in humans. Our results indicate that genetic *AGTR1* variants leading to reduced receptor expression may contribute to prolong human lifespan. These findings are of particular relevance as they disclose AT_1R as a peculiar pharmacological target for the prevention and treatment of disabilities and diseases that occur with increasing age.

Acknowledgments The generous support of Fondazione ART per la Ricerca sui Trapianti ONLUS (Milan, Italy) and of Fondazione Aiuti per la Ricerca sulle Malattie Rare, Bergamo, Italy, and of Istituto Edizioni ATLAS SpA, Bergamo, Italy is acknowledged. The authors thank the Istituto Italiano Edizioni ATLAS S.p.A for the grant for printing colour figures. The authors are indebted to Dr. Rossella Piras, recipient of a fellowship of Fondazione ART, for the invaluable help in gene sequencing and to Dr. Antonietta Chianca, Ph.D., for elrm analysis.

References

Alba G, El Bekay R, Chacon P, Reyes ME, Ramos E, Olivan J, Jimenez J, Lopez JM, Martin-Nieto J, Pintado E, Sobrino F (2008) Heme oxygenase-1 expression is down-regulated by angiotensin II and under hypertension in human neutrophils. J Leukoc Biol 84(2):397–405. doi:10.1189/jlb.0108035

Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21(2):263–265. doi:10.1093/bioinformatics/bth457

Benigni A, Corna D, Zoja C, Sonzogni A, Latini R, Salio M, Conti S, Rottoli D, Longaretti L, Cassis P, Morigi M, Coffman TM, Remuzzi G (2009) Disruption of the Ang II type 1 receptor promotes longevity in mice. J Clin Invest 119(3):524–530. doi:10.1172/JCI36703

Benigni A, Cassis P, Remuzzi G (2010) Angiotensin II revisited: new roles in inflammation, immunology and aging. EMBO Mol Med 2(7):247–257. doi:10.1002/emmm.201000080

Bilal E, Rabadan R, Alexe G, Fuku N, Ueno H, Nishigaki Y, Fujita Y, Ito M, Arai Y, Hirose N, Ruckenstein A, Bhanot G, Tanaka M (2008) Mitochondrial DNA haplogroup D4a is a marker for extreme longevity in Japan. PLoS One 3(6): e2421. doi:10.1371/journal.pone.0002421

Ceolotto G, Papparella I, Bortoluzzi A, Strapazzon G, Ragazzo F, Bratti P, Fabricio AS, Squarcina E, Gion M, Palatini P, Semplicini A (2011) Interplay between miR-155, AT1R A1166C polymorphism, and AT1R expression in young untreated hypertensives. Am J Hypertens. doi:10.1038/ajh.2010.211

Corder EH, Lannfelt L, Basun H (1996) Apolipoprotein E genotype and the rate of decline in probable Alzheimer disease. Arch Neurol 53(11):1094–1095

de Cavanagh EM, Inserra F, Ferder M, Ferder L (2007) From mitochondria to disease: role of the renin–angiotensin system. Am J Nephrol 27(6):545–553. doi:10.1159/000107757

El Bekay R, Alvarez M, Monteseirin J, Alba G, Chacon P, Vega A, Martin-Nieto J, Jimenez J, Pintado E, Bedoya FJ, Sobrino F (2003) Oxidative stress is a critical mediator of the angiotensin II signal in human neutrophils: involvement of mitogen-activated protein kinase, calcineurin, and the transcription factor NF-kappaB. Blood 102(2):662–671. doi:10.1182/blood-2002-09-2785

Erdmann J, Riedel K, Rohde K, Folgmann I, Wienker T, Fleck E, Regitz-Zagrosek V (1999) Characterization of polymorphisms in the promoter of the human angiotensin II subtype 1 (AT1) receptor gene. Ann Hum Genet 63(Pt 4):369–374

Flachsbart F, Caliebe A, Kleindorp R, Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc Natl Acad Sci U S A 106(8):2700–2705. doi:10.1073/pnas.0809594106

Gu D, Kelly TN, Hixson JE, Chen J, Liu D, Chen JC, Rao DC, Mu J, Ma J, Jaquish CE, Rice TK, Gu C, Hamm LL, Whelton PK, He J (2010) Genetic variants in the renin–angiotensin–aldosterone system and salt sensitivity of blood pressure. J Hypertens 28(6):1210–1220

Guasti L, Marino F, Cosentino M, Maio RC, Rasini E, Ferrari M, Castiglioni L, Klersy C, Gaudio G, Grandi AM, Lecchini S, Venco A (2008) Prolonged statin-associated reduction in neutrophil reactive oxygen species and angiotensin II type 1 receptor expression: 1-year follow-up. Eur Heart J 29(9):1118–1126. doi:10.1093/eurheartj/ehn138

Inukai T, Yoshida N, Wakabayashi S, Inukai Y, Matsutomo R, Takanashi K, Nakamachi T, Takebayashi K, Aso Y, Takahashi K (2005) Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers effectively and directly potentiate superoxide scavenging by polymorphonuclear leukocytes from patients with type 2 diabetes mellitus. Am J Med Sci 329 (5):222–227

Jurewicz M, McDermott DH, Sechler JM, Tinckam K, Takakura A, Carpenter CB, Milford E, Abdi R (2007) Human T and natural killer cells possess a functional renin–angiotensin system: further mechanisms of angiotensin II-induced inflammation. J Am Soc Nephrol 18(4):1093–1102. doi:10.1681/ASN.2006070707

Kumar A, Prater A, Li Y, Jain S (2005) A haplotype of the angiotensin-II receptor subtype-1 is associated with hypertension in Caucasian women. Circulation 111(4):E-52

Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 4(7):1073–1081. doi:10.1038/nprot.2009.86

Marino F, Guasti L, Cosentino M, Ferrari M, Rasini E, Maio RC, Cimpanelli MG, Cereda E, Crespi C, Simoni C, Restelli D, Venco A, Lecchini S (2007) Angiotensin II type 1 receptor expression in polymorphonuclear leukocytes from high-risk subjects: changes after treatment with simvastatin. J Cardiovasc Pharmacol 49(5):299–305. doi:10.1097/FJC.0b013e31803d35ce

Marino F, Guasti L, Tozzi M, Consuelo Maio R, Castiglioni L, Rasini E, Schembri L, Maroni L, Legnaro M, De Leo A, Piffaretti G, Castelli P, Venco A, Lecchini S, Cosentino M (2009) Angiotensin type 1 receptor expression and interleukin-8 production in polymorphonuclear leukocytes of patients with peripheral arterial disease. J Cardiovasc Pharmacol 54(6):520–525. doi:10.1097/FJC.0b013e3181bfadfd

Martinez-Rodriguez N, Posadas-Romero C, Cardoso G, Rodriguez-Perez JM, Perez-Hernandez N, Vallejo M, Vargas Alarcon G (2011) Association of angiotensin II type 1-receptor gene polymorphisms with the risk of developing hypertension in Mexican individuals. J Renin Angiotensin Aldosterone Syst. doi:10.1177/1470320311419175

Min LJ, Mogi M, Iwai M, Horiuchi M (2009) Signaling mechanisms of angiotensin II in regulating vascular senescence. Ageing Res Rev 8(2):113–121. doi:10.1016/j.arr.2008.12.002

Mottl AK, Shoham DA, North KE (2008) Angiotensin II type 1 receptor polymorphisms and susceptibility to hypertension:

a HuGE review. Genet Med 10(8):560–574. doi:10.1097/GIM.0b013e3181809613

Ng PC, Henikoff S (2002) Accounting for human polymorphisms predicted to affect protein function. Genome Res 12(3):436–446. doi:10.1101/gr.212802

Nikiforovich GV, Zhang M, Yang Q, Jagadeesh G, Chen HC, Hunyady L, Marshall GR, Catt KJ (2006) Interactions between conserved residues in transmembrane helices 2 and 7 during angiotensin AT1 receptor activation. Chem Biol Drug Des 68(5):239–249. doi:10.1111/j.1747-0285.2006.00444.x

Noris M, Ruggenenti P, Todeschini M, Figliuzzi M, Macconi D, Zoja C, Paris S, Gaspari F, Remuzzi G (1996) Increased nitric oxide formation in recurrent thrombotic microangiopathies: a possible mediator of microvascular injury. Am J Kidney Dis 27(6):790–796

Noris M, Azzollini N, Mister M, Pezzotta A, Piccinini G, Casiraghi F, Cugini D, Perico N, Orisio S, Remuzzi G (1999) Peripheral donor leukocytes prolong survival of rat renal allografts. Kidney Int 56(3):1101–1112. doi:10.1046/j.1523-1755.1999.00631.x

Oliverio MI, Kim HS, Ito M, Le T, Audoly L, Best CF, Hiller S, Kluckman K, Maeda N, Smithies O, Coffman TM (1998) Reduced growth, abnormal kidney structure, and type 2 (AT2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT1A and AT1B receptors for angiotensin II. Proc Natl Acad Sci U S A 95(26):15496–15501

Palatini P, Ceolotto G, Dorigatti F, Mos L, Santonastaso M, Bratti P, Papparella I, Pessina AC, Semplicini A (2009) Angiotensin II type 1 receptor gene polymorphism predicts development of hypertension and metabolic syndrome. Am J Hypertens 22(2):208–214. doi:10.1038/ajh.2008.319

Paragh G, Szabo J, Kovacs E, Keresztes T, Karpati I, Balogh Z, Pall D, Foris G (2002) Altered signal pathway in angiotensin II-stimulated neutrophils of patients with hypercholesterolaemia. Cell Signal 14(9):787–792

Pawlikowska L, Hu D, Huntsman S, Sung A, Chu C, Chen J, Joyner AH, Schork NJ, Hsueh WC, Reiner AP, Psaty BM, Atzmon G, Barzilai N, Cummings SR, Browner WS, Kwok PY, Ziv E (2009) Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. Aging Cell 8(4):460–472. doi:10.1111/j.1474-9726.2009.00493.x

Rasini E, Cosentino M, Marino F, Legnaro M, Ferrari M, Guasti L, Venco A, Lecchini S (2006) Angiotensin II type 1 receptor expression on human leukocyte subsets: a flow cytometric and RT-PCR study. Regul Pept 134(2–3):69–74. doi:10.1016/j.regpep.2006.01.007

Rosenbaum DM, Rasmussen SG, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. Nature 459(7245):356–363. doi:10.1038/nature08144

Sethupathy P, Borel C, Gagnebin M, Grant GR, Deutsch S, Elton TS, Hatzigeorgiou AG, Antonarakis SE (2007) Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3′ untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. Am J Hum Genet 81(2):405–413. doi:10.1086/519979

Sirtori CR, Calabresi L, Franceschini G, Baldassarre D, Amato M, Johansson J, Salvetti M, Monteduro C, Zulli R, Muiesan ML, Agabiti-Rosei E (2001) Cardiovascular status of carriers of the apolipoprotein A-I(Milano) mutant: the Limone sul Garda study. Circulation 103(15):1949–1954

Sunyaev S, Ramensky V, Koch I, Lathe W 3rd, Kondrashov AS, Bork P (2001) Prediction of deleterious human alleles. Hum Mol Genet 10(6):591–597

Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD (2008) FOXO3A genotype is strongly associated with human longevity. Proc Natl Acad Sci U S A 105(37):13987–13992. doi:10.1073/pnas.0801030105

Wilson SK (1990) Role of oxygen-derived free radicals in acute angiotensin II-induced hypertensive vascular disease in the rat. Circ Res 66(3):722–734

Zamar D, Brad McNemey, Graham J (2007) elrm: software implementing exact-like inference for logistic regression models. J Stat Softw 21(3)