Università degli Studi di Milano

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Summary of PhD thesis

Longevity-associated variant (LAV)-BPIFB4 characterization and its role in the modulation of eNOS signaling through Ca^{2+}/PKC-alpha dependent mechanism.

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Abstract

***Rationale**: Endothelial nitric oxide synthase (eNOS) is a crucial enzyme for vascular physiology and its reduced activity during aging leads to increased cardio and cerebrovascular disease susceptibility1-4. Caloric restriction, which delays aging and increases life-span in all species, doesn't exert any effect in mice lacking eNOS5. Long Living Individuals (LLIs) have a favourable genetic profile, characterized by an enrichment of alleles that protect from aging and cardiovascular disease6, 7.*
We have recently shown that LLIs of three populations are enriched for the minor allele rs2070325 (I229V) of the bactericidal/permeability-increasing fold-containing family B member 4 (BPIFB4) and that rs2070325 is part of a four SNPs haplotype that codified for a Wild Type (WT) and a longevity-associated variant (LAV) of BPIFB4, that is able to potentiate eNOS activity. This unique ability is correlated with its higher efficiency, as compared to WT-BPIFB4, in binding 14-3-3 through a BPIFB4 atypical binding site for 14-3-3, which correlates with its level of phosphorylation at serine 75, a phosphorylation site for stress kinase PERK. HSP90 recruitment into the complex is part of the eNOS activation machinery triggered by LAV-BPIFB4. Indeed HSP90 is co-immunoprecipitated together with BPIFB4 and a specific HSP90 inhibitor blocks LAV-BPIFB4 potentiation on endothelial function and eNOS activation.
The data so far reported clearly demonstrate that LAV-BPIFB4 activates eNOS function trough 14-3-3 and HSP90 recruitment and, as underlined by William Sessa in its editorial, further characterization is needed to define how LAV-BPIFB4 transduce upstream signals to eNOS

***Objective**: To further characterize the molecular signaling regulated by LAV-BPIFB4 to modulate vascular function.*

***Methods and Results**: Here we show that eNOS activation by LAV-BPIFB4 is mediated by Ca2+ mobilization and PKC-alpha activation. In LAV-BPIFB4 transfected HEK293 cells, there was an enhancement of ATP-induced Ca2+ mobilization and PKC-alpha translocation on plasma membrane. In vascular reactivity studies, LAV-BPIFB4 failed to potentiate eNOS and endothelial function upon PKC-alpha inhibition by Gö6976. Moreover, when vessels were exposed to external Ca2+-free conditions, LAV-BPIFB4 lost the ability to activate PKC-alpha and eNOS. In these conditions, though, and in eNOS knockout vessels, LAV-BPIFB4 still potentiated endothelial activity, through mechanisms alternative to eNOS, and this function was blunted by the inhibitors of the endothelium-derived hyperpolarizing factors (EDHF). Notably, peripheral blood mononuclear*

cells from subjects carrying the a/a genotype for rs2070325 had a significant increase of phosphorylation of PKC-alpha (T497).

***Conclusions**: We have identified novel molecular determinants of the beneficial effects of LAV-BPIFB4 on endothelial function, showing the role of Ca2+ mobilization and PKC-alpha in eNOS activation and of EDHF activation upon eNOS inhibition. These results highlight the role of LAV-BPIFB4.*

Nonstandard Abbreviations and Acronyms

BPIFB4 = bactericidal/permeability-increasing fold-containing-family-B-member-4
HSP90 = heat shock protein 90
LAV = longevity-associated variant
LLIs = long living individuals
MNC = mononuclear cells
PERK = protein kinase R–like endoplasmic reticulum kinase
NO = nitric oxide
ACh =acetylcholine
EDHF= endothelium-derived hyperpolarizing factors
[Ca2+]i = intracellular calcium

1. INTRODUCTION

1.1 Definition of aging

Aging is a complex process ruled by stimuli of different nature and belonging to several field of life. Stochastic events contribute to this process by producing casual damages to essential molecules, the external environment interfere through the life style (diet and/or caloric restriction), while the genetic asset provides a background of intrinsic alterations that are protective or detrimental for the onset of aging related pathologies. The attention on this latter point increased in the last years, promoting the search for genetic variations connected with protective features against typical age-related disease like hypertension, diabetes and cancer.
Therefore, longevity is defined as that phenotype of long-living subjects that approach old age under the effects of these several factors.

1.2 The longevity phenotype

The Birth Cohort Study found that in the United State at the beginning of twentieth century life expectancy was 51.5 years for males and 58.3 years for females; currently 1 person each 10,000 reach 100 years of age, and this prevalence is quickly changing and will probably soon approach 1 person each 5,000[8]. It was estimated that the increased ability to reach 100 years old in industrialized countries over the last 160 years reflects a rise in life expectancy — quantified as 3 months per year for females — subsequent to improvements in diet and a reduced exposure to infection and inflammation[9]. The improvement of healthy care and the attention to a balanced and quality food intake speedup the process towards a diffused

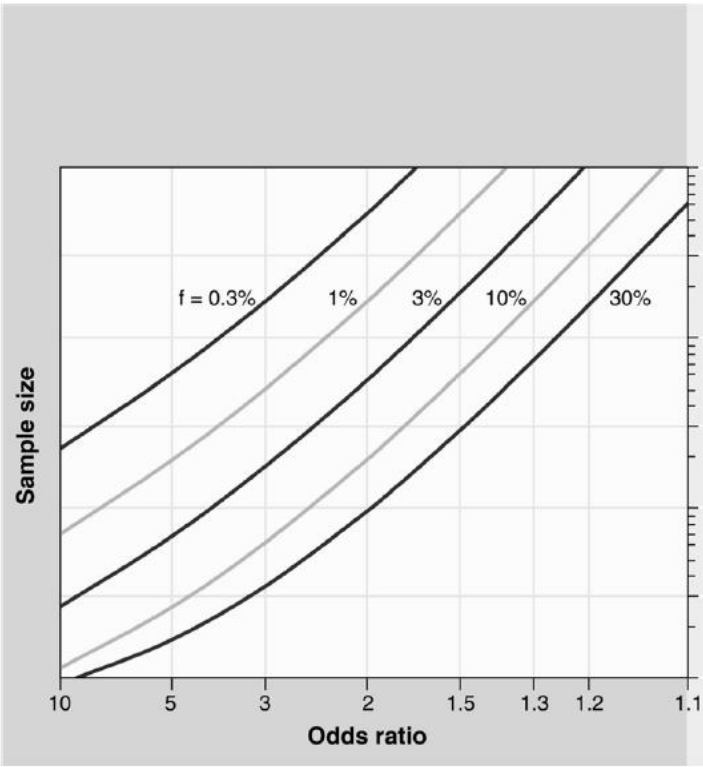
longevity. In favor of diet as a modulator of longevity, the Elderly Prospective Cohort Study (EPIC) identified a reduced overall mortality among the elderly consuming a modified Mediterranean diet in which saturated fatty acids were substituted for monounsaturated ones[10].

Centenarians, despite being exposed to the same environmental conditions as members of the average population, manage to live much longer. Long-living individuals (LLIs), i.e. those that approach 100 years of age, are a model of successful aging: most of them have a compression of disability and morbidity towards the end of their life and tend not to show changes normally observed during aging, such as a decrease in insulin sensitivity and in heart rate variability (HRV)[11-13]. The exceptional longevity of LLIs is to some extent genetically driven, as underlined by the familial clustering effect for extreme longevity and the reduced mortality of centenarians' siblings compared to those of non-centenarians. Under a genetic point of view, the compression in morbidity and mortality is correlated with the enrichment of protective alleles and the depletion of detrimental ones. It has been estimated that genetic variants account for at least 25% of human life-span, and for even a larger proportion in individuals living to extreme age[6, 14].

Ultimately, aging is an independent risk factor associated with endothelial dysfunction, impaired angiogenesis and loss of protein homeostasis, or proteostasis, the decline of which is contrasted by adaptive cellular responses[15]. The mechanisms that help cells adapt and survive under stressful conditions include the activation of heat shock factor protein 1 (HSF1)-controlled heat shock proteins (HSPs), ribosomal biogenesis and protein synthesis, and are at least in part orchestrated by growth factors, as observed in *Caenorhabditis elegans*, in which HSF-1 is under the control of insulin/IGF-1-like signaling (IIS)[16].

1.3 Genome-Wide Association Studies

In our studies on longevity phenotype, we follow the genetic trait by trying to characterize the functional aspects of the genetic background that make a person a centenarian. Genome Wide Association Studies (GWASs), that analyze the differences in the frequencies of presence of common genetic variants in two populations - cases and controls - generate findings that need to be replicated in independent populations. In the case of exceptional longevity, success in replicating initial findings is negatively influenced by the differences in participant ages, gender and disease status distribution across the analyzed populations. Furthermore, the GWAS approach suffers from the multiple-testing statistical penalty that forces the adoption of very low p-values of significance, hence favouring the phenomenon of the winning course, i.e. the enrichment of false-positive associations among the dozens of top findings[17]. Other potential problems that generate false positive and false negative results include the low power of studies using small sample sizes, and a lack of a suitable control for the genetic admixture. On the first point, an extremely instructive review has been written by Altshuler, Daly and Lender, who calculate the power of a study based on the number of individuals genotyped, the number of tested hypotheses, and the frequency of the allele tested for a specific OR[18]. From the graph given in their review (**Figure 1.1**), it is clear that for the OR expected in human exceptional longevity (between 1.2 and 2), the power of a study is highly dependent upon the number of hypotheses tested.



	GWAS			Rare variant	Nominal
	90%	50%	10%	90%	90%
	100,000	66,790	40,260	66,040	21,370
	30,000	20,037	12,078	19,812	6411
	10,000	6679	4026	6604	2137
	3000	2004	1208	1981	641
	1000	668	403	660	214
	300	200	121	198	64
	100	67	40	66	21

Figure 1.1 Sample sizes required for genetic association studies.
The graph shows the total number N of samples (consisting of N/2 cases and N/2 controls) required to map a genetic variant as a function of the increased risk due to the disease-causing allele (x axis) and the frequency of the disease-causing allele (various curves). The required sample size is shown in the table on the right for various different kinds of association studies[18]. Reproduced with permission from The American Association for the Advancement of Science.

For these reasons, recent GWASs have failed to find variants that cross-validate across populations — with the exception of few cases — pointing to the need of much larger studies or alternative study designs in order to discover common polymorphisms with smaller genetic effects and rare variants with high penetrance that influence exceptional longevity[19, 20].

Thus, a GWAS on exceptional longevity can be considered only a hypothesis-generating effort to be used in conjunction with other studies.

To date, the most remarkable genetic findings for exceptional longevity observed in the genome of centenarians are the decreased frequency of detrimental alleles of apolipoprotein E (*APOE*) and the increase of protective alleles of forkhead box O3A (*FOXO3A)*[21-26]*.*

Recently, we found associated with longevity three more variants in *CAMKIV*, *ATXN1* and *DCAMKL1* (**Table 1.1**)[27].

APOE is involved in lipid transport to the lymph system and was the first gene to be successfully replicated as a gene important in exceptional longevity[28]. FOXO3A mediates a pro-survival activity by regulating the cellular response to stimuli ranging from radical stress to growth factors [Insulin growth factor 1 (IGF1)], and by acting on the transcription of genes involved in radical stress response, cell cycle arrest, proliferation, and apoptosis[29, 30]. Protein Kinase B (AKT)-dependent phosphorylation and sirtuin 1 (SIRT1)-dependent deacetylation of FOXO3A induce pro-survival activity by activating genes linked to the stress response and cell cycle arrest and by inhibiting pro-apoptotic genes[31]. In humans, genetic alterations of the IGF1 receptor that alter the IGF signaling pathway confer an increase in propensity for longevity[32]. *AKT* polymorphisms have also been associated with human longevity[26].

Table 1.1 Genes and variants found correlated with longevity in humans

Gene	**Variant**	**Occurrence in centenarians**	**Previous disease correlations**	**Potential role in longevity**
APOE	ε4	reduced	Alzheimer's	Maintenance

			and cardiovascular diseases	of vascular integrity
FOXO3A	rs2802292*	more present	none	Control of cell homeostasis
CAMK4	rs10491334†	more present	hypertension	Modulation of CREB, SIRT and FOXO3A
ATXN1	rs697739	more present	amyotrophic lateral sclerosis (age of onset)	Modulation of CREB
DCAMKL1	rs9315385	more present	heart rate variability	Modulation of CREB

**, result obtained by a candidate gene approach and replicated in other studies; †, result obtained from a GWAS and confirmed in a replication cohort.*

For the above reasons, in our recently published GWAS on individuals enrolled in the Southern Italian Centenarian Study (SICS), we identified CAMK4 rs10491334, a variant that had been already established among the top 5 SNPs in the Framingham Heart Study on diastolic high blood pressure[33]. The fact that CAMK4 rs10491334 associates also with hypertension is reassuring in that hypertension and longevity are regulated by common pathways. In fact, mice with genetic ablation of the angiotensin II type1 receptor — the key regulator of blood pressure — had increased expression of the longevity gene Sirt3 and improved survival[34].

Interestingly, rs10491334 correlated with CAMK4 protein expression, and functional studies revealed the ability of CAMK4 protein to modulate SIRT1 and FOXO3A (**Figure 1.2**).

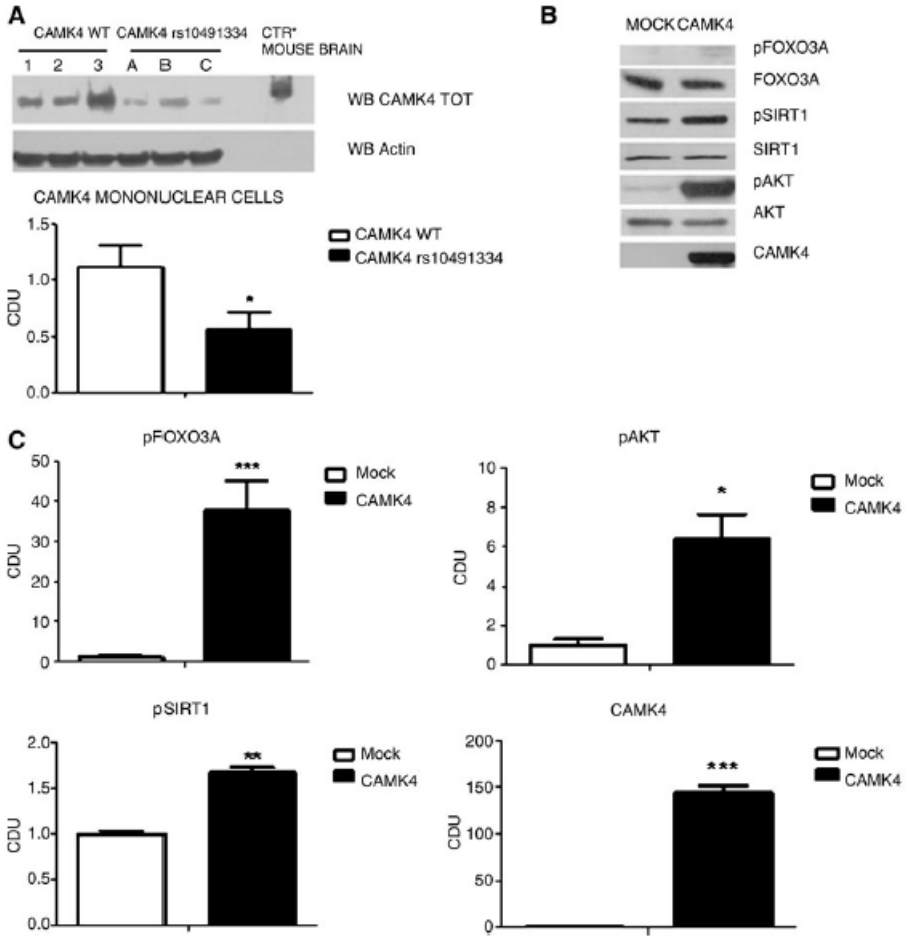


Figure 1.2 Western blot analysis of calcium/calmodulin-dependent protein kinase IV (CAMKIV).

(A) Western blot analysis of CAMKIV revealing higher CAMKIV levels in mononuclear cells of subjects expressing wild-type CAMKIV (CAMKIV WT) compared to subjects homozygous for the CAMKIV minor allele (CAMKIV rs10491334). CAMKIV levels were normalized to actin (corrected densitometry units, CDU; n=10). (B and C) Western blot analyses of HT29

cells expressing CAMKIV revealing a significant increase of phosphorylated FOXO3A, AKT, and SIRT1 compared with cells infected with empty vector (MOCK). Data from immunoblots were quantified by densitometric analysis. Statistics were performed with analysis of variance (ANOVA). (*) p<0.05; (**) p<0.005; (***) p<0.0005.

The ataxin-1 (ATXN1) rs697739 allele was another variant found among the top findings of our GWAS on SICS individuals. This polymorphism had been previously associated with the age of onset of sporadic amyotrophic lateral sclerosis, a disease of unknown cause characterized by slowly progressive degeneration of motor neurons and that usually occurs in patients aged 40–60 years[35]. ATXN1 is the gene responsible for spinocerebellar ataxia type 1 andantagonizes the neuronal survival function of myocyte enhancer factor-2 (MEF2)[36]. MEF2 transcription repression by cabin1-HDAC4 is removed by CAMKIV activation, and this suggests that MEF2 is a common downstream target of CAMKIV and ATXN1[37, 38].

In addition to CAMKIV rs10491334 and ATXN1 rs697739, the rs9315385 allele of doublecortin and Ca^{2+}/calmodulin-dependent kinase-like-1 (DCAMKL1) was a third top finding of our study. DCAMKL1 has structural similarity with CAMKIV, but despite this, it represses CAMKIV-induced activation of cAMP response element-binding (CREB) protein via phosphorylation of transducer of regulated CREB activity 2 (TORC2) at Ser171[39]. DCAMKL1 rs9315385 was previously associated with total power of HRV[40]. A reduced HRV is a marker of autonomic dysfunction and is associated with an increased risk of cardiovascular morbidity and mortality[41]. HRV-parasympathetic function decreases up to the eighth decade of life, followed by an increase to higher levels — similar to those found in a younger population — in nonagenarians and centenarians[13].

Similarly to CAMKIV, DCAMKL1 and ATXN1 are expressed mainly in brain. These data support the importance of the CAMKIV/CREB pathway in regulating the aging process.

A brief mention needs to be made here on the cutting edge, genetic signature paper by Sebastiani et al. that very elegantly proved that a complex analysis on 281 SNPs allowed to define clusters of individuals that aged differently based on their genetic signature[42].

The identification and the study of genetic variants that influence exceptional longevity in humans are important, since novel targets for prevention and therapy of a large spectrum of age-related diseases could be discovered[43]. Genome-wide association studies (GWAS) are hypothesis-generating studies that can be used to this end.

1.4 Aging process theories

1.4.1 Free radicals theory

One of the theory that tempts to explain the aging process is the free radicals theory. Responsible for oxidative stress, free radicals are defined as atoms or molecules that contain one or more unpaired electrons. Biological systems depend on these kind of molecules: the most important are the reactive oxygen species (ROS) superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$) and hydroxyl ($^{\cdot}OH$) radicals, and hypochlorous acid (HOCl)[44]. In 1956 Denham Harman proposed the “free-radical theory of aging” (FRTA)[45] that postulated that accumulation of free radicals was the prime cause of the sequential alterations characterizing advancing age and the progressive increase in disease and death rates[46]. This hypothesis was based on the “rate of living” theory formulated by

Raymond Pearl explaining longevity variation within species in terms of combined mass-specific resting metabolic rate and the “lifetime energy potential”: it held that the pace of life is inversely related to the length of life[47].

In the oxygen managing system of the cell, mitochondria play an important role and physiological or pathological dysfunctions of them are associated with aging or age-related diseases[48]. The aging process, indeed, is associated with the improvement of the mitochondrial production of ROS, specially in heart and in the vasculature system. In the heart, the last evidences are cardiac hypertrophy, fibrosis and apoptosis. In the vasculature system the mitochondrial oxidative stress and the presence of ROS contribute to the development of chronic low-grade vascular inflammation in aging[49] by activating redox signaling pathways. In addition, the ROS activation of Akt pathway increases the development of the senescent phenotype in endothelial cells characterized by an impairment in the ability of regeneration and angiogenesis, and a general reduction in vascular reactivity and increasing in atherosclerosis events due to less cytokines vessel production[50].

A proved way to overcome to the rapid aging of mitochondria and consequently of cardiovascular system, is exercise. It’s clear that the physical activity brings to a stress–induced NO production able to restore vascular activity and endothelial cells protein homeostasis[51]. A similar effect is achieved by a caloric restriction lifestyle or by the administration of caloric restriction mimetics, in which the energy sensor AMPK upregulates SIRT activity restoring the vessels activity.

1.4.2 Lipid metabolism and aging

In apparent contrast with the FRTA is the finding that the cell membranes of offspring of nonagenarians have an accumulated amount of endogenous *trans* fatty acids[52]. The peroxidation index of erythrocyte membrane lipids was significantly lower in nonagenarians' offspring than in a group of matched controls[52]. It is of particular interest that we found significantly increased levels of palmitoleic acid (C16:1n-7) in the nonagenarians' offspring, similarly to what was later observed in genetically modified long-living worms[53]. Because these worms were genetically modified in homologue genes of the insulin-like growth factor 1 (IGF1)/forkhead box O3 (FOXO3A) axis, a possible explanation for this finding is that IGF-1 signalling modulates, or is modulated by, the membrane fatty-acid composition[54]. Moreover, it was reported that after chronic thermal or saline stress of yeast, the induced increase in the level of membrane palmitoleic acid was responsible for a reset of heat shock protein (Hsp) release to higher levels[55]. Thus, the high C16:1n-7 detected in the offspring of nonagenarians could be correlated to the low serum level of Hsp70 detected in centenarians' offspring[56]. Endogenous *trans* fatty acids are an index of endogenous free-radical cellular stress and are produced by endothelial nitric oxide synthase (eNOS)-generated nitrates ($NO_2^{\cdot}$), as shown by the lack of *trans*-arachidonic acids in the retinas of $eNOS^{-/-}$ mice[57]. Moreover, calorie restriction, which is known to increase longevity, induces the expression of eNOS, the activation of telomerase and the biogenesis of mitochondria. Thus, the increase in ROS observed with calorie restriction suggests a role for free-radical stress: the induction of endogenous defense mechanisms, maybe through *trans* fatty acids participation, that culminate in increased resistance to stress and longevity. This adaptive response was named hormesis[58]. From this

point of view, the FRTA formulated by Harman doesn't appear consistent and these findings, taken together, suggest that ROS can act as essential signaling molecules for the promotion of metabolic health and longevity.

The degree of oxidative stress could possibly explain this apparent paradox: a low stress situation results protective for organism, while massive stress becomes deleterious. In support of this hypothesis, deletion in worms of mitochondrial proteins, such as ISP-1 and NUO-6, induces the oxidative stress necessary and sufficient for promoting longevity: in fact, this effect is abolished by antioxidants and is induced by mild treatment with oxidants (**Figure 1.3**)[59].

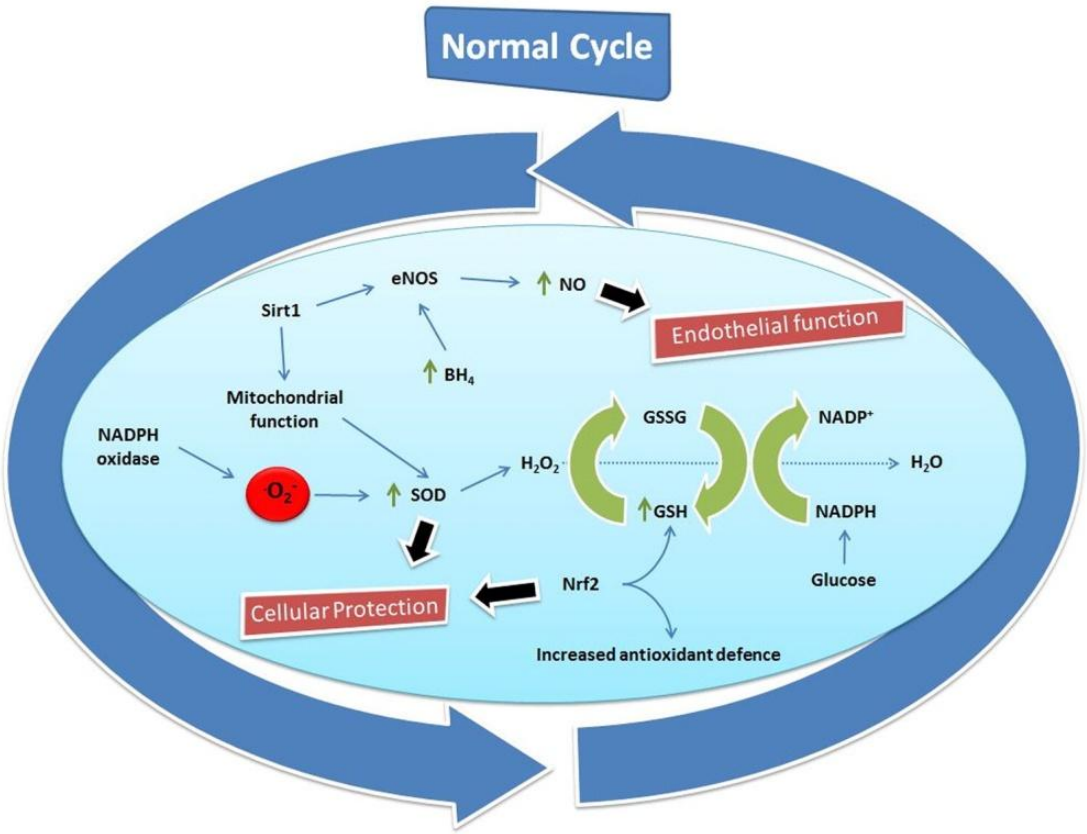


Figure 1.3 Schematic of vascular physiological function

1.5 Nitric Oxide and its prevalent role in the vascular system

The beneficial effects of calorie restriction are multiple: it reduces the incidence of tumors and diabetes and the age-related decline in T-lymphocyte proliferation[60]. The effects of calorie restriction can be explained by increased IGF1-insulin signal (IIS) efficiency: in fact, findings on patients with growth hormone receptor deficiency suggest that their high insulin sensitivity could account for the absence of diabetes and very low incidence of cancer seen in these individuals[61]. Furthermore, calorie restriction can be mimicked by genetic manipulation aimed at blocking IIS (i.e., the IGF1/PI3K/AKT/FOXO3A axis): for example, the FIRKO mouse − a carrier of a fat-specific insulin receptor knockout − and *C. elegans* models carrying null mutations of daf-2 − an IGF1 homologue − and age-1 − a homologue of the catalytic subunit of mammalian PI3K− all live longer than wild-type animals[62, 63]. To be noted, the beneficial effects of daf-2 and age-1 null mutations are antagonized by null mutation of daf-16, which encoder three members of the FOXO family of transcription factors[63]. Thus, via AKT the IIS is important for controlling eNOS and, hence, human longevity[64].

Exercise is inversely correlated with total mortality[65]. An elegant report on athletes undergoing marathon training identified a combination of metabolites (i.e., glycerol, niacinamide, glucose-6-phosphate, pantothenate and succinate) that increased in the plasma in response to exercise; in vitro, these metabolites were able to up-regulate the expression of NUR77, a transcriptional regulator of glucose utilization and lipid metabolism genes[66]. NUR77 is under the control of Ca^{2+}/calmodulin-dependent protein kinase (CAMKIV), which is activated by AMPK and has been associated with human exceptional longevity[27, 38]. Furthermore, AMPK controls eNOS phosphorylation, which explains the potentiation of eNOS activity by both calorie restriction and physical exercise.[64] AMPK is activated acutely at

exercise intensities above ≈ 60% of maximal aerobic capacity[67]. Calorie restriction and exercise both activate mitochondrial biogenesis through activation of AMPK with an eNOS-dependent mechanism, as shown by experiments on eNOS knockout mice[68]. Thus, the beneficial effects on longevity of calorie restriction, genetic makeup and exercise can be explained, at least in part, through eNOS-dependent activation of mitochondrial biogenesis.

During the aging process, oxidative stress increases in the arterial system either of humans and experimental models[69]. This phenomenon has been linked directly to the development of atherosclerotic vascular diseases (**Figure 1.4**).

We think that the state of the vascular system is the most important factor in determining health during aging. In fact, vascular system is responsible for the transport of oxygen and nutrients throughout the body and, therefore, is responsible of when and how the organs and systems could encounter suffering and ultimately fail. In the end, oxidative-stress-induced endothelial dysfunction is probably the key mechanism linking older age to increased risk of clinical cardiovascular disease and death[70].

Free radicals play a physiological role in the vessel wall as well: they participate as second messengers in endothelium-dependent functions, in smooth muscle cell and endothelial cell growth and survival, and in remodeling of the vessel wall[71]. When these processes are out of control, they contribute to vascular alterations characterized by mitochondrial dysfunction and increased ROS production,[72] and lead, ultimately, to the development of cardiovascular pathologies, such as hypertension, stroke and coronary artery disease.[73, 74].

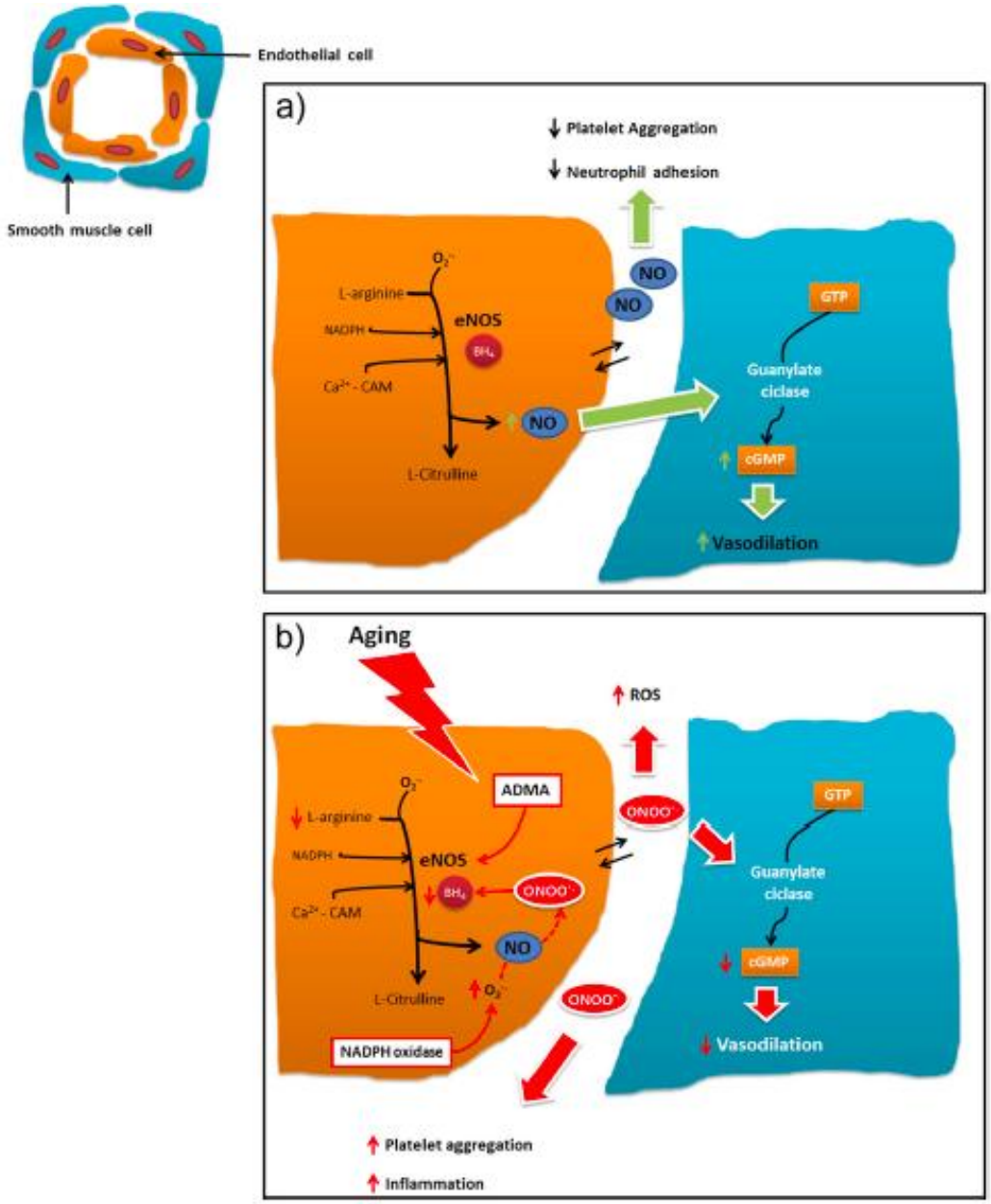


Figure 1.4 a) Representative nitric oxide pathway. b) Effects of aging on nitric oxide pathway.

BH4 = tetrahydrobiopterin; cGMP = cyclic guanosine monophosphate; GTP = guanosine triphosphate; NADPH = nicotinamide adenine dinucleotide phosphate; ONOO- = Peroxynitrite; ADMA = Dimethylarginine

One of the most important mediators in the cardiovascular system is nitric oxide (NO). During aging, increased oxidative stress leads to a progressive decrease in NO production. In particular, this is caused by eNOS uncoupling − a phenomenon that promotes the formation of superoxide rather than NO − and decreased expression of the essential eNOS cofactor tetrahydrobiopterin (BH4)[3]. In fact, superoxide produced mainly by NADPH oxidase may react with NO to produce peroxynitrite that, promoting eNOS uncoupling, contribute to the increase of superoxide and to the reduction of NO that may cause an acceleration of the atherosclerotic process[75]. Thus, abnormalities in eNOS signalling underlie endothelial dysfunction, a common finding in aging.

Several studies emphasize the importance of mitochondrial oxidative stress, which represents a typical characteristic of endothelial dysfunction that develops during aging and it is associated with the over-activation of an important enzyme, which is localized both in the cytoplasm that in membrane, namely NADPH oxidase[76, 77].

Deshpande et al. demonstrated that Rac1 − a regulatory component of plasma membrane NAD(P)H oxidases − plays an important role in premature aging of the endothelium and in the development of associated vascular pathologies[78, 79]. In addition, other molecular mechanisms responsible for age-related mitochondrial oxidative stress in the vasculature involve dysregulation of antioxidant defences, such as peroxynitrite-mediated Nrf2/ARE (antioxidant response elements) dysfunction , nitration and inhibition of manganese superoxide dismutase (MnSOD), declines in glutathione (GSH) content and a dysfunctional electron transport chain[69].

Nrf2 is a redox-sensitive transcription factor that upregulates the expression of numerous ARE genes that encode proteins that detoxify ROS and mediate the anti-aging effects of calorie restriction[80]. Recent findings demonstrate that Nrf2 dysfunction in the vasculature is associated with

aging and contributes to the age-related dysregulation of GSH synthesis in various tissues.

Van der Loo et al. provided evidence for association between the formation of peroxynitrite and age-associated vascular dysfunction by demonstrating selective nitration of MnSOD − the major antioxidant enzyme in the mitochondria of all mammals − with increased age[81]. This finding was supported by a recent report showing that genetic inactivation of MnSOD in mutant mice resulted in premature death and that treatment with a SOD mimic dramatically prolonged survival.

Moreover, the activities of various electron transport chain oxido-reductases are deleteriously affected during aging. The mitochondria generate ATP from nutrients, and its synthesis via the mitochondrial respiratory chain is the result of a proton potential generated by the electron transport chain. Damage to this latter can cause breakdown of the proton potential, apoptosis and the generation of free radicals in a vicious cycle[82].

According to the FRTA, ROS are the major candidates responsible for senescence and age-related diseases in which the redox balance is disturbed and generates oxidative stress. Indeed, senescence of endothelial cells has been proposed to be responsible for endothelial dysfunction and atherogenesis during aging. In fact, vascular cells exhibiting the morphological features of cellular senescence have been found in atherosclerotic lesions[83].

Relatively recent studies have demonstrated that inhibition of Sirt1 induces premature senescence and that Sirt1 overexpression reverts premature senescence induced by hydrogen peroxide. In macrophages in vitro and in rats in vivo, Sirt1 inhibits NF-kB and so hinders pro-inflammatory mediator release, protecting from the development of oxidative stress[84].

Finally, there are several findings that relate telomere shortening to aging in vivo. Telomeres are regions of DNA, characterized by G-rich sequences located at the ends of linear chromosomes that can form four-stranded structures: they are considered a biological clock that measures mitotic time and determines the senescence of cell replication. There appears to be a correlation between telomere length, age and oxidative stress in human arteries at sites of elevated hemodynamic stress and, presumably, with a high cell turnover. Data suggest that G-rich strands are more vulnerable to oxidative damage and that faster telomere shortening rates are observed in cell strains that also have higher peroxide levels. Thus, telomere loss is clearly implicated in oxidative damage, accumulation of senescent cells in elderly individuals and reduction in lifespan[85] (**Figure 1.5**).

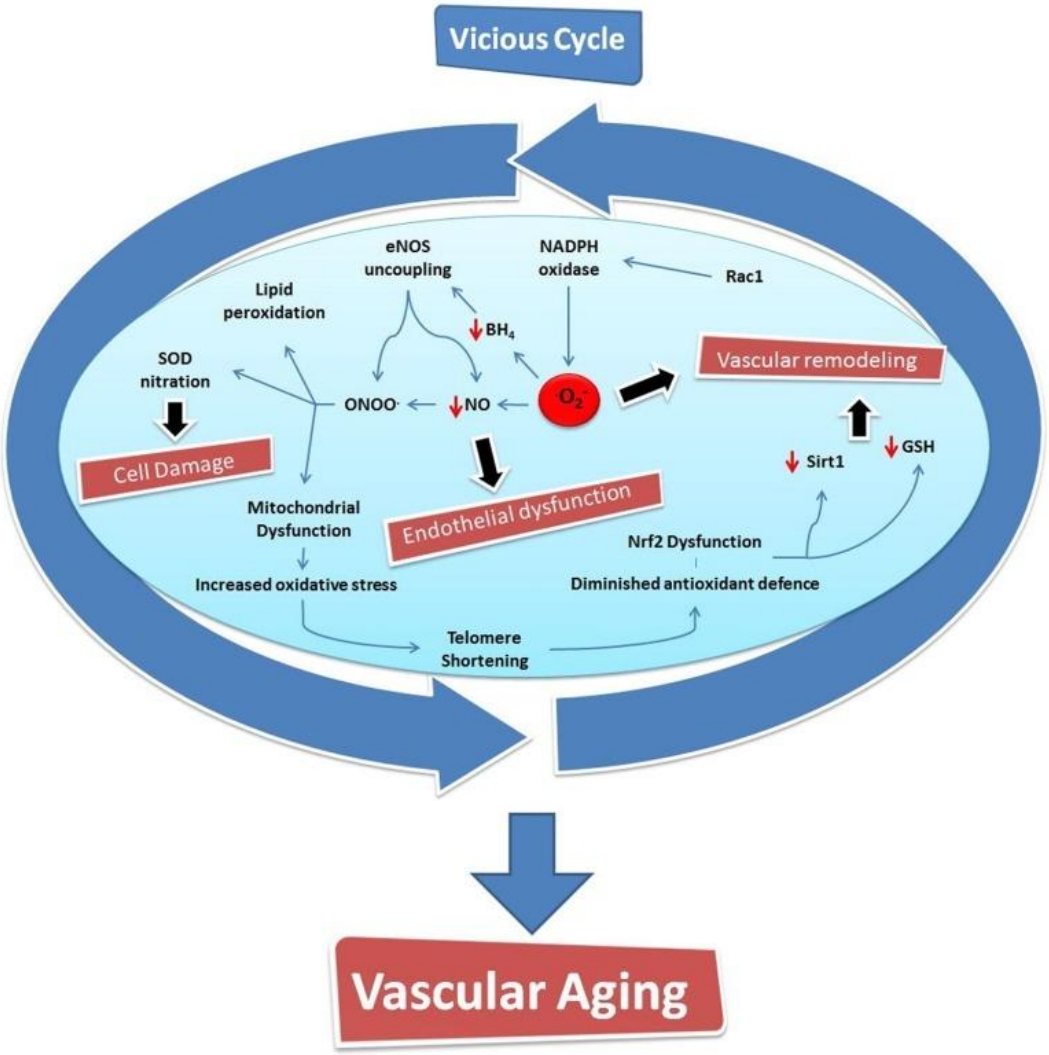


Figure 1.5 Schematic of the involvement of free radicals in vascular ageing.

1.6 Longevity populations

We recruited and genotyped 582 LLIs (age range 90-109 years) and 784 young control individuals (age range 18-45 years) as part of the Southern Italian Centenarian study (SICS). SICS LLIs were thoroughly investigated for demographic characteristics, medical history (past and present diseases), level of independence (Barthel Score)[86],and cognitive status (Cognitive Score) (**Table 1.2**).

Table 1.2 Characteristics of SICS screening-set (N=410) and SICS replication-set LLIs (N=116)

	SICS LLIs - Screening (N=410)						SICS LLIs - Replication (N=116)					
Variable/Disease	**N**	**%**	**Mean**	**SD**	**Min**	**Max**	**N**	**%**	**Mean**	**SD**	**Min**	**Max**
Demographics												
Gender (Males)	240	58.82					54	46.96				
Age			96.62	3.64	90	109			96.14	3.52	90	109
Patient's History												
Deceased, Age at Death	313	76.72	96.95	3.59	90	109	87	75.65	96.08	3.32	90	109
Smoking History	97	23.77					30	26.09				
Diabetes Mellitus	36	8.82	79.74	11.69	54	94	19	16.52	73.18	12.12	50	91
Overweight (BMI >25) [a]	74	25.00					23	24.73				

		8.3	74.	12.				6.9	77.	15.		
Osteoporosis	34	3	92	96	50	91	8	6	25	39	55	90
		7.8	83.	11.			1	9.5	81.	10.		
Heart Attack	32	4	07	39	50	97	1	7	73	55	60	93
Cardiac		19.	86.	9.5		10	2	18.	80.	16.		
Arrythmia	81	85	09	1	50	2	1	26	35	8	20	96
		1.7	76.	12.				0.8				
Angina	7	2	4	03	60	92	1	7	80	NA	80	80
Congestive		4.1	81.	10.				5.2		7.2		
Heart	17	7	77	25	70	97	6	2	86	1	77	95
High Blood	12	30.	79.	11.		10	5	49.	74.	14.		
Pressure	4	39	47	27	38	4	7	57	31	58	20	96
		9.3	87.	7.3			1	11.	85.	7.4		
Stroke	38	1	35	2	70	98	3	3	69	9	69	93
		4.4	81.	16.				2.6	93.	4.1		
Kidney Disease	18	1	06	42	27	93	3	1	33	6	90	98
PVD Circulatory		8.3	85.	7.0		10	1	10.	80.	14.		
Problems	34	3	97	7	70	0	2	43	75	85	40	96
	14	36.	84.	9.3		10	4		84.	8.0		
Cataracts	8	27	01	6	40	0	6	40	3	3	65	98
		3.1	86.	8.3				5.2				
Glaucoma	13	9	11	3	66	93	6	2	94	NA	94	94
Macular		2.4		7.7				7.8	78.	3.5		
Degeneration	10	5	85	8	77	93	9	3	33	1	75	82
Thyroid		1.2	55.	15.				2.6	75.	20.		
Condition	5	3	6	44	31	68	3	1	5	51	61	90

	SICS LLIs - Screening (N=410)						**SICS LLIs - Replication (N=116)**					
Variable/Diseas	**N**	**%**	**Me**	**SD**	**Mi**	**Ma**	**N**	**%**	**Me**	**SD**	**Mi**	**Ma**

e			an		n	x			an		n	x
Emphysema/Bro		21.	74.	15.		10	2	22.	76.	14.		
nchitis	89	81	92	81	35	2	6	61	21	81	50	94
		8.3	75.	25.		10	1		67.	29.		
Other Illnesses	34	3	5	54	10	3	0	8.7	29	49	15	90
		6.6	80.	13.				4.3	80.	4.2		
Cancer	27	2	88	25	48	95	5	5	2	1	73	83
Parkinson's		3.1	85.	8.2				3.4	79.	1.5		
Disease	13	9	08	4	65	95	4	8	67	3	78	81
		3.6		5.6				1.7				
Dementia	15	8	86	6	78	94	2	4	90	NA	90	90
		3.6	85.	3.3				0.8				
Depression	15	8	23	5	80	90	1	7	94	NA	94	94
		3.1	85.	11.				2.6	81.	14.		
Anxiety	13	9	82	92	60	98	3	1	33	01	70	97
Independence and Cognitive Score												
			65.	37.		10			69.	37.		10
Barthel Score [c]			93	66	0	0			52	14	0	0
Cognitive Score			28.	10.					27.	11.		
[c]			85	33	0	34			65	79	0	34

Abbreviations: Variable/Disease, analyzed variable/disease; N = number of LLIs affected/carrying the disease/trait; %, percentage of LLIs carrying the disease/trait; Mean, SD, Min, Max: mean, standard deviation, minimum, maximum of the age at onset for each disease.

[a] Overweight condition: Body Mass Index (BMI) > 25. BMI has been calculated as Weight (kg) / (height (m) x height (m))

[b] Peripheral Vascular Disease Circulatory Problems

[c] *Mean, standard deviation, minimum, maximum of the Barthel and Cognitive Scores distribution are reported instead of mean, standard deviation, minimum, maximum of the age at onset.*

The control populations were constituted by a German and a US cohort. The German sample comprised 1,628 LLIs (age range, 95–110 years; mean age, 98.8 years) and 1,104 younger controls (age range, 60–75 years old; mean age, 66.8 years), and was first described by Nebel et al[20]. The study protocol was approved by the Ethics Committee of University Hospital Schleswig–Holstein (Campus Kiel), Germany, and local data protection authorities. The US-American study sample consisted of 1,461 LLIs with an age range of 91–119 years (mean age, 100.8 years) and 526 controls with an age range of 0–35 years (mean age, 28.2 years); these individuals were recruited by Elixir Pharmaceuticals, either directly or through the New England Centenarian Study[87, 88].The study protocol was approved by the Boston Medical Center's Institutional Review Board and by the Western Institutional Review Board.

1.7 Genotype determination and statistical data interpretation

Genotyping was carried out using the Illumina BeadChip 317K. All genotypes were evaluated using a quantitative quality score called GenCall that assigns a score values from 0 to 1 and reflects the proximity within a cluster plot of the intensities of that genotype to the centroid of the nearest cluster. Samples and SNPs showing Call Rate lower than respectively 93% and 95 % as well as SNPs deviating from Hardy Weinberg Equilibrium (HWE) ($p < 1.72 \times 10^{-7}$) or with Minor Allele Frequency (MAF) lower than 0.05 were removed. Principal Component Analysis (PCA) has been

applied on a reduced set of autosomal markers showing r^2 estimates < 0.2, using the default outliers removal threshold (sigma=6) as implemented in Eigenstrat[89]. We looked for evidence of genetic population stratification on a subset of 454 LLIs and 591 young controls by applying PCA: a graphical inspection of the first two principal components revealed genetic homogeneity between LLIs and controls (**Figure 1.3**).

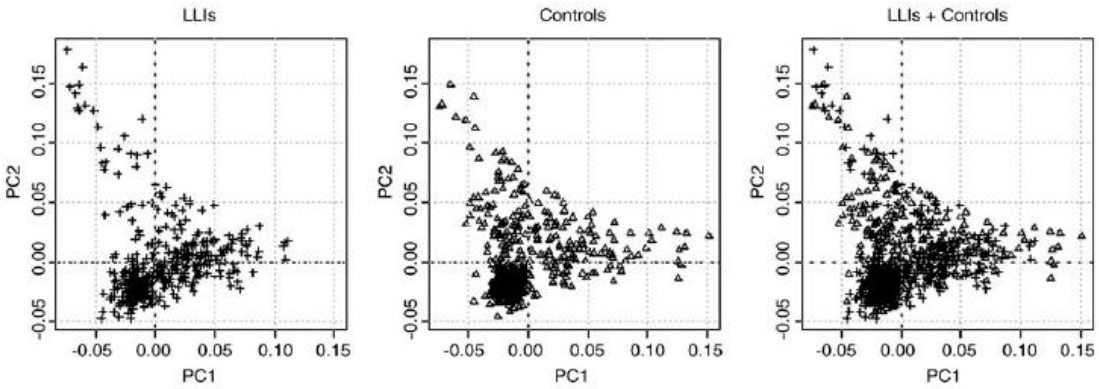


Figure 1.3 Population structure

Population structure of long-living individuals (LLIs) (+) and control subjects (∆). Each scatter plot shows the first two principal components that were estimated using genotype data for Southern Italian Centenarian Study (SICS) LLIs and control subjects using the EigenSoft program. The two populations are ethnically identical, as shown in the right scatter plot.

1.8 rs2070325 in BPIFB4 associates with exceptional longevity in three independent populations

In order to follow up our previous hypothesis-generating genome-wide association study, we designed a two-stage replication effort on four

variations reported among the top findings (P<1x10^{-4}) of that study. To this end, two non-synonymous SNPs — i.e., rs2070325 and rs571391 — and two intronic markers — i.e., rs7583529 and rs285097, which tagged the functional variants rs7917 and rs16955011 (r^2>0.8 in the HapMap CEU panel), respectively — were tested for association in two independent cohorts, the first of which was recruited for the German Longevity Study,[20] and the second for a US-based effort.

Of the four variations tested with TaqMan assays, only rs2070325 — which induces the amino acid change Ile229Val in BPIFB4 (identifier: P59827-2) — replicated the association observed in the screening cohort under the recessive genetic model (OR = 2.42; 95% CI = 1.56–3.77; P = 5.8 x 10^{-5}; power >0.85) in the German Longevity Study (OR = 1.43; 95% CI = 1.12–1.80; P = 0.0036). This variant resulted associated with the longevity phenotype also in the USA replication set (OR = 1.60; 95% CI = 1.14–2.24; P = 0.0063). Meta-analysis of the two populations confirmed this finding (3,060 LLIs and 1,609 controls: OR = 1.49; 95% CI = 1.22–1.81; P= 7.59 x 10^{-5}; power >0.90) and that of the screening and replication sets combined (3,464 LLIs and 2,160 controls; Bonferroni-adjusted significance threshold: P<3.22 x 10^{-7}; OR = 1.61; 95% CI = 1.34-1.92; P= 2.4 x 10^{-7}; power >0.80)

1.9 rs2070325 is associated with a quadruple-SNP haplotype

Haplotype analysis revealed patterns of strong linkage disequilibrium (LD: r^2>0.8, D′>0.9) within the *BPIFB4* genomic locus, delimiting a region highly enriched in non-synonymous SNPs: the rs2070325 variation of *BPIFB4* tagged rs2889732 (Asn281Thr), rs11699009 (Leu488Phe) and rs11696307 (Ile494Thr), codifying respectively for wild-type (WT) (allele frequency, 66%) and longevity-associated variant (LAV) (allele frequency, 29.5%)

isoforms.

BPIFB4 belongs to the superfamily of bactericidal BPI/PLUNC proteins, which are central to the host innate immune response against bacteria in regions of significant bacterial exposure, like the mouth, nose and lungs. The expression of the activity-enhanced polymorphic variant LAV-BPIFB4 might initially produce privileged survival through better resistance to infectious diseases.

1.10 BPIFB4 overexpression induces an adaptive stress response and proteostasis

In order to gain information on the role of BPIFB4 and its LAV in gene expression regulation, we performed genome-wide transcriptional profiling of HEK293T cells transfected with an empty-, WT- or LAV-BPIFB4-encoding vector. BPIFB4 isoforms activated adaptive stress responses and proteostasis, two key aspects for improved organism survival[5] and stem cell maintenance,[90] and potentiated small noncoding RNAs supportive of the spliceosome, genomic integrity machinery and telomere maintenance, indicative of a role of the protein in organism homeostasis

1.11 PERK modulates the complexing of LAV-BPIFB4 with 14-3-3

To further dissect the molecular determinants underlying the mechanism of action of BPIFB4, we analyzed the structure of the protein motifs in its sequence. We identified a protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) substrate motif (amino acids 73–80: EXSXRXXR/EGSIRDLR)[91, 92]. PERK is a known transducer of the unfolded

protein response, reducing endoplasmic reticulum (ER) protein loading through the inhibition of protein synthesis mediated by phosphorylation of eukaryotic translation initiation factor 2 (eIF2)-alpha[93]; thus, a PERK substrate motif on BPIFB4 would indicate a role of BPIFB4 as a downstream effector. Specifically, we observed a reduction of eIF2-alpha phosphorylation upon transfection with BPIFB4. These findings suggest that BPIFB4 is part of a cascade of events orchestrated by PERK aimed at reducing ER stress[94].

Further sequence analysis revealed the presence also of an atypical 14-3-3 binding motif (amino acids 80–86: RXSXXXS/RNSGYRS)[95]. 14-3-3 modulates cell signaling by binding and/or retaining proteins within the cytoplasm based on their phosphorylation status[96].

We next compared LAV-BPIFB4 and WT-BPIFB4 for potential interaction with 14-3-3 *in vitro*. Immunoprecipitation and confocal analyses revealed that LAV-BPIFB4 was mainly localized in the cytoplasm and efficiently formed a complex with 14-3-3, whereas WT-BPIFB4 was mostly nuclear. Cells transfected with LAV-BPIFB4 mutated either at serine 75 (LAV-BPIFB4^{mutPERK}) or at serine 82 (LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$), which is part of the 14-3-3 binding motif, failed to immunoprecipitate 14-3-3, indicating a role of these sites in 14-3-3 recruitment.

Further characterization of LAV-BPIFB4 interactions revealed that it forms a complex with heat shock protein 90 (HSP90), a phenomenon that does not take place in cells transfected with LAV-BPIFB4^{mutPERK} or LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$. Pharmacological inhibition of PERK with GSK2606414 after transfection with LAV-BPIFB4 impeded the immunoprecipitation of 14-3-3 and HSP90. These findings link PERK-mediated phosphorylation of LAV-BPIFB4 to its 14-3-3 binding activity, which is central for the recruitment of HSP90, a known eNOS activator[97].

Of note, WT-BPIFB4 co-immunoprecipitated with HSP90, but not with 14-3-3, as detected by Western blotting. However, WT-BPIFB4$^{mut14\text{-}3\text{-}3}$ failed to

immunoprecipitate with HSP90. This indicates that WT-BPIFB4 has a reduced, rather than no, ability to recruit 14-3-3, which is a step needed for forming a complex with HSP90.

1.12 eNOS is phosphorylated in homozygotic rs2070325 MNCs

Aging is generally associated with a significant reduction in NO bioavailability, which leads to endothelial dysfunction[3]. Based on the previous observations of HSP90 recruitment by BPIFB4, we assessed the effect of the A/A (homozygotic major allele), A/a (heterozygotic allele) and a/a (homozygotic minor allele) genotypes of rs2070325 on the activity status of eNOS in MNCs from healthy blood donors. We found increased eNOS phosphorylation at serine 1177 — an activation site of the enzyme — in a/a carriers[94].

1.13 BPIFB4 is present in the vessel wall and modulates vascular tone

Based on the evidence of enhanced eNOS phosphorylation in MNCs with an rs2070325 genotype, we explored the role of BPIFB4 in the modulation of vascular tone, a process in which NO plays a prominent role[3]. In mouse mesenteric arteries — a typical resistance vessel involved in blood pressure homeostasis — expression of the protein was upregulated upon application of a biomechanical stress (i.e., increased intraluminal pressure) (**Figure 1.13A**). Inhibition of BPIFB4 expression had a detrimental effect on vascular function: indeed, siRNA-mediated knockdown of BPIFB4 induced marked reductions in phenylephrine- and potassium-evoked

vasoconstrictions (**Figure 1.13B**); in addition, acetylcholine-evoked endothelial vasorelaxation was impaired, clearly indicating endothelial dysfunction (**Figure 1.13B**). These effects were associated with the inhibition of eNOS phosphorylation at serine 1177 (**Figure 1.13C**). These findings on eNOS phosphorylation status and vascular tone modulation by BPIFB4 could be related to the role of BPIFB4 in recruiting 14-3-3/HSP90 and facilitating eNOS phosphorylation. So we next investigated the impact of BPIFB4 variants at the vascular level.

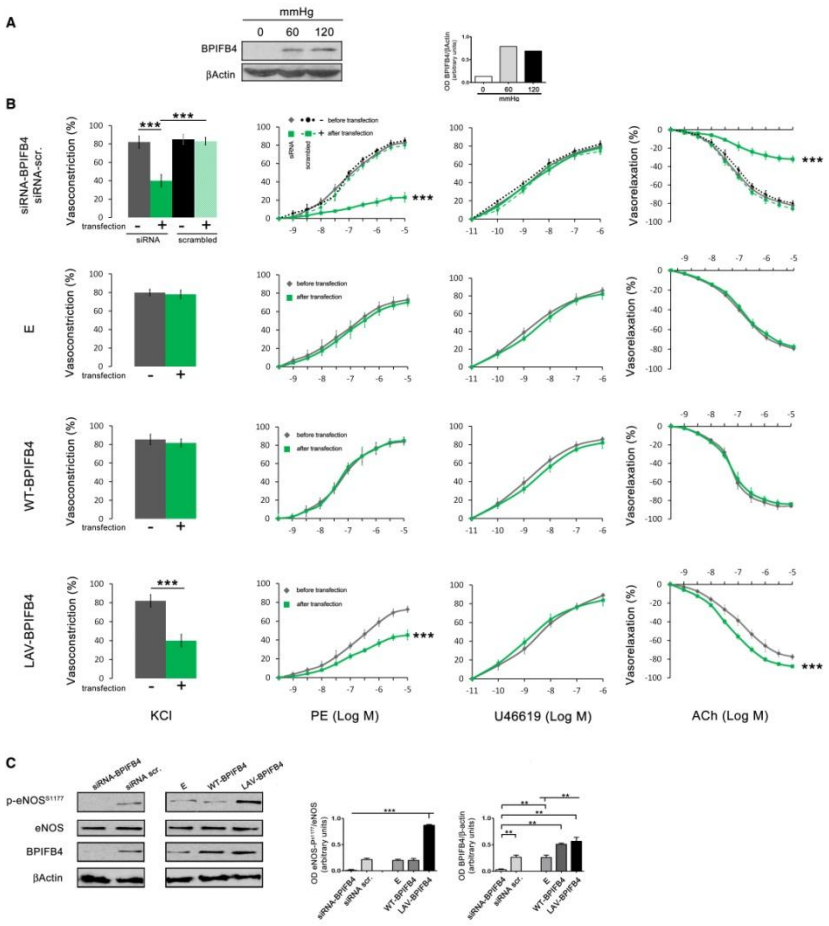


Figure 1.13. Expression of BPIFB4 in perfused vessels and effects of its variants on vascular reactivity and on phosphorylation of eNOS and PKC-alpha in mesenteric arteries. A, *BPIFB4 protein expression in ex vivo mouse mesenteric arteries perfused with increasing pressure levels. The right graph gives quantification of BPIFB4 protein.* ***B,*** *The graphs show, from left to right, the vascular response of ex vivo mouse mesenteric arteries to potassium (80mM KCl), and the dose–responses to phenylephrine (PE), to the thromboxane agonist U46619, and to acetylcholine (ACh). In the first row, the vascular responses are measured before (◆-) and after (■ +) transfection with short interfering (si)RNA for BPIFB4, and before (••-) and after (■ +) transfection with scrambled*

*siRNA. In the consecutive rows, before (-) and after (+) transfection with an empty (E) plasmid, or with wild-type (WT) or the longevity associated variant (LAV) BPIFB4-encoding plasmids. Values are means ± SEM. N=7 experiments per group. Statistics was performed using ANOVA; ***, p<0.001, before vs after transfections.* ***C****, Western blot of seven pooled experiments on ex vivo mouse mesenteric arteries transfected with a BPIFB4 siRNA or a scrambled siRNA or with an empty (E) plasmid, with wild-type (WT), longevity associated variant (LAV) BPIFB4-encoding plasmids. Right graphs show quantification of eNOS phosphorylation and BPIFB4. Values are means ± SEM, N=2 pools of experiments. Statistics was performed using ANOVA; **, p<0.01; ***, p<0.001*

1.14 LAV-BPIFB4 enhances eNOS phosphorylation and endothelial function

We transfected mesenteric arteries *ex vivo* with plasmids encoding a variant tagged with green fluorescent protein (GFP). Overexpression of WT-BPIFB4 did not interfere with vascular reactivity (**Figure 1.13B**), whereas the expression of LAV-BPIFB4 significantly enhanced acetylcholine-evoked vasorelaxation and promoted phosphorylation of eNOS at serine 1177 (**Figure 1.13C**). Of note, acetylcholine-evoked vasorelaxation was more efficiently inhibited with N^G-nitro-L-arginine methyl ester (L-NAME) in LAV-BPIFB4-expressing resistance vessels than in vessels exposed to an empty vector, indicating a higher presence of NO in the former[94].

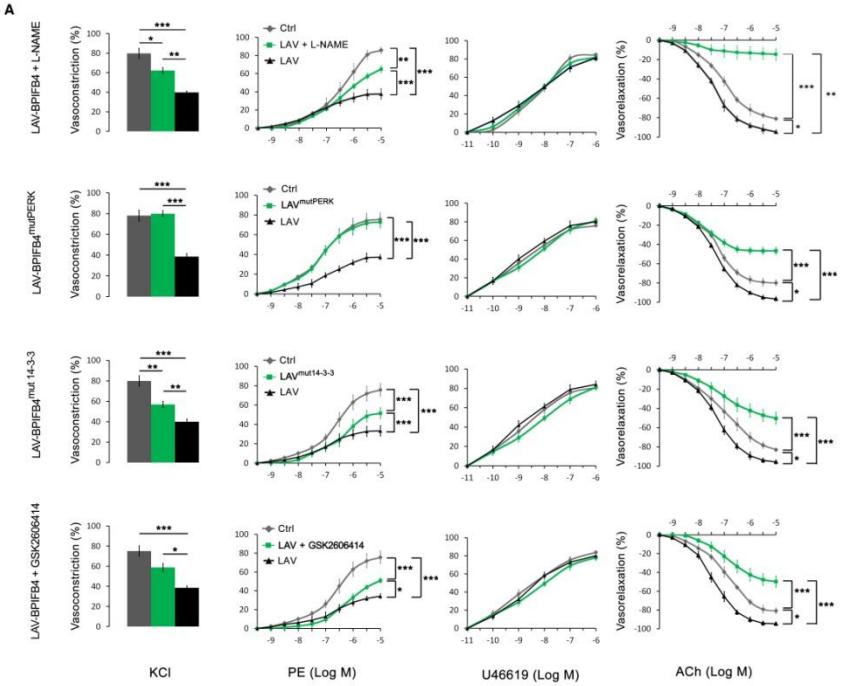
We then assessed the importance of LAV-BPIFB4 phosphorylation and 14-3-3 binding for activation of eNOS. When we transfected mouse mesenteric

arteries *ex vivo* with LAV-BPIFB4^{mutPERK} or LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$, we encountered endothelial dysfunction associated with inhibition of eNOS (**Figure 1.14A** and **1.14B**). Pharmacological inhibition of PERK with GSK2606414 after transfection with LAV-BPIFB4 also resulted in blunted eNOS phosphorylation and impairment of endothelial function (**Figure 1.14A, 1.14B and 1.14C**)[94].

Phosphorylation of BPIFB4 at serine 75 was higher in mesenteric vessels transfected with LAV-BPIFB4 than in those transfected with WT-BPIFB4 (**Figure 1.14D**). Treatment with GSK2606414 reduced the level of phosphorylation at serine 75 on LAV-BPIFB4, as well as at serine 1177 on eNOS, supporting the role of PERK in potentiating the phosphorylation status of LAV-BPIFB4 and eNOS (**Figure 1.14C**). Because mesenteric vessels transfected with WT-BPIFB4$^{mut14\text{-}3\text{-}3}$ also displayed endothelial dysfunction, it is plausible that WT-BPIFB4, similarly to LAV-BPIFB4, requires binding to 14-3-3 for activation of eNOS[94].

Further evidence in support of a role of HSP90 in mediating LAV-BPIFB4's potentiation of eNOS came from LAV-BPIFB4's failure to modulate eNOS/endothelial function upon inhibition of HSP90 with SNX5422 . As a result, we hypothesize a model in which BPIFB4 needs to be phosphorylated by PERK to recruit 14-3-3, a step required to allow a complex to form with HSP90 and, hence, the activation of eNOS; this cascade of events is potentiated in the presence of LAV-BPIFB4.

We also observed apparently paradoxical reductions in potassium- and phenylephrine-evoked vasoconstrictions by LAV-BPIFB4 compared with WT-BPIFB4. This effect was partially rescued by the eNOS inhibitor L-NAME, suggesting that LAV-BPIFB4-mediated enhancement of NO production modulated adrenergic and potassium vascular responses (**Figure 1.14B**)[94].


A
LAV-BPIFB4 + L-NAME
LAV-BPIFB4mutPERK
LAV-BPIFB4mut 14-3-3
LAV-BPIFB4 + GSK2606414
Vasoconstriction (%)
Vasorelaxation (%)
Ctrl
LAV + L-NAME
LAVmutPERK
LAVmut14-3-3
LAV + GSK2606414
LAV
KCl
PE (Log M)
U46619 (Log M)
ACh (Log M)

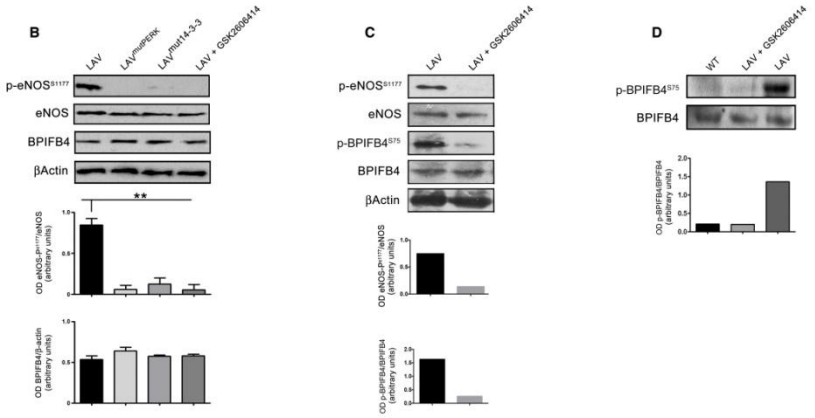

B
C
D
LAV
LAVmutPERK
LAVmut14-3-3
LAV + GSK2606414
WT
p-eNOSS1177
eNOS
BPIFB4
p-BPIFB4S75
βActin
OD eNOS-P1177/eNOS (arbitrary units)
OD BPIFB4/β-actin (arbitrary units)
OD p-BPIFB4/BPIFB4 (arbitrary units)

Figure 1.14. Effect of the NO inhibitor L-NAME, LAV-BPIFB4^{mutPERK}, LAV-BPIFB4$^{mut14-3-3}$, and the PERK inhibitor GSK2606414 on LAV-BPIFB4-mediatedvascular reactivity and on phosphorylation of eNOS in mesenteric arteries. A, *The graphs show, from left to right, the vascular response of ex vivo mouse mesenteric arteries to potassium (80mM KCl), and the dose–responses to phenylephrine (PE), to the thromboxane agonist U46619, and to acetylcholine (ACh), before transfection (◆), after transfection with LAV-BPIFB4 (▲), or after transfection with LAV-BPIFB4 plus L-NAME (300μM) in the first row, LAV-BPIFB4^{mutPERK} in the second row, LAV-BPIFB4$^{mut14-3-3}$ in the third row, and LAV-BPIFB4 plus GSK2606414 (0.5 μM) (■) in the last row. Values are means ± SEM. N=7 experiments per group. Statistics was performed using ANOVA; *, p<0.05; **, p<0.01; ***, p<0.001 before vs after transfection or treatment.* ***B,*** *Western blot of seven pooled experiments on ex vivo mouse mesenteric arteries transfected with LAV-BPIFB4, LAV-BPIFB4^{mutPERK}, LAV-BPIFB4$^{mut14-3-3}$, or LAV-BPIFB4 plus GSK2606414. Graphs show quantification of eNOS phosphorylation and BPIFB4. Values are means ± SEM, N=2 pools of experiments. Statistics was performed using ANOVA; **, p<0.01.* ***C,*** *Western blot of four pooled experiments on ex vivo mouse mesenteric arteries with LAV-BPIFB4 or LAV-BPIFB4 plus GSK2606414. Graphs show quantification of eNOS and BPIFB4 phosphorylation.* ***D,*** *Western blot of four pooled experiments on ex vivo mouse mesenteric arteries with WT-BPIFB4, LAV-BPIFB4 plus GSK2606414, or LAV-BPIFB4. Graphs show quantification of eNOS and BPIFB4 phosphorylation. LAV-BPIFB4, mutated variant; LAV-BPIFB4^{mutPERK}, LAV-BPIFB4 with a Ser75Ala variation; LAV-BPIFB4$^{mut14-3-3}$, LAV-BPIFB4 with a Ser82Asn variation. N=1 pool of experiments.*

1.15 Forced expression of LAV-BPIFB4 in vivo enhances eNOS function and reduces blood pressure

To evaluate the *in vivo* relevance of the findings obtained through plasmid transfection, we generated WT-BPIFB4-, LAV-BPIFB4- and GFP-encoding adeno-associated viral vectors (AAV serotype 9 with a TBG promoter) and used them to transduce normotensive mice through the femoral artery.[98]
We found that AAV-LAV-BPIFB4 enhanced NO-mediated vasorelaxation in femoral and mesenteric arteries explanted after seven days; this effect was associated with potentiation of eNOS phosphorylation and with a reduction of both systolic and diastolic blood pressure. Transduction with AAV-WT-BPIFB4 did not influence NO-mediated vasorelaxation at either the functional or molecular level.
AAV-LAV-BPIFB4 localized throughout the whole vessel, enhancing eNOS phosphorylation.
There were no significant quantitative differences in BPIFB4 from rats receiving AAV-LAV-BPIFB4 or AAV-GFP in serum (507± 92 vs 551±56 pg/mL *P*=0.339) and MNCs (as measured by ELISA (Cusabio) and Western blot, respectively. Thus, we conclude that the effect observed was due to LAV-BPIFB4 expression in the whole vessel[94].

2. AIM OF THE WORK

The scope of this thesis is the disclosure of molecular mechanisms that regulate aging process. In particular, the mechanisms involved in BPIFB4 pathway that we found mutated in three centenarians populations.

A recent paper pointed out that BPIFB4 plays a crucial role in maintenance of vascular homeostasis and that LAV-BPIFB4 exerts extra protection. It shows that biomechanical stress due to increased arterial pressure up-regulated BPIFB4 expression, and that BPIFB4 silencing induced eNOS/endothelial dysfunction.

LAV-BPIFB4 potenciate eNOS and endothelial function, compared to WT-BPIFB4 treated vessels. In agreement, LAV-BPIFB4 showed higher serine 75 phosphorylation and improved ability to bind 14-3-3 as compared to WT isoform.

In order to better clarify the mechanisms involved in LAV-BPIFB4 potentiation of eNOS and endothelial function, here we further investigated additional potential players, focusing our attention on PKC-alpha, which is one of the major PKC expressed in endothelial cells able to regulate eNOS function.

3. MATERIALS AND METHODS

3.1 Ex vivo transfection of mouse vessels and evaluation of vascular reactivity

Second-order branches of the mesenteric arterial tree were removed from C57BL6 mice and transfected as described previously[99]. Briefly, vessels were placed in a Mulvany pressure system filled with Krebs solution supplemented with 20 µg of the pRK5 vector encoding either WT-BPIFB4, LAV-BPIFB4, LAV-BPIFB4^{mutPERK}, or LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$, or with an empty plasmid as a negative control. All vessels were perfused at 100 mmHg for 1 hr and then at 60 mmHg for 5 hrs. Endothelium-dependent relaxations was assessed by measuring the dilatory responses of mesenteric arteries to cumulative concentrations of acetylcholine (from 10^{-9} M to 10^{-5} M) in vessels pre-contracted with U46619 at a dose necessary to obtain a similar level of pre-contraction in each ring (80% of initial KCl-evoked contraction)[79]. The maximal contraction evoked by U46619 was considered as the baseline for subsequent evoked vasorelaxations. Acetylcholine vasorelaxation was tested in the presence of Gö6976 (0.5 µM), a PKC inhibitor. Another experimental series was performed in absence of external Ca^{2+} using Ca^{2+}-free Krebs and in presence of apamin (Sigma-Aldrich), a potent inhibitor of ATP-type Ca^{+2}-activated K^+ channels and charybdotoxin (Sigma-Aldrich), a potent and selective inhibitor of the Ca^{2+}-activated K^+ channel and voltage-gated (Kv1.3) channel. Caution was taken to avoid endothelial damage; the corresponding values are reported as the percentage of lumen diameter change after drug administration. Responses were tested before and after transfection.

3.2 Cell culture and Co-immunoprecipitation

Human embryonic kidney cells (HEK293T) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum and 1% non-essential amino acids at 37° C in a 5% CO2 atmosphere. For co-immunoprecipitation assay 1,4x106 cells were plated in 10cm dish and transfected with pRK5 vector encoding LAV-BPIFB4 or with an empty plasmid6, using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Twenty-four hours post-transfection, HEK293T cells were incubated with PKC-alpha inhibitor Go6976 (0.3 μM) for other twenty-four hours, harvested and solubilized in lysis buffer (20 mMTris-HCl, pH7.5, 650 mM sodium chloride, 500 mM EDTA, 250 mM EGTA, and Triton X-100). The cell lysates were cleared at 13,000 rpm for 20 min at 4ºC and 700 μg protein were incubated overnight with 2 μg of anti-GFP (Invitrogen, mouse mAb), anti-14-3-3 (Abcam, mouse mAb), and anti-IgG (Millipore, mouse pAb) for control. The antibody–antigen complexes were precipitated with protein Glinked Sepharose (GE Healthcare) for 4 hr at 4°C and the beads were washed three times with lysis buffer. The denatured co-immunoprecipitation products were resolved with SDS-PAGE, electro-blotted onto PVDF membranes, and hybridized with anti-14-3-3 (Abcam, rabbit pAb, 1:1000).

3.3 Western blotting

Western blotting was performed on pooled protein extracts from transfected perfused vessels, or from MNCs. Protein extracts were separated on 10% SDS-PAGE at 100V for 1 hr or on 4–12% SDS-PAGE at 100V for 2 hrs and then transferred to a nitrocellulose or PVDF membrane. The membranes were incubated overnight with the following primary antibodies: anti-

phospho-eNOS Ser1177 (Cell Signaling Technology, rabbit mAb, 1:1000), anti-eNOS (Cell Signaling Technology, mouse mAb, 1:800), anti-phospho-BPIFB4 Ser75 (developed by Prof. Annibale Puca in collaboration with Areta International)[100] anti-BPIFB4 (Abcam, rabbit pAb, 1:200), anti-beta-actin (Cell Signaling Technology, mouse mAb, 1:3000), anti-PKC-alpha phospho-T497 (Abcam, rabbit mAb, 1:10000), anti-PKC-alpha (Abcam, rabbit mAb, 1:1000). After a triple wash, membranes were incubated for 1 or 2 hrs with the secondary antibody (Amersham Life Science, horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG, 1:3000). The membranes were then washed four times and specific protein bands were detected with ECL Prime chemiluminescent agents (Amersham Life Science). Western-blot data were analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, USA) to determine optical density (OD) of the bands. The OD readings of phosphorylated proteins were expressed as a ratio relative to total protein or to beta-actin. All other protein expressions were normalized to beta-actin to account for variations in loading.

3.4 Ca^{2+}-transient recordings

HEK293 cells were grown in DMEM (Gibco) supplemented with 10% fetal calf serum. For transfections, 100 µl DMEM was mixed with 2 µl Magnetofection NeuroMag (OZ Bioscience, France) and 1 µg of the *hBPIFB4* cDNA subtype or empty vector. The mixture was incubated for 15–20 min at room temperature and thereafter distributed drop-wise over the culture. Cells were then placed on a magnetic plate (OZ Bioscience) and incubated for 15 min at 37°C. Cells were used for the experiments 48–76 h after transfection. Efficacy of transfection was assessed by

fluorescence microscopy, monitoring the amount of cells expressing vector-encoded GFP.

Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) recordings were obtained by time-resolved digital fluorescence microscopy in HEK cells loaded with the Ca^{2+} indicator X-rhod-1 AM (excitation 550 nm; emission 610 nm)[101] to avoid overlapping of fluorescence signals due to the presence of GFP. Briefly, cells were serum-starved for 2 h and incubated 45 min at 37°C with the cell-permeant X-rhod-1 AM (2 μM). Cells were then placed in standard mammalian Ringer's solution (in mM: NaCl, 140; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 2; Hepes-NaOH, 10; and glucose, 10; pH 7.3), and continuously superfused with a gravity-driven fast-perfusion system (BioLogique 100, France). Most cells were transfected and displayed clear GFP fluorescence that did not interfere with the fluorescent signal of the Ca^{2+} dye X-rhod-1. Ca^{2+} transients were elicited by applying ATP 100 μM for 10 s. The time courses of Ca^{2+} transients were quantified by measuring at each time point the fluorescence emission in each cell, and then transforming the obtained values as $\Delta F/F = [F(t)-F(0)] / F(0)$. The overall ATP-induced Ca^{2+} mobilization was evaluated as $Q\text{-}Ca^{2+}_{6min} = \int \Delta F/F dt$, calculated 6 min from ATP application. All cells in the optical fields were averaged, given that a detailed analysis discriminating GFP-positive and -negative cells yielded no statistical difference between the two subsets (not shown), indicating that HEK293 extensive electrical coupling[102] underlies the Ca^{2+} response.

3.5 Immunofluorescence and confocal microscopy

HEK293T grown in multichamber slides were transfected with pRK5 vector encoding WT- or LAV-BPIFB4 or with an empty plasmid. Twenty-four hours post-transfection, the cells were fixed in 4% Para formaldehyde in PBS for 20 min, washed twice in 50 mM NH4Cl in PBS, and permeabilized for 5 min in 0.2% Triton X-100 in PBS. Fixed cells were treated as described[103]. Immunofluorescence analysis was performed at a confocal laser-scanning microscope LSM 510 Meta (Zeiss, Gottingen, Germany). The λ of the two HeNe lasers was set at 543 and at 633 nm. Fluorescence emission was revealed by 560–615 band pass filter for Alexa Fluor 546, and by 615 long pass filter for DRAQ5. Double-staining immunofluorescence images were acquired separately in the red, and infrared channels at a resolution of 1024 x 1024 pixels, with the confocal pinhole set to one Airy unit and then saved in TIFF format.

3.6 Statistical analyses

Vessel reactivity results are given as mean ± standard error of mean (SEM). Data were analyzed with Student's t-test or two-way ANOVA followed by Bonferroni post-hoc analysis, as appropriate, using dedicated software (GraphPad Prism Software, version 5.0). Densitometry data were analyzed with Student' s *t*-test or two-way ANOVA, as appropriate, using a dedicated software (GraphPad Prism Software, version 5.0). Unpaired Student' s t-test was used to compare the mean (± SEM) of the OD of MNC data about the wild-type, homozygous and the heterozygous carriers of the rs2070325 variation.

4. RESULTS

4.1 LAV-BPIFB4 activates PKC-alpha.

We previously reported that LAV-BPIFB4 enhanced acetylcholine evoked nitric oxide vasorelaxation94. Acetylcholine-evoked vasodilation of isolated mesenteric vessels has been reported to require an intact PKC-alpha activity[104]. Here, we found that PKC-alpha was more phosphorylated (at threonine [497], an activation site of the enzyme) in LAV-BPIFB4-expressing vessels than in those treated with an empty or WT-vector **(Figure 1A).** Treatment with the PKC-alpha inhibitor Gö6976 significantly blunted the acetylcholine-induced vasorelaxation in control vessels (Figure 2A) and abolished both enhanced eNOS phosphorylation and endothelial vasorelaxation of LAV-BPIFB4-expressing vessels (Figure 2B-C) Based on these results we can assert that PKC-alpha is recruited by LAV-BPIFB4 to modulate eNOS and vascular tone.

4.2 BPIFB4 isoforms modulate Ca^{2+} and PKC-alpha membrane translocation

Based on the modulatory actions of BPIFB4 on vascular function and on PKC-alpha activity, which are well-known Ca^{2+}-dependent processes,[104] we investigated how agonist-induced Ca^{2+} mobilization was influenced by the expression of these proteins. Thus, we studied the ATP-induced Ca^{2+} transients in HEK293T cells transfected with different BPIFB4 isoforms.
The expression of WT-BPIFB4 promoted a slight increase in Ca^{2+} influx, with an enhancement of the mean Ca^{2+} mobilization and a higher number of responding cells to ATP stimuli compared to empty vector (**Figure 3A-B-**

C). The expression of LAV-BPIFB4 further and strongly enhanced the percentage of responding cells and Ca^{2+} mobilization (**Figure 3A-B-C**), indicating a facilitation of agonist-induced Ca^{2+} release. Fluorescence analyses of HEK293T cells revealed that only—after LAV-BPIFB4 transfection there is an increase of PKC-alpha localized to the plasma membrane -a known hallmark of activated PKC-alpha[105]- compared to empty or WT-BPIFB4 transfected cells (**Figure 3D)**. PKC-alpha activation occurs in all cells, despite only a fraction of them were efficiently transfected with LAV-BPIFB4, further supporting a role of Ca^{2+} that is known to be able to propagate among cells through tight junctions.[106]
Taken together, these data clearly suggest that LAV-BPIFB4 is able to enhance Ca^{2+} influx leading to PKC-alpha activation.

4.3 LAV-BPIFB4 fails to modulate PKC-alpha and eNOS in absence of external Ca^{2+}

Acetylcholine induced eNOS phosphorylation requires Ca^{2+} influx.[104] To clarify the role of Ca^{2+} in LAV-BPIFB4 vascular action we performed vascular reactivity studies in mesenteric arteries in absence of external Ca^{2+}. In external Ca^{2+}-free condition, LAV-BPIFB4 was hypo-phosphorylated and not able to enhance PKC-alpha and eNOS phosphorylation **(Figure 4A).** In this experimental setting, LAV-BPIFB4 was still able to enhance endothelial vasorelaxation **(Figure 4B).** It is well know that in addition to NO, endothelium generates other mediators involved in the regulation of vascular tone and among these endothelium-derived hyperpolarizing factors (EDHF) plays a prominent role.[107] Thus, we aimed to inhibit EDHF release using apamin plus charybdotoxin. In particular, we showed that during the inhibition of EDHF release, in absence of external

Ca^{2+}, acetylcholine evoked vasorelaxation was abolished and LAV-BPIFB4 failed to enhance endothelial vasorelaxation (**Figure 4C**). Taken together our data demonstrate that external Ca^{2+} mobilization is necessary to LAV-BPIFB4 to activate PKC-alpha and eNOS signalling, while in its absence LAV-BPIFB4 up-regulate additive mechanisms (EDHF) to enhance endothelial function.

Our conclusion is supported by experiments performed in eNOS knockout murine vessels that demonstrated that LAV-BPIFB4, in absence of eNOS signalling, was still able to enhance endothelial vasorelaxation, again blunted by apamin plus charybdotoxin (**Figure 4D**).

4.4 Feed-forward mechanism of PKC-alpha vascular action

We have previously showed that BPIFB4 phosphorylation by stress kinase PERK at serine 75 induced its binding to 14-3-3. The phosphorylation at serine 75 and the 14-3-3 binding was higher in LAV-BPIFB4 than in WT-BPIFB4 and its phosphorylation and 14-3-3 binding status correlated with its ability to activate eNOS and endothelial function[94] As further support of the role of phospho-BPIFB4 and BPIFB4-14-3-3 binding in eNOS activation, mutagenesis of the serine 75 (LAV-BPIFB4^{mutPERK}) and the serine in the 14-3-3 binding site (LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$) or pharmacological treatment with GSK2606414, a PERK inhibitor, showed a blunted eNOS phosphorylation at Ser1177 (**Figure 5A**) and reduced acetylcholine endothelial vasorelaxation associated with a loss of 14-3-3 immunoprecipitation[94]. Overall, these data indicate that BPIFB4 is crucial for eNOS activation, which is activated by phosphorylation of BPIFB4 at serine 75 and 14-3-3 binding.

A detailed amino acid sequence analysis showed that serine 75 of BPIFB4 is also a potential phosphorylation substrate motif for PKC-alpha (amino acids 73-75: SXR/SIR).

Thus, we hypothesized that PKC-alpha contributes to eNOS activation also through BPIFB4 phosphorylation. Here, we demonstrate that treatment with the PKC-alpha inhibitor Gö6976 significantly reduced LAV-BPIFB4 phosphorylation at serine 75 and eNOS phosphorylation as compared to vessels treated with LAV-BPIFB4 alone (**Figure 2C**), indicating that PKC alpha activation is needed for LAV-BPIFB4 phosphorylation and eNOS activation. Finally, in presence of PKC-alpha inhibitor, LAV-BPIFB4 lost its ability to co-immunoprecipitate 14-3-3 in vitro, as a consequence of its hypo-phosphorylation (**Figure 5B**), further supporting the role of PKC-alpha in modulating serine 75 phosphorylation and the dependent 14-4-3 binding.

To further characterize PKC-alpha/LAV-BPIFB4 interaction, we evaluated PKC-alpha phosphorylation in mesenteric vessels transfected with LAV-BPIFB4^{mutPERK}, LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$, or with LAV-BPIFB4 plus GSK2606414. Surprisingly, in this experimental setting, while eNOS phosphorylation was diminished, PKC-alpha signaling was still activated (**Figure 5A**).

Overall, these data, together with the described LAV-BPIFB4 activation of Ca^{2+} and PKC-alpha, with the latter inhibited in Ca^{2+}-free conditions, suggest that LAV-BPIFB4, enhancing Ca^{2+} influx, leads to PKC-alpha activation through alternative mechanisms that do not require serine 75 phosphorylation.

Thus, we envision a model in which Ca^{2+}/PKC-alpha (and PERK) allow hyper-phosphorylation of LAV-BPIFB4, increase of its binding to 14-3-3, HSP90 (as previously demonstrated)[94] and eNOS interaction enhancing its phosphorylation by PKC-alpha.

4.5 PKC-alpha is phosphorylated in MNC of LLIs

Our data obtained in experimental models candidate PKC-alpha as a crucial signalling molecule recruited by LAV-BPIFB4 to enhance endothelial nitric oxide release. Interestingly, mononuclear cells from homozygous-rs2070325 individuals had significantly increased phosphorylation of PKC-alpha at threonine 497 (Figure 6A-B), thus confirming the signalling recruited by LAV-BPIFB4, identified in experimental models, also in human cells.

5. DISCUSSION

BPIFB4 belongs to the superfamily of bactericidal BPI/PLUNC proteins, which are central to the host innate immune response against bacteria in regions of significant bacterial exposure, like the mouth, nose and lungs. The expression of the activity-enhanced polymorphic variant LAV-BPIFB4 might initially produce privileged survival through better resistance to infectious diseases. The variant may offer additional advantages because of its ability to activate the nitric oxide (NO)-releasing enzyme eNOS. NO and peroxynitrite, which is generated by the interaction of NO with $O2^-$ during the respiratory burst, are mediators of the bactericidal effects of macrophages through inactivation of heme-containing enzymes, including cytochrome oxidases and cytochrome P450. Interestingly, development and refining of this prosurvival mechanism has facilitated exceptional longevity by ensuring better adaptation to stress conditions through improved function of ribosomal biogenesis, protein synthesis and cardiovascular homeostasis[94].

Endothelial dysfunction is a systemic pathological state characterized by a reduction in the bioavailability of and/or responsiveness to vasodilators, and altered vascular wall metabolism. It is well known that cardiovascular risk is markedly increased in aged individuals. Because of its major causal role in cardiovascular diseases, such as hypertension and ischemia, therapeutic agents that restore endothelial function are of clinical interest, expecially in the context of aging. In this context, a body of evidence pinpoints eNOS as a major player in blood pressure homeostasis and eNOS downregulation as a hallmark of cardiovascular disease[108]. A recent study evaluated the effect of LAV-BPIFB4 in a rat model of essential hypertension characterized by impaired endothelial function[94]. The *in vivo* administration of LAV-BPIFB4 normalized blood pressure levels and endothelial function in genetically hypertensive rats, without influencing vascular remodeling.

The same study showed the association of dysfunctional endothelial-dependent vasorelaxation, reduced eNOS phosphorylation and BPIFB4 downregulation in old mice. Importantly, it demonstrated that forced BPIFB4 expression by LAV-BPIFB4 restores endothelium-dependent vasorelaxation, eNOS phosphorylation and reduces blood pressure levels in old mice[94].

The main finding of my thesis is that LAV-BPIFB4 is able to enhance NO production trough PKC-alpha signaling activation. In HEK293 cells, LAV-BPIFB4 *per se* evokes Ca^{2+} mobilization and PKC-alpha translocation on plasma membrane, a step necessary for its activation[105]. The removal of external Ca^{2+} from vessels abolishes the ability of LAV-BPIFB4 to enhance both PKC-alpha and eNOS phosphorylation, although it is still able to enhance endothelial vasorelaxation which depends on its action on EDHF signaling.

Mammalian PKC-alpha consists of 672 amino acids and is distributed in all tissues, in contrast to other PKC proteins whose expression is restricted in the particular cell types. PKC-alpha is activated by a variety of signals, including signals binding to guanine-nucleotide-binding protein-coupled receptors and to tyrosine kinase receptors, and also physical stresses like hypoxia and mechanical strain.

Diacylglycerol (DG) and Ca^{2+} increased in the cell upon stimulation synergistically drive the release of a pseudosubstrate region from the active site, leading to activation. Also, activity of PKC-alpha is regulated by phosphorylation of three conserved residues in its kinase domain: the activation-loop site Thr-497, the autophosphorylation site Thr-638, and the hydrophobic C-terminal site Ser-657. PKC-alpha exhibits almost no activity without phosphorylation at these sites. After stimulation, PKC-alpha moves from cytosol to a so-called particulate fraction. This movement was first identified by western blotting using a specific antibody against PKC-alpha after fractionation of cells and later by immunofluorescent study. Recently,

new approaches to analyze the localization of the protein in cells, such as fluorescence resonance energy transfer (FRET) and confocal microscopy, provide a tool for identifying the exact location of PKC-alpha.

Stimulation with phorbol myristate acetate (PMA), a non-specific activator o f PKCs, cause the traslocation of PKC-alphato the membrane of the cell where DG is produced[109].

There are at least 10 distinct lipid-regulated isoforms of PKC that can be divided into three categories: conventional Ca^{2+}-dependent (cPKCs), novel Ca^{2+}-independent (nPKCs), and atypical Ca^{2+}- and diacylglycerol/phorbol ester-independent (aPKCs). A comparison of the amino acid sequence of these isoforms points out that the Ca^{2+}-dependent cPKCs contain a conserved C2 domain that is absent in the Ca^{2+}-independent nPKCs and aPKCs. Therefore, it is generally thought that the C2 domain plays a critical role in the Ca^{2+}-dependent activation of cPKCs[110].

Calcium is a ubiquitous second messenger in eukaryotic cells and it's the regulator of a wide variety of signaling pathways such as fertilization, gene transcription, cell division, differentiation, muscle contraction, neuronal signaling, inflammation, and programmed cell death[111, 112].

Especially, intracellular Ca^{2+} is an important second messenger in endothelial cells. It acts to link external stimuli with the synthesis and release of the regulatory factors in endothelial cells. However, excessive intracellular Ca^{2+} in endothelial cells is detrimental. Because $[Ca^{2+}]i$ plays a dual role as the cause of both physiological and pathological events, fine control of $[Ca^{2+}]i$ in endothelial cells is obligatory[113].

The endothelium is the thin inner layer (tunica intima) of a blood vessel regulates the vascular tone by releasing relaxing factors in response to both mechanical stimuli (shear stress and cyclic strain) and soluble agonists (bradykinin and acetylcholine (ACh)[114]. It has been established that the calcium influx through the plasma membrane is one of the proximal

signaling factors in the production of relaxing factors such as nitric oxide (NO) and prostaglandins in response to external stimuli[115].

The endothelial cells provide a pathway for oxygen distribution from the blood to the tissue and also regulate the permeation of various metabolites, macromolecules and autocrine and paracrine factors. In addition, the endothelium has its own functions. To this end, endothelial cells produce many unique molecules, including NO, plasminogen activator inhibitor, adhesion molecules, growth factors and monocyte chemoattractant protein. It is known that endothelial cells secret endothelium-derived relaxing factor (EDRF), EDHF and prostacyclin in response to hormones, chemical signals or mechanical stimuli. The release of EDRF was first discovered by Furchgott in 1980[116], who found that endothelial cells produced EDRF in response to acetylcholine (ACh) stimulation[117]. In 1987, Moncada et al.[118] and Ignarro et al.[119] suggested that the EDRF was NO. Nitric oxide circulates from the endothelial cells to the underlying smooth muscle cells and causes vascular relaxation, which, in turn, modulates blood pressure and blood flow[113].

The $[Ca^{2+}]i$ is the link between external stimuli to the synthesis and release of regulatory factors by endothelial cells. Changes in $[Ca^{2+}]i$ modulate the releasing of dilators agents from endothelial cell . For example, an increase in $[Ca^{2+}]i$ activates endothelial NO synthase (eNOS) to generate NO. Nitric oxide then activates guanylyl cyclases (GCs) to produce cGMP, which itself or via PKG modulates the activity of Ca^{2+} channels and pumps in a feedback manner[114]. A rise in $[Ca^{2+}]i$ also stimulates EDHF release and this release may be partly attributed to the fact that increases in $[Ca^{2+}]i$ activate the small- and intermediate-conductance Ca^{2+}-sensitive K^+ channels in endothelial cells.

In our recent paper we have demonstrated that LAV-BPIFB4 potentiated eNOS and endothelial function as compared to WT-BPIFB4 treated vessels and that serine 75 phosphorylation of BPIFB4 by PERK is fundamental for

eNOS activation and endothelial function.[100] In agreement, LAV-BPIFB4 showed higher serine 75 phosphorylation and improved ability to bind 14-3-3 as compared to WT isoform[94].

In order to better clarify the mechanisms involved in LAV-BPIFB4 potentiation of eNOS and endothelial function, here we further investigated additional potential players, focusing our attention on PKC-alpha, which is one of the major PKC expressed in endothelial cells able to regulate eNOS function.[115, 120]

PKC-alpha stimulates NO production in endothelial cells by increasing phosphorylation of Ser^{1179} while having no effects on Thr^{497} of eNOS.

Pretreatment of bovine aortic endothelial (BAEC) cells with Gö6976, an inhibitor of calcium-dependent PKC inhibits FGF-2 induced eNOS- Ser^{1179} phosphorylation[115].

Here we showed that PKC-alpha inhibitor Gö6976 impeded LAV-BPIFB4 potentiation of eNOS and endothelial function, thus positioning PKC-alpha between LAV-BPIFB4 and eNOS. To be noted, transfection of vessels with LAV-BPIFB4^{mutPERK}, and with LAV-BPIFB4$^{mut14\text{-}3\text{-}3}$, or with LAV-BPIFB4 plus PERK inhibitor didn't interfere with LAV-BPIFB4 ability to potentiate PKC-alpha although blunted eNOS activation. These data indicate that PKC-alpha activation is not dependent on LAV-BPIFB4 phosphorylation and binding to 14-3-3.

In our experiments in vitro, we showed that WT- and LAV-BPIFB4 both improved mobilization of Ca^{2+}, with the latter being more efficient than the former. This effect of LAV-BPIFB4 is associated with higher amount of PKC-alpha translocation on plasma membrane and the data obtained in vessels, in external Ca^{2+}-free condition, clearly demonstrate that external Ca^{2+} is crucial for the mechanisms recruited by LAV-BPIFB4 to enhance eNOS signaling, including PKC-alpha activation.

To be noted, serine 75 of BPIFB4, which is a phosphorylation motif for PKC[121] (amino acids 73-75: SXR/SIR) is hypo-phosphorylated by PKC-

alpha inhibitor, indicating that PKC-alpha is also upstream of BPIFB4, as also shown by the loss of LAV-BPIFB4 ability to immunoprecipitate 14-3-3, which is dependent on serine 75 phosphorylation, after PKC-alpha inhibition in vitro.

Overall, these data suggest that of LAV-BPIFB4, modulating Ca^{2+} influx, leads to PKC-alpha activation that in-turn increases phosphorylation in serine 75 of BPIFB4. The consequence of this hyper-phosphorylation results in the improvement of LAV-BPIFB4 binding to 14-3-3 and HSP90[100] that can interacts with eNOS, thus enhancing its phosphorylation by PKC-alpha (**Figure 7**).

However, a careful analysis of our data show that in presence of LAV-BPIFB4 and absence of external Ca^{2+} in vessels, although PKC-alpha and eNOS phosphorylation are blunted, there was still enhancement of endothelial vasorelaxation. It is well known that during eNOS dysfunction, EDHF substitutes for nitric oxide[122, 123]. In agreement, we demonstrated the enhancement of endothelial vascular effect of LAV-BPIFB4 under the above conditions is mediated by a recruitment of EDHF.

To definitively clarify the concept that LAV-BPIFB4 is able to recruit alternative mechanisms to protect endothelial function in absence of eNOS, we performed experiments in eNOS deficient mice. Also in this experimental model we showed an EDHF-release dependent LAV-BPIFB4 enhancement of endothelial vasorelaxation. More studies needed to clarify the mechanism involved in LAV-BPIFB4 action on EDHF release, an experimental condition present only in absence of eNOS signaling (**Figure 7**).

Our data highlight Ca^{2+} mobilization and PKC-alpha activation as key signals necessary for LAV-BPIFB4 to enhance endothelial NO release. To be noted, LAV-BPIFB4 activation of PKC-alpha was shown also in HEK293T cells and in humans MNCs with a a/a homozygous genotype for rs2070325 (LAV).

These data corroborate the observation made on vessels and indicate a more broader role of LAV-BPIFB4 in modulating other functions where PKC-alpha is involved, such as control of stem cell maintenance, development, differentiation, functions that are lost during ageing and that could be preserved in long-living individuals.[124,125] Our further characterization of the molecular determinants of LAV-BPIFB4 protective effects puts the foundation to investigate on it's a possible translation in clinical field.

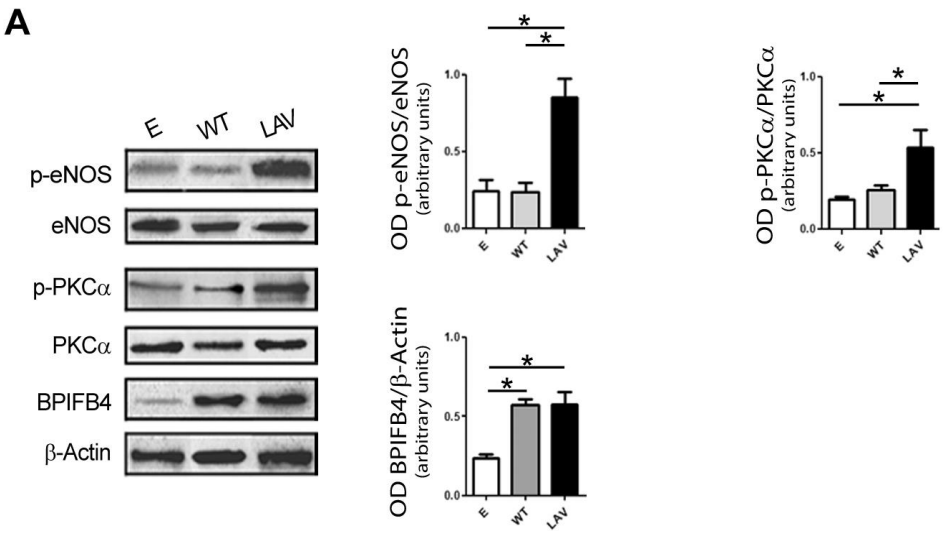


Figure 1. *Western blot of 7 pooled experiments on ex vivo C57BL/6 mouse mesenteric arteries transfected with Empty vector (E), Wild-type BPIFB4 (WT), LAV-BPIFB4 (LAV) vectors. Right graphs show quantification of p-eNOS (S1177), p-PKC-alpha (T497) and BPIFB4. Values are means±SEM, n =3 pools of experiments. Statistics was performed using ANOVA; *p<0.05.*

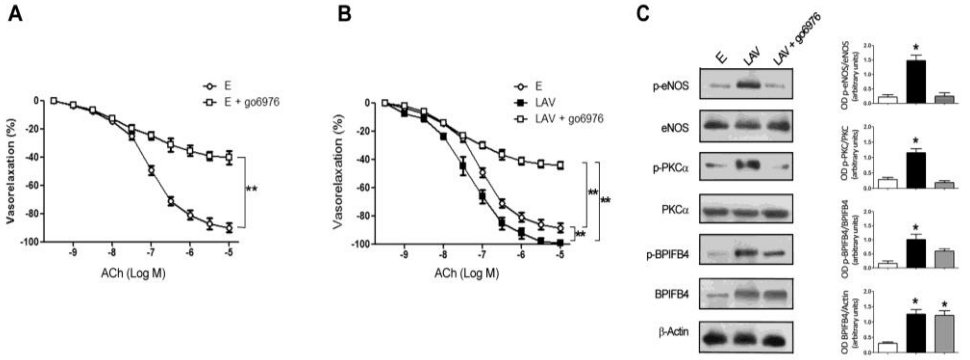


***Figure 2. A**, Dose–response curve to acetylcholine in ex vivo C57BL/6 mouse mesenteric arteries transfected with empty vector (E) either untreated or treated with the PKC-alpha inhibitor Gö6976; **B**, Dose–response curve to acetylcholine in ex vivo C57BL/6 mouse mesenteric arteries transfected with Empty vector (E), or with LAV-BPIFB4 (LAV) untreated or treated with the PKC-alpha inhibitor Gö6976. Values are means±SEM. n =5 experiments per group. Statistics was performed using ANOVA; ** p<0.01.**C**, Western blot of seven pooled experiments on ex vivo mouse mesenteric arteries from control mice, transfected with Empty vector, with LAV-BPIFB4 or with LAV-BPIFB4 plus PKC-alpha inhibitor (Gö6976). Right graphs show quantification of p-eNOS (S1177), eNOS, p-PKC-alpha (T497), PKC-alpha, p-BPIFB4 (S75), BPIFB4 and Actin. Values are means ± SEM, n=3 experiments. Statistics was performed using ANOVA; * p<0.05*

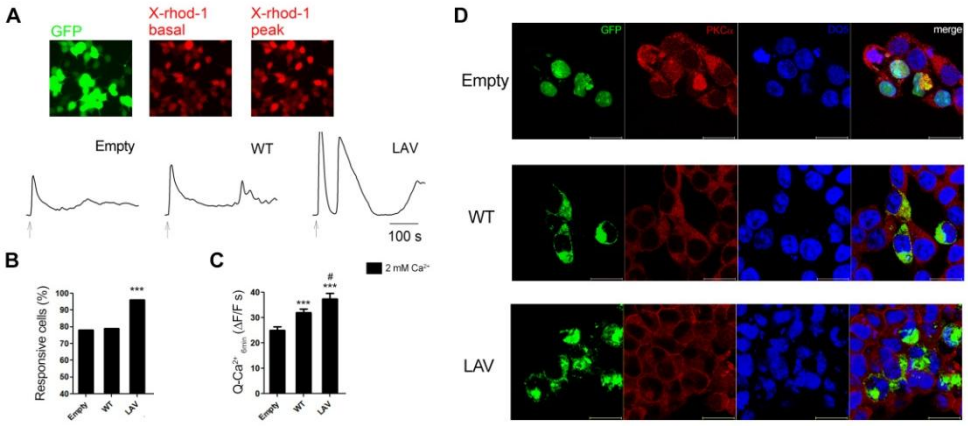


***Figure 3. A**, top, Digital fluorescence images of the same HEK293T cells showing expression of GFP after transient transfection with control GFP-encoding vector (left, 480 nm excitation, 510 nm emission), X-rhod-1 fluorescence in basal [Ca^{2+}]i conditions (middle, 550 nm excitation, 610 nm emission), and at [Ca^{2+}]i peak (right). Bottom, time-courses of [Ca^{2+}]i changes elicited by 100 μM ATP (arrows, 2-second application), in individual HEK293T cells transiently transfected with empty vector (Empty), WT, LAV isoforms as indicated, in the presence of 2 mM external Ca^{2+}. **B**, Histogram representing the percentage of cells responding with a detectable Ca^{2+} transient to ATP application. Values are averaged from empty, WT- and LAV-transfected cells in the presence of 2 mM external Ca^{2+}(n=454, 589, 530, and 474, respectively). Statistics was performed with χ^2 test; *, p< 0.05; ***, p< 0.001. **C**, Histogram of Q-Ca^{2+}6min values (i.e. ΔF/F time integral, see methods), representing Ca^{2+} mobilization in the first 6 min after ATP application. Values are averaged from empty, WT- and LAV-transfected cells in the presence of external Ca^{2+}. Statistics was performed using ANOVA; ***, P < 0.001; #, p< 0.05. **D**, Subcellular localization of PKC-alpha in transfected HEK293T cells. PKC-alpha (red) was mainly cytosolic in HEK293T cells transfected with an empty vector (Empty, green); with a plasmid encoding wild-type-BPIFB4 (WT, green); in*

contrast, PKC-alpha was located mainly on the plasma membrane in HEK293T cells forced to express LAV-BPIFB4 variant (LAV, green), which is a clear hallmark of PKC-alpha activation. Nuclei (blue) were stained with DRAQ5.

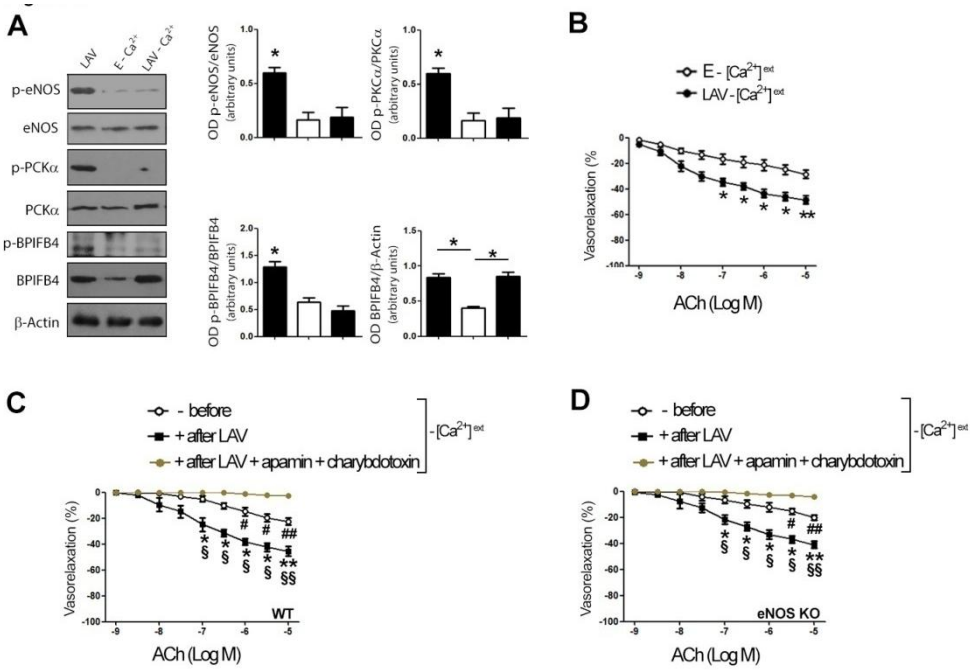


Figure 4. A, *Western blot of 7 pooled experiments on ex vivo C57BL/6 mouse mesenteric arteries transfected with LAV-BPIFB4 in presence of Ca^{2+} (LAV); with empty vector in absence of external Ca^{2+} (E - Ca^{2+}) or with LAV-BPIFB4 in absence of external Ca^{2+} (LAV - Ca^{2+}). Values are means ± SEM, n=3 experiments. Statistics was performed using ANOVA; *, p<0.05.* ***B****, Dose–response curve to acetylcholine in ex vivo C57BL/6 mouse mesenteric arteries transfected with Empty vector (E) or with LAV-BPIFB4 (LAV) in absence of external Ca^{2+} (-[Ca^{2+}]ext). Values are means ± SEM, n=3 experiments. Statistics was performed using ANOVA; *, p<0.05; **, p<0.01.* ***C****, Dose–response curve to acetylcholine in ex vivo C57BL/6 wild-type mouse mesenteric arteries (WT) in absence of external Ca^{2+} before (basal condition) and after transfection with LAV-BPIFB4 (LAV) or LAV-BPIFB4 plus apamin and charybdotoxin. Values are means ± SEM, n=4 experiments. Statistics was performed using ANOVA *, p<0.05; **, p<0.01 after LAV + apamin + charybdotoxin; #, p<0.05; ##, p<0.01 vs. after LAV +*

apamin + charybdotoxin; §, p<0.05; §§, p<0.01 vs. before. ***D****, Dose–response curve to acetylcholine in ex vivo mesenteric arteries from eNOS knockout mouse (eNOS KO) in absence of external* Ca^{2+} *before and after transfection with LAV-BPIFB4 (LAV) or LAV-BPIFB4 plus apamin and charybdotoxin. Values are means ± SEM, n=4 experiments. Statistics was performed using ANOVA; *, p<0.05; **, p<0.01 vs. before; #, p<0.05; ##, p<0.01 vs. after LAV + apamin + charybdotoxin; §, p<0.05; §§, p<0.01 vs. after LAV + apamin + charybdotoxin.*

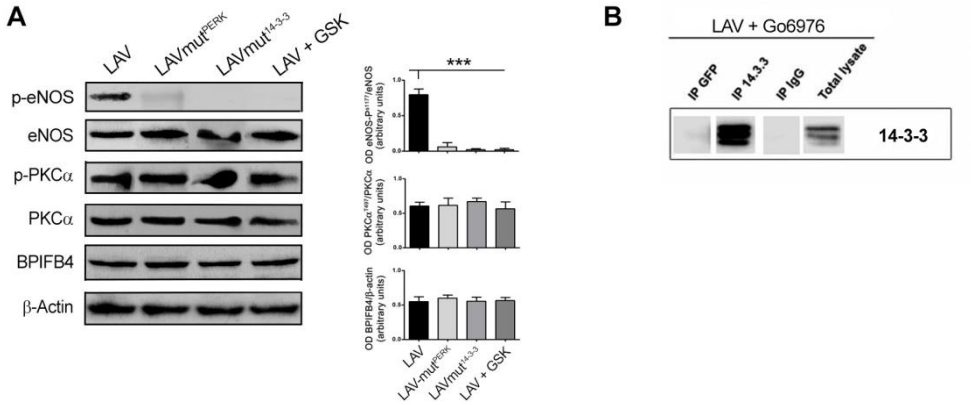


***Figure 5. A**, Western blot of 7 pooled experiments on ex vivo mouse mesenteric arteries transfected with LAV-BPIFB4, LAV-BPIFB4mutPERK (Ser75Ala variation), LAV-BPIFB4mut14-3-3 (Ser82Asn variation) or LAV-BPIFB4 plus GSK2606414 (PERK inhibitor). Right graphs show quantification of p-eNOS (S1177), p-PKC-alpha (T497), and BPIFB4. Values are means ± SEM, n=3 experiments. Statistics was performed using ANOVA; ***, p<0.001. **B**, Co-immunoprecipitation of BPIFB4 with 14-3-3 in extracts of HEK293T cells overexpressing LAV-BPIFB4 tagged with GFP protein and treated with PKC-alpha inhibitor Gö6976. Immunoprecipitation was performed with anti-GFP (directed toward LAV-BPIFB4-GFP), anti-14-3-3 and anti-IgG (as negative control) antibody followed by immunoblotting with anti-14-3-3.*

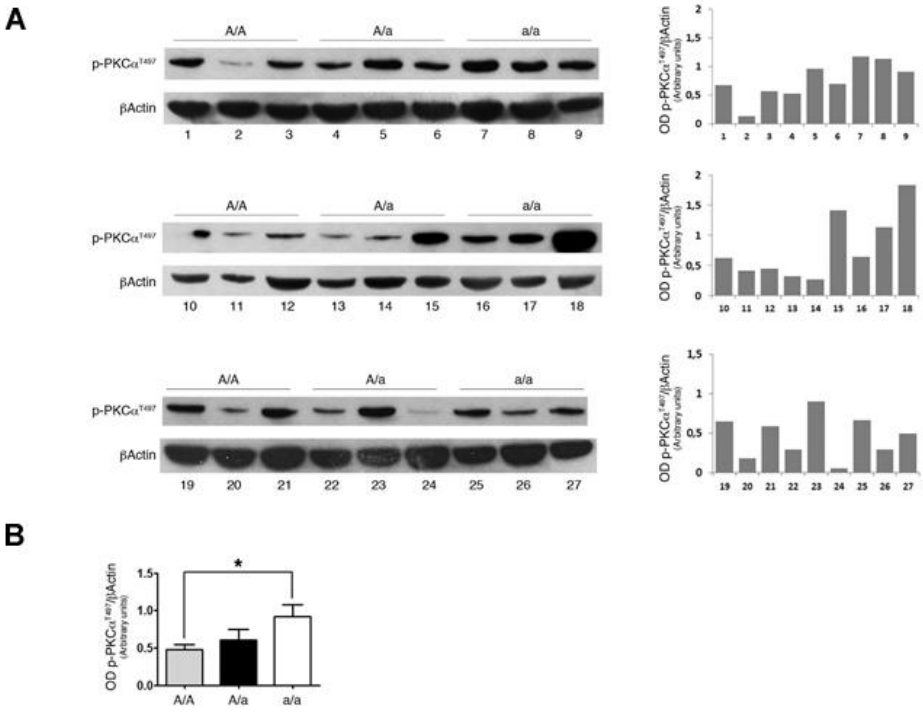


***Figure 6**. **A**, Evaluation of PKC-alpha activation in subjects carrying mutations on BPIFB4.a, Phosphorylation at threonine 497 of PKC-alpha in ex vivo mononuclear cells (MNC) of subjects A/A (n=9), A/a (n=9), or a/a (n=9) for rs2070325. The plots on the right show the OD ratios between phosphorylated PKC-alpha and beta-actin. **B**, Plot of the average values. Statistics was performed with Student's t-test comparing homozygous carriers; *, p< 0.05.*

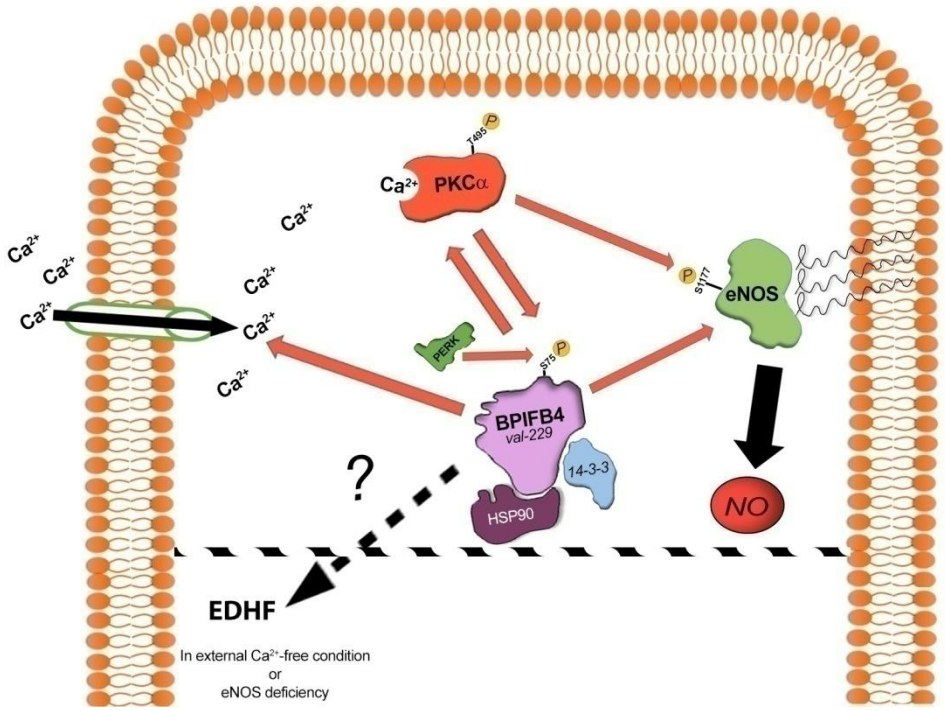


***Figure 7**. Representative mechanisms recruited by LAV-BPIFB4 to modulate eNOS. LAV-BPIFB4, modulating Ca^{2+} influx, leads to PKC-alpha activation that in-turn increases phosphorylation in serine 75 of BPIFB4. The consequence of this hyper-phosphorylation results in the improvement of LAV-BPIFB4 binding to 14-3-3 and HSP90 that can interacts with eNOS, thus enhancing its phosphorylation by PKC-alpha. Alternatively, in external Ca^{2+}-free condition or in absence of eNOS enzyme LAV-BPIFB4 is able to stimulate EDHF signaling. BPIFB4 val-229 = LAV-BPIFB4; PKCα = Protein kinase C-alpha; HSP90 = heat shock protein 90; eNOS = endothelial nitric oxide synthase; NO = nitric oxide; EDHF = Endothelium-derived hyperpolarizing factors; Ca^{2+} = Calcium ion.*

6. WORK IN PROGRESS AND FUTURE PERSPECTIVES

The way toward pharmacology of aging still follows the perception of the discovery of a mimetic drug for dietary restriction (DR), which is the only recognized efficient way to delay aging.

Indeed, none of the alternative anti-aging therapies has been tested in humans, except for caloric restriction. To be noted, we, as others, are interested in health-span more than lifespan, and to this end, it's important to understand when it's better to start a treatment that could reduce or delay the effects of ageing on a particular body district.

The results reported in our recent work, which has been summarized in this thesis, about the impact on endothelial vasorelaxation of the administration of Adeno-Associated Viral vectors carrying the LAV isoform of BPIFB4 in mouse models, encouraged us to start clinical trials. Viral mediated gene therapy could represent a realistic and nearby achievement and maybe it's suitable for a longer and more performing solution of age-related diseases.

The benefits of using AAV for gene therapy include long-term gene expression, the inability to autonomously replicate without a helper virus, transduction of dividing and nondividing cells, and the lack of pathogenicity from wild-type infections.

On the other hand, alternative roads of administration, such as recombinant protein administered orally or intravenous, are better from a pharmacological and industrial prospective but it requires an enormous technical and economic support.

We are going to set up the preliminary characterization of the recombinant protein, expressing LAV-BPIFB4 in mammalian cells as they are the best host for the expression of recombinant vertebrate proteins because they

produce the same posttranslational modifications and recognize the same signals for synthesis, processing, and secretion utilized in the organism from which the sequence was originally derived. Once expressed, the recombinant protein will be purified using Ni-NTA affinity chromatography under native conditions and will be eluted. The quality and purity of LAV-BPIFB4 recombinant protein will be checked.

Human administration must be preceded by the pre-clinical evaluation of the AAV vector and recombinant protein including toxicology, safety, minimal dose administrable, and bio-distribution of the serotype, and these are very long and expensive steps.

Based on the data obtained in my thesis that suggest a more broader role of LAV-BPIFB4 in modulating other functions where PKC-alpha is involved, such as control of stem cell maintenance, development, differentiation, functions that are lost during ageing and that could be preserved in long-living individuals, we envision oa broad anti-aging application for LAV-BPIFB4.[124,125]

Indeed, BPIFB4 is a multitasking protein involved in processes that are important for cellular functions, whose role in modulating eNOS is potentiated by the variations harbored by the LAV isoform.

The additional action of LAV-BPIFB4 on EDHF release open new scenarios for still unexplored therapeutic targets that could benefit by the alternative mechanisms other than eNOS for activating endothelial function.

Briefly, our further characterization of the molecular determinants of LAV-BPIFB4 protective effects puts the foundation to investigate on it's a possible translation in clinical field.

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SCIENTIFIC PRODUCTION RELATIVE TO THE PRESENT WORK

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