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"Identification of molecular and genetic markers common to multifactorial diseases (cardiovascular diseases, metabolic disorders, tumors) associated with aging and linked to metabolic syndrome."

IL DOTTORE
Dott.ssa Loredana Vaccarino

IL COORDINATORE
Prof. Calogero Caruso

IL TUTOR
Prof. Domenico Lio

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Inflammageing: the centenarians lesson

The extraordinary increase of the elderly in developed countries underscore the importance of studies on ageing and longevity and the need for the prompt spread of knowledge about ageing in order to satisfactorily decrease the medical, economic and social problems associated to advancing years for the increase of the subjects which are not autonomous and are affected by invalidating pathologies (Christensen K. et al. 2008).

Ageing is a post-maturational process that, due to a diminished homeostasis and increased organism vulnerability, causes a reduction of the response to environmental stimuli.

The progressive decrease in physiological capacity and the reduced ability to respond to stresses lead to increased susceptibility and vulnerability to diseases. Thus, mortality due to all causes increases exponentially with ageing.

This relentless process affects all cells, tissues, organs, and organisms, diminishing homeostasis and increasing organism vulnerability (Franceschi et al., 2008).

In particular, ageing progression causes a reduction of the response to environmental stimuli and, in general, is associated with an increased predisposition to illness and death (Troen, 2003; Candore et al., 2006a).

The main characteristic of aging is a chronic low-grade inflammation state clearly showed by 2–4-fold increase in serum levels of inflammatory mediators such as cytokines and acute phase proteins in aged population, which act as predictors of mortality independent on pre-existing morbidity (Vasto et al. 2006).

The group of Franceschi C. conceptualized for the first time in 2000the term **"Inflammageing"** a neologism, that indicates the tight relation between the role of inflammation and successful ageing.

In particular, this term indicates the chronic low-grade inflammation typical of ageing, that seems to be the common biological factor responsible for the decline and the onset of disease in the elderly.

The peculiar chronic inflammatory status which characterizes ageing, is under genetic control and is detrimental for longevity (Chung, H.Y et al. 2002; Zanni, F. et al. 2003). This age-related chronic inflammatory activity, leading to long term tissue damage, is related to mortality risk from all causes in old persons. A wide range of age-related dis-

eases, such as neurodegeneration, atherosclerosis, diabetes, osteoporosis and sarcopenia, among others, share an inflammatory pathogenesis (Pawelec, G. et al.2002; Lio, D. et al 2003; Roubenoff, R. 2003; Barbieri, M. et al. 2003; Licastro, F. et al.2003; Abbatecola, A.M et al. 2004; Roubenoff, R. et al. 2004).

Inflammation is considered a response set by the tissues in response to injury elicited by trauma or infection. It is a complex network of molecular and cellular interactions that facilitates a return to physiological homeostasis and tissue repair. The individual response against infection and trauma is also determined by gene variability.

Actually, inflammation is defined, as a localized response with systemic consequences, elicited by injury or tissue damage, which helps to destroy, reduce or sequester both the harmful agent and the wounded tissue. It is characterized in the acute form by the classical signs of pain, heat, redness, swelling and loss of function. In case the healthy tissue is not restored, or in response to stable low-grade irritation, inflammation becomes a chronic condition that continuously damages the surrounding tissues. In fact, chronic inflammation may have a rapid or slow onset but is characterized primarily by its persistence and lack of clear resolution; it occurs when the tissues are unable to overcome the effects of the harmful agent. During chronic inflammatory events, immune responses, tissue injury and healing proceed simultaneously. The inflammatory response directs immune system components to the site of injury or infection.

The inflammation is not *per se* a negative phenomenon: it is the response of the immune system to the aggression of viruses or bacteria. Nowadays the immune system must be active for more decades than in past centuries. This very long activity leads to a chronic inflammation that slowly but inexorably damages all the organs: this is a typical phenomenon linked to aging considered the major risk factor for all the chronic age-related diseases (Licastro, F. *et al.* 2005) .

Therefore, through inflammation and its mediators the IS influences not only the immunological defense reactions, but also exerts detrimental effects on muscle, bone, cardiac function, hematopoiesis and cognition (Barbieri, M et al.2003; Licastro, F. et al. 2003; Abbatecola, A.M. et al. 2004). Furthermore, the production of chemokines (RANTES, MIP-1a, IL-8, MCP-1) is also increased in the elderly, as a consequence of inflammation. Elevated IL6 serum levels are associated with diseases, disability and mortality in the elderly.

So, the ageing of immune system, definite as **immunosenescence** (IS), is the consequence of the continuous attrition caused by chronic antigenic overload. Immunosenescence and probably morbidity and mortality will be accelerated in those subjects who are exposed to an extra burden of antigenic load, such as chronic infections.

Immunosenescence represents a complex remodelling, whereby some parameters decrease with age while others increase or remain unchanged (Franceschi, C., et al. 1998; Franceschi, C., et al. 2000; Franceschi, C., et al. 2000; Franceschi, C.,et al.1999). Remodelling suggests that not only a loss of function, but also complex changes in immune function, occur with age. In particular, some functions appear to be up-regulated with aging; particularly important among these are the inflammatory response and the effector system of T lymphocytes.

In particular, this phenomenon is characterized by inflamm-ageing, accumulation of memory and effector T cells, reduction of naive T cells, shrinkage of T cell repertoire, modification of CD4/CD8 T lymphocytes proportion, the involution of the thymus, reduction of the immunological space.

The changes associated with immunosenescence are playing a more and more important role in the emergence of a series of age-related pathologies, conditioning the present epidemiology of old people (Franceschi, C. 2003). Old people have to cope with a lifelong antigenic burden encompassing several decades of evolutionary unpredicted antigenic exposure. This chronic antigenic stress and the subsequent inflammatory burden have a major impact on survival and frailty.

The quality of ageing and the peculiar remodeling of the IS in more advanced age are the results of the individual immunological history, which therefore heavily influences both longevity and successful ageing (Franceschi, C. and Bonafe`,M. 2003) . Individual immunological history derives from the interaction between genetic background and specific lifelong antigenic burden (Cooper, R.S. 2003;Hasty, P. et al. 2003).

Inflammaging appears to be a universal phenomenon that accompanies the aging process, and which is related to frailty, morbidity and mortality in the elderly.

However, very marked individual variability is observed: on the one hand, there are people who become frail and suffer early in life from age-related diseases that have an inflammatory pathogenesis; on the other, we observe healthy centenarians in whom

high levels of inflammatory mediators are present, thus suggesting that inflammaging is compatible with very old age.

Centenarians represent a cohort of select survivors who have, at least, markedly delayed diseases that normally cause mortality in the general population at earlier age. So, centenarians may be a human model of disease-free or at the least, disease delayed ageing (Franceschiet al., 1995; Franceschi and Bonafe, 2003).

Centenarians are the best model in which to study human longevity.

Thus centenarians have avoided or survived the most important pathologies that affect old people and are responsible for their morbidity and mortality. However, at the same time, centenarians are by definition extremely old people, and show all the signs and the characteristics of a prolonged aging process.

In general, centenarian women outnumber centenarian men, and this is true all over the world. In Italy, a gradient exists from North to South, and the ratio between centenarian women and men decreases from about 7 : 1 in Northern Italy to 3 : 1 in Southern Italy (Passarino, G. et al. 2002).

Centenarians as a model to study the determinants of aging and longevity in humans.

Studies performed on centenarians compared to old subjects (usually about 60 years, when mortality goes up dramatically, in order to avoid cohort effects) have evidenced that centenarians escaped the major age-related diseases, and a minority of them is still in quite good health.

As well known, life expectancy is a familial trait and longevity is determined by different factors. In particular, the environmental milieu and **genetic background** play a central role.

As demonstrated by many epidemiological studies, family members of long-lived subject shave a significant survival advantage compared to general population.

In this context, the study of centenarian offspring (CO), a group of healthy elderly people with a familiar history of longevity, might help gerontologists to better identify the correlation between genetic profile and hope of a healthy ageing.

Previous studies have reported that CO, like their centenarian parents, have genetic and immune system advantages, which reflect a minor risk to develop major age-related diseases, such as cardiovascular diseases, hypertension or diabetes mellitus as well as cancer (Terry DF. et al. 2004a; Terry DF. et al. 2004b).

In these last years, some researchers have speculated about the distinctive immunological profile of offspring enriched for longevity respect to the immunological features of coeval elderly.

During ageing, B cell compartment shows significant modifications in numbers and functions (Colonna Romano G. et al 2003; Colonna Romano G. et al. 2002; Listì F. et al. 2006; Candore G. et al. 1997).

In fact, advanced age is per se a condition characterized by lack of B clonotypic immune response to new extracellular pathogens. In any event, data are suggesting that the loss of naive B cells could represent a hallmark of immunosenescence (Colonna-Romano G. et al. 2008). On the other hand, a B cell population lacking of both IgD and CD27 resulted increased in healthy elderly (Colonna-Romano G. et al. 2009).

It is well documented that the quality and the size of the humoral immune response declines with age (Bulati M. et al 2011; Frasca D. et al 2005; Dunn-Walters DK. et al. 2010; Cancro MP. et al 2009; Kumar R. and Burns E.A. 2008;Gibson KL. et al 2009; Grubeck-Loebenstein B. et al 2009).

This change is characterized by lower antibody responses and decreased production of high affinity antibodies.

The evaluation of IgM secreted in CO serum shows that the values are within the range of the levels observed in young subjects (Colonna-Romano G. et al 2010).

In this way, CO could have a bigger advantage to fight against new infections and appropriately respond to vaccinations, giving them a selective advantage for longevity in healthiness.

Whereby, individuals genetically enriched for longevity possess immune different signatures respect to those of the general population . This suggests the idea of the “familiar youth” of the immune system.

Ageing in good condition seems directly correlated with a good functioning of the immune system, suggesting that genes regulating the immune inflammatory responses are involved in longevity (Candore et al., 2006b; Franceschi et al., 2008).

1.2 Genetic determinants of successful or unsuccessful ageing.

The idea that the longevity is based on genetic background is supported by many scientific studies.

Data obtained in a study of Scandinavian twins suggest that lifespan is influenced by genetics for 25% (Ljungquist B. *et al.* 1998).

But these studies have not been carried out on nonagenarians and centenarians, so they do not have information about the maximum lifespan. However, centenarian siblings have an increased probability of a prolonged existence and the age at death of the centenarian parents is higher than the expectancy of their birth cohort. It is perhaps unsurprising that parents and siblings of centenarians are themselves relatively long-lived. Given the rarity of life-long mortality differences between social groups defined in other ways, these findings suggest a substantial familial component in differentiating exceptionally long-lived individuals from the rest of the population.

Obviously, as previously stated, a familiar trait could be environmental, behavioral, or genetic.

In the present study we focus on the role of the genes that control immune-inflammatory responses.

According to evolutionary ageing theories, most of the parameters influencing immunosenescence appear to be under genetic control (Capri M. et al. 2008; Troen BR. 2003; Ostan R. et al 2008).

An example is given by the innate immune system, involved in neutralizing infectious agents (Candore G. et al.2006). It plays a beneficial role until the time of reproduction and parental care. In old age, a period largely not foreseen by evolution, it can determine an opposite and detrimental effect through chronic inflammatory responses ("antagonistic pleiotropy") (Ostan R. et al 2008; Williams GC. 1957).

Genetic pro-/anti-inflammatory variations in innate immune response are, indeed, thought to influence the susceptibility of age-related human diseases, by altering ost response to environmental and endogenous stress (Vasto S. et al. 2007). Thus, they are able to determine a negative or positive control of inflammation, by affecting both interactions between host and microbes and survival of the individual and attainment of longevity. Furthermore, they appear both to be responsible, at least in large part, for dif-

ferent men and women strategies to achieve longevity, and to contribute to the preferential sex dimorphism of the age-related diseases (Candore G. et al.2010; Candore G. et al.2006; Lio D. et al. 2002).

Indeed, the **genes involved in the inflammation** process are numerous, as well as the genomic variations within most of these genes.

The role of an individual genetic background and predisposition for the extent of an inflammatory response is determined by variability of genes encoding endogenous mediators that constitute the pathways of inflammation.

The genetically determined capacity of mediator production and release might contribute to a wide range of clinical manifestations of an inflammatory disease (Feghali and Wright, 1997; Imahara and O'Keefe, 2004; Hollegaard and Bidwell, 2006; Licastro et al., 2005).

The individual genetic background and the eventual influence of counteracting cytokines could therefore play a determinating role in the onset of age-related diseases.

Genetic variations located within the promoter regions of pro-inflammatory and regulatory cytokines could influence inflamm-ageing and the susceptibility to age-related diseases (Lio D. et al. 2003).

Centenarians show a complex and peculiar balancing between pro-inflammatory and anti-inflammatory characteristics, either phenotypically or genetically (Franceschi C. et al. 2007) whose net result is a slower, more limited and balanced development of inflamm-ageing in comparison to other old people, who are characterized by either faster or inadequately counteracted anti-inflammatory responses (Franceschi C. and Bonafè M. 2003).

A potentially important consequence of the age related impairment of the balancing between inflammatory and anti-inflammatory agents is a profound, systemic modification of cellular microenvironment(s), which in turn can determine a different rate of ageing of organs and tissues, leading to the so-called 'mosaic of ageing' (Cevenini E. et al. 2008).

Furthermore, the age-related and inflammation-related change of cell microenvironment can also cause a remodelling in epigenetic and gene expression at different ages.

Within this perspective, **healthy aging** and **longevity** are likely the result not only of a lower propensity to mount inflammatory responses but also of efficient anti-

inflammatory networks, which in normal aging fail to fully neutralize the inflammatory processes consequent to the lifelong antigenic burden and exposure to damaging agents. Such a global imbalance can be a major driving force for frailty and common age-related pathologies.

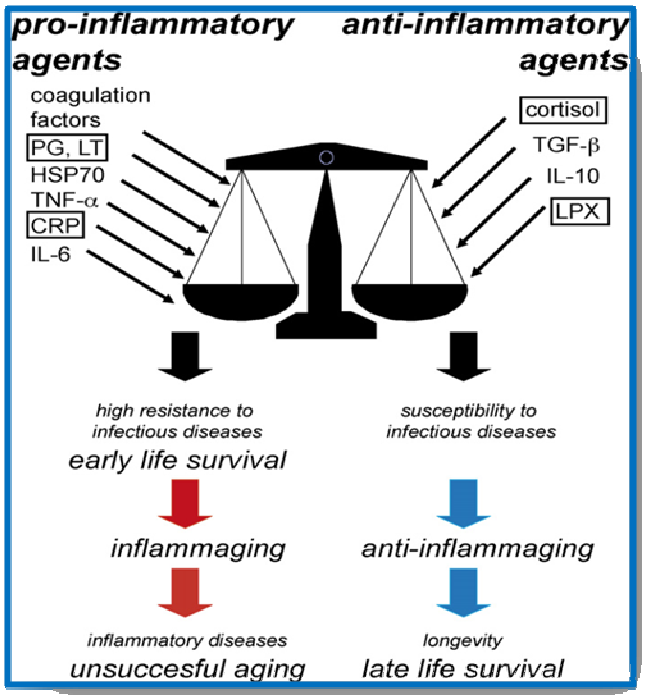

Fig. 1. The balancing between pro- and anti-inflammatory agents. Efficient inflammatory responses can confer high resistance to infectious diseases, but also an increased susceptibility to inflammation-based diseases later in life. On the other side, low inflammatory responses, while rendering more susceptible to infectious diseases, can confer a survival advantage in old age. PG: prostaglandins; LT: leukotrienes, LPX: lipoxins. Squares indicate factors whose contribution to longevity have not been studied yet.

These studies support the existence of buffering mechanisms operating in the determination of human longevity, probably through the presence of favorable genotypes contrasting the deleterious effect of age-related disease genes: as a result, the frequency of deleterious genotypes may increase among individuals with extreme lifespan because

their protective genotype allows disease-related genes to accumulate (Bergman A. et al. 2007).

A better understanding of the functional genes that affect healthy longevity in humans may lead to a rational basis for intervention strategies that can delay or prevent age-related diseases.

Genetic variations (gene polymorphisms) located within the promoter regions of pro-inflammatory cytokines have been shown to influence the susceptibility to age-related diseases, by increasing gene transcription and therefore cytokine production. Conversely, genetic variations determining increased production of anti-inflammatory cytokines or decreased production of pro-inflammatory cytokines have been shown to be associated with successful ageing, suggesting a role for the control of the inflammatory state in the attainment of healthy longevity (Lio et al., 2003; Carrieri et al., 2004).

So, it is emergent evidence that polymorphic alleles of inflammatory cytokines, involved in high cytokine production, are related to unsuccessful aging as atherosclerosis and Alzheimer's disease; reciprocally, controlling inflammatory status may allow to us better attain successful aging (Candore G. et al. 2006).

1.3 Cytokine profile: a biomarker for successful ageing

Cytokines are considered key players in maintaining lymphocyte homeostasis (Sanjabi S. et al.2009; Iannitti T and Palmieri B 2011). Their function is not limited to induce response after an immune insult, but they can modulate the nature of response (cytotoxic, humoral, cell mediated, inflammatory or allergic) or, in contrast, they may cause non-responsiveness and active immunesuppression (Iannitti T and Palmieri B 2011).

Cytokines interact in networks in which the functions of one cytokine are modified, modulated or substituted by another one.

For example, IL-10 and TNF-α have complex and predominantly opposing roles in the inflammatory responses (Hajeer and Hutchinson, 2001; Girndt et al., 2002). IL-10 limits and ultimately terminates inflammatory responses, whereas TNF-α determines strength, effectiveness and duration of inflammatory reactions.

TNF-α is an independent prognostic marker for mortality in persons aged 100 years (Bruunsgaard et al., 2003b).

Furthermore, sequence variations in several cytokine genes, such as IFN-γ and IL-10 genes, have been demonstrated to be associated with successful ageing and longevity (Iannitti T. and Palmieri B 2011).

On the other hand, individual changes in type and intensity of immune response affecting life span expectancy and health ageing seem to have a genetic component. A well-preserved immune function characterizing the successful ageing has been found in centenarians (Ostan R. et al.2008).

Recent evidence suggests that centenarians seem to be genetically equipped gene polymorphism for overcame the major age-related diseases and polymorphisms in immune system genes involved in regulation of immune responses have been found associated to longevity.

In particular, associations between both cytokine gene polymorphisms and longevity, and differential gender longevity in males and females, and reciprocally to age-related diseases have been demonstrated (Ostan R. et al. 2008; Iannitti T. and Palmieri B 2011; Caruso C. et al. 2005).

Interpersonal differences in the regulation of cytokines production, as IL10 and TNFα, may be critical with respect to the final outcome of an inflammatory response.

The increased production of IL-6 and TNF-α and the reduced production of growth hormone and IGF-I are directly involved in the loss of fat-free mass during ageing.

Low-grade elevations in levels of circulating pro inflammatory cytokines and their receptors, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, interleukin (IL)-1 receptor antagonist (IL-1Ra), soluble TNF receptors, etc., are strong independent risk factors of morbidity and mortality in the elderly (Bruunsgaard and Pedersen, 2003; Bruunsgaard et al.,2003a). A further consequence of hyper production of pro inflammatory cytokines in the elderly is the increased production of diverse chemokines (RANTES, MIP-1α, IL-8, MCP-1) (Appay and Rowland-Jones, 2002; Tracy, 2003) and the increase in C reactive protein (CRP) levels (Di Iorio et al.,2003; Mariani et al., 2002; Bo et al., 2005).

Frailty and disability in the elderly represent the complex and cumulative expression of the altered production of such inflammatory cytokines (Di Iorio et al., 2003; Sandmand etal., 2003). In support of this hypothesis, increased levels of inflammatory serum markers in the elderly are associated with dementia, Parkinson's disease, atherosclerosis, type 2 diabetes, sarcopenia, functional disability and high mortality risk (Franceschi et al., 2001; Abbatecola et al., 2004; Barbieri etal., 2003).

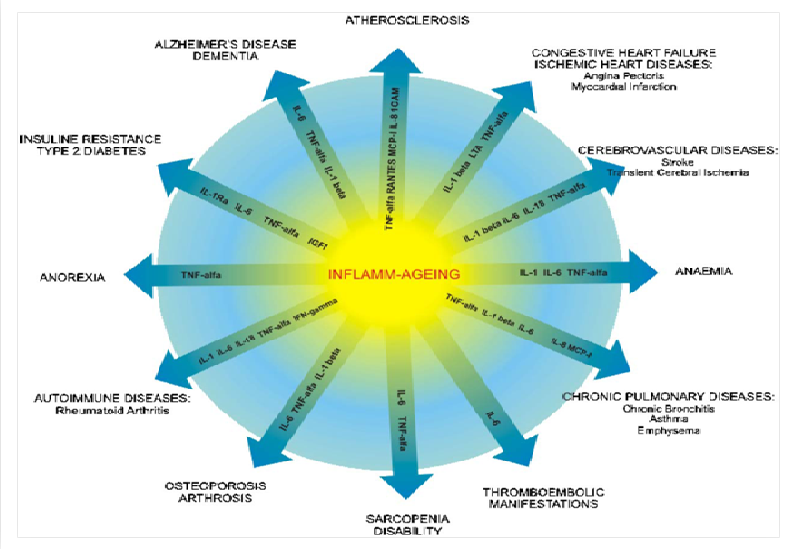

Fig. 2. Inflamm-ageing, cytokine network and age-related diseases. Cytokines mainly involved in the pathogenesis and progression of specific disease entities are sho

Furthermore, it seem that those individuals who are genetically predisposed to produce low levels of inflammatory cytokines or high levels of anti-inflammatory cytokines have an increased capacity to reach the extreme limits of the human life-span. (Pawelec et al. 2002, Candore et al. 2003, Lio et al. 2003).

In particular, a considerable body of data indicates that particular cytokine polymorphisms, especially those involving IL-6, TNF-α, and IL-10 genes, may influence susceptibility, and in some cases prognosis in age-related disease.

In this regard, a study performed on Italian centenarians reported that those individuals who are genetically predisposed to produce high levels of IL-6 during aging, homozygous for the IL-6 −174GG, that is, C-negative men at IL-6 –174 C/G single nucleotide polymorphism (SNP) have a reduced capacity to reach the extreme limits of human lifespan (Bonafè , M. et al. 2001).

Independent studies found that high IL-6 levels are the major predictor of disability and mortality in the elderly.

Further studies have shown that the IL-6 −174GG genotype is underrepresented in centenarians, thus to be consider that the IL-6−174 genotype may be a major modulator of inflammaging.

In particular it was shown that this phenomenon to be restricted primarily to males, suggesting that the two genders follow different trajectories to attain longevity.

Several data suggest that IL-6 −174 C/G locus variability is capable of modulating the individual susceptibility to common causes of morbidity and mortality among elderly, such as type 2 diabetes, cardiovascular diseases, Alzheimer'sdisease and dementia, among others, thus influencing individual life span (Licastro et al., 2003; Olivieri et al., 2003; Franceschiet al., 2005).

Similar data on the decreased frequency of IL-6 −174 C-carriers in Irish octogenarian subjects from the BELFAST elderly longitudinal ageing study have been reported (Rea et al., 2003; Ross et al., 2003).

Some of recent evidence has, in fact, linked cytokine polymorphisms with longevity and differential longevity between males and females in the Italian population.

Moreover, other data indicate that –1082G IL-10 SNP, associated with a high production of the cytokine, is increased among Italian male centenarians (Lio D. et al.2004; Lio, D. *et al.* 2002; Lio D. et al.2003).

In a study on 190 Italian centenarians, the IL-10 −1082G/G polymorphism has been demonstrated to be a specific marker for longevity (Lio et al.,2002). This genotype, associated with high IL-10 production, was argued to confer an anti-inflammatory status, thus enhancing the possibility to attain extreme longevity.

IL-10 gene codifies for IL-10 cytokine. IL-10 is produced by macrophages, T and B cells. It is one of the major immune-regulatory cytokines, usually considered to mediate potent down-regulation of inflammatory responses. IL-10 production, independently on interaction with other cytokine gene products, is generally controlled by several polymorphic elements in the 5'flanking region of IL-10 gene. Multiple SNPs have been identified in human IL-10 5'flanking region and some of these (i.e. -592, -819, -1082) combine with microsatellite alleles to form haplotype associated with differential IL-10 production.

These three SNPs in the IL-10 proximal gene region (considered potential targets for transcription regulating factors) might be involved in genetic control of IL-10 production.

In particular, the homozygous -1082GG genotype seems to be associated with higher IL-10 production respect to G/A heterozygous and AA homozygous genotypes.

An interferon γ polymorphism was found to be a likely marker for inflammaging in women (Lio D. et al. 2002).

It is interesting to note that the IL-10 −1082 GC genotype, that likely confers an advantage to become a centenarian, is much less frequent in patients affected by Alzheimer's disease.

IFN-γ gene codifies for a cytokine involved in defense against viruses and intracellular pathogens, and in induction of immune mediated inflammatory responses.

Its production is genetically regulated. A variable length CA repeat sequence in the first intron of IFN-γ gene has been described to be associated with high IFN-γ production.

Furthermore, a SNP, T to A (+874 T/A), at 59 end of the CA repeat region has been described and T presence has been related to high-producing microsatellite allele 2. This SNP coincides with a putative NF-_B binding site, which might have functional consequences for transcription of IFN–γ gene. Thus, this SNP might directly influence IFN-g production levels associated to CA microsatellite marker (Lio D. et al. 2002) .

In centenarians, our group first reported that possession of the +874A allele conferred an overall anti-inflammatory status promoting longevity, particularly in centenarian females (Lio et al. 2002a).

Alongside our recent findings allow us to suggest that different alleles at different genes coding for pro- or anti-inflammatory molecules may affect individual life-span expectancy by influencing the type and intensity of the immune-inflammatory responses against environmental stressors (Caruso et al., 2005; Franceschi et al., 2005; Rea et al., 2006).

Moreover, centenarian offspring have a marked increased likelihood of surviving up to 100 years and show a reduced prevalence of age-associated diseases, in particular related to cardiovascular diseases and less prevalence of cardiovascular risk factors (Terry et al., 2003). So, genes involved in CVD, the leading worldwide cause of morbidity and death (Nabel, 2003), should play an opposite role in human longevity.

1.4. Metabolic Syndrome (MetS), Cardiovascular diseases and Cancer

The **metabolic syndrome** (MetS) is defined by a constellation of interconnected physiological, biochemical, clinical, and metabolic factors that directly increases the risk of cardiovascular disease, type 2 diabetes mellitus, and all cause mortality.

Insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hyper coagulable state, and chronic stress are the several factors which constitute the syndrome.

Chronic inflammation is known to be associated with visceral obesity and insulin resistance which is characterized by production of abnormal adipocytokines such as tumor necrosis factor (TNFα), interleukin-1 (IL-1), IL-6, leptin, and adiponectin.

The interaction between components of the clinical phenotype of the syndrome with its biological phenotype (insulin resistance, dyslipidemia, etc.) contributes to the development of a pro inflammatory state and further a chronic, subclinical vascular inflammation which modulates and results in atherosclerotic processes.

The metabolic syndrome confers a 5-fold increase in the risk of type 2 diabetes mellitus (T2DM) and 2-fold the risk of developing cardiovascular disease (CVD) over the next 5 to 10 years (Alberti K. G. M. M. et al. 2009).

Further, patients with the MetS are at 2- to 4-fold increased risk of stroke, a 3- to 4-foldincreased risk of myocardial infarction (MI), and 2-foldthe risk of dying from such an event compared with those without the syndrome (Alberti K. G.M.M. and Zimmet P. 2005) regardless of a previous history of cardiovascular events (Olijhoek, J. K. et al. 2004).

It further increases with age (10% in individuals aged 20–29, 20% in individuals aged 40–49, and 45% in individuals aged 60–69) (Ford E.S. et al.2002).

The prevalence of MetS (based on NCEP-ATP III criteria, 2001) varied from 8% to 43%in men and from 7% to 56% in women around the world (Cameron J.E. et al. 2004).

Park Y.W. et al. (2003) noticed that there is an increase in the prevalence of MetS from 20 years old through the sixth and seventh decade of life for males and females, respectively.

MetS is a state of chronic low grade inflammation as a consequence of complex interplay between genetic and environmental factors.

Insulin resistance and inflammation are postulated among the important underlying pathophysiologies of the syndrome (Reaven GM. 1988).

Increased levels of inflammation markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) are observed in subjects with MetS (Dandona P. et al. 2004; Ridker PM. et al.2003; Wannamethee SG. et al. 2005) as well as in people with components of MetS (Frohlich M. et al.2000; Lakoski SG. et al 2005;Piche ME. et al 2005; Sesso HD. et al 2007).

Insulin Resistance is defined as a pathophysiological condition in which a normal insulin concentration does not adequately produce a normal insulin response in the peripheral target tissues such as adipose, muscle, and liver. Under this condition, pancreatic beta cell secretes more insulin (i.e., hyperinsulinemia) to overcome the hyperglycemia among insulin-resistant individuals.

Although hyperinsulinemia may compensate for insulin resistance to some biological actions of insulin, that is, maintenance of normoglycemia, however, it may cause an over expression of insulin activity in some normally sensitive tissues. This accentuation of some insulin actions coupled with a resistance to other actions of insulin results in the clinical manifestations of MetS (Gill H. et al 2005).

The great variations in the susceptibility and age of onset in individuals with a very similar risk profile suggest a major interaction between genetic and environmental factors (Ordovas J.M. et al 2007). It is recognized that some people who are not obese by traditional measures nevertheless are insulin resistant and have abnormal levels of metabolic risk factors.

Examples are seen in individuals with 2 diabetic parents or 1parent and a first- or second-degree relative (Perseghin G. 1997).

It is likely that the expression of each metabolic risk factor falls partially under its own genetic control, which influences the response to different environmental exposures. For example, a variety of polymorphisms in genes affecting lipoprotein metabolism are associated with the worsening of dyslipidemia among obese people (Laakso M. 2004).

Similarly, a genetic predisposition to the defective insulin secretion when combined with insulin resistance can raise the plasma glucose to abnormal levels (Poulsen P. et al 2005).

The metabolic syndrome is a constellation of risk factors that co-segregateand is associated with increased cardiovascular events (McNeill AM et al 2006; Wilson PW et al 2005).

It is shown that inflammation markers and metabolic syndrome are associated with risk of congestive heart failure (CHF).

Suzuki T. et al.(2008) studied 4017 men and women > or =65 years old, without baseline CHF or diabetes, participating in the Cardiovascular Health Study, an observational study with 12.2 years follow-up and 966 cases of incident CHF. They observed that inflammation markers provided additive information on CHF risk in this elderly cohort. So, inflammation markers and MetS together may be useful in clinical and research settings.

Several studies have shown that centenarians have better cardiovascular risk profiles compared to younger old people. Some reports have revealed that cardiovascular diseases (i.e. hypertension, diabetes, angina and/or myocardial infarction) are less common in centenarians respect to 70 and 80 years old persons.

Among older population, centenarians may be considered the best example of successful cardiovascular aging.

The capacity to avoid, delay or limit cardiovascular damage associated with aging-related diseases has been proposed as one of the mechanisms that may help to explain the successful aging in centenarians. In particular, lower incidence of cardiovascular diseases has been observed in centenarians, which represent the most frequent causes of death at younger ages (70–80 years).

The examination of death certificates has shown a decreased frequency of diabetes and myocardial infarction as causes of death in centenarians in contrast with the accepted concept that atherosclerosis and congestive heart failure increase in frequency with age (Gessert et al., 2002). These findings suggest that centenarians appear to ‘‘outlive’’the cardiovascular risk factors for many of the conditions that are frequent causes of death at the age of 70, 80, or 90 years.

The advantages of a favorable cardiovascular risk profile in centenarians seem to be transmitted to their descendants, who exhibit better cardiovascular risk profiles compared to age-matched people without centenarian relatives (Perls and Terry, 2003). This supports the existence of genetic determinants in the genesis of arteriosclerosis and its complications. In addition, a more favorable inflammatory atherogenic profile has been reported in centenarians together with a better antioxidant profile (Fletcher et al.,2003), which is in accordance with the inflammatory and oxidative stress hypothesis of cardiovascular aging (Chunget al., 2001).

Together with a favorable genetic profile, a key aspect that seems to be present in most of those who have reachedan exceptional old age is to have conducted a healthy lifestyle (Perls and Terry, 2003).

The incidence of atherosclerosis is markedly linked to the presence of a pro-inflammatory status (Viles-Gonzalezet al., 2006). Thus, control of inflammatory reactions may also decrease the incidence of cardiovascular diseases.

The predictive validity of traditional cardiovascular risk factors diminishes with increasing age (Cesari et al., 2004; Casiglia and Palatini, 1998).

Various reports have indicated that inflammatory markers appear to be predictive of cardiovascular events, especially in aged populations (Harris et al., 1999; Volpato et al., 2001; Bruunsgaard et al., 2000; Vasan et al., 2003; Ridker et al., 2000).

CRP is increasingly measured to stratify coronary artery disease risk and guide clinical management (Ridker, 1998; Bogaty et al., 2005). Simultaneous assessment of markers of inflammation and lipid metabolism may improve cardiovascular risk stratification in patients with stable coronary artery disease (Hoffmeister et al., 2005).

Gene polymorphisms for pro-inflammatory cytokines seem to contribute significantly to the risk of atherosclerosis related diseases (Candore et al., 2006a; Lio et al., 2004).

The linkage between inflammation, genetic factors and atherosclerosis has been demonstrated by the analysis of the frequency of pro-inflammatory and anti-inflammatory genotypes in Italian centenarians. In this population, the frequency of the genotype associated with interleukin 10 (-1082GG) is related to a significant increased production of this anti-inflammatory cytokine. Conversely, the frequency of the genotype associated with low production of IL 10 is significantly higher in patients with acute myocardial infarction compared to controls.

Although a low production of interleukin 10 is associated with an increased resistance to pathogens, increased concentrations of this cytokine seems to be linked to a better control of inflammatory responses induced by chronic vessel damage, and with a reduced risk for atherogenic complications (Lio et al., 2004).

A 7-year prospective cohort study assessed the incidence of coronary heart disease, stroke and congestive heart failure events according to serum levels of CRP, IL-6 and TNF-α in a large sample of older, well-functioning subjects (Cesari et al.,2003).

It has been shown that IL-6 is significantly associated with all outcomes, whereas TNF-αshowed significant associations with coronary heart disease and CRP was associated with congestive heart failure events. Also, IL-1β serum levels are associated with congestive heart failure and angina, supporting the hypothesis that IL-1β is mainly involved in the functional alterations of cardiomyocytes (Di Iorio et al., 2003).

It has been suggested that different genotypes of inflammatory molecules (pro- and anti-inflammatory cytokines) may have opposite effects on predisposing to atherosclerosis and accelerated vascular aging or longevity (Candore et al., 2006a).

In addition to cardiovascular disease, individual components of the metabolic syndrome have been linked to the development of cancer, particularly to colorectal cancer (Kreger BE et al. 1991) .

Colorectal cancer is an important health problem since one million new cases are diagnosed world-wide each year with half million related deaths (Boyle P and Leon ME 2002).

This association is sustained by many epidemiological studies. Recent reports suggest that individuals with metabolic syndrome have a higher risk of colon or rectal cancer. Moreover, the clusters of metabolic syndrome components increase the risk of associated cancer.

Accumulating evidence suggests that systemic inflammation might be a mechanism that play a major role in colon carcinogenesis.

Studies have shown that genetic variations in inflammation-related genes, such as interleukin (IL)-6, IL-8, and IL-10, are associated with susceptibility to colorectal cancer and adenomas.

IL-6 appears to enhance tumorigenesis by a paracrine and autocrine mechanism, to stimulate cell growth and inhibit apoptosis. Also IL-6 concentrations reflected disease sta-

tus and were commonly associated with metastatic disease (Chung YC and Chang YF 2003).

There are several studies which demonstrated the correlation between high levels of IL-6, TNF-α, C-reactive proteins (CRP) and colorectal carcinogenesis.

Moreover, a Greek study demonstrated that high levels of serum IL-6, TNF-α and CRP were correlated with larger tumor size.

The relation to tumor size could be related to the fact, that larger tumors may trigger a more potent immunological response manifested by the circulation of proinflammatory cytokines such as TNF-α (Nikiteas NI et al. 2005).

Understanding the pathological mechanism that links metabolic syndrome and its components to carcinogenesis has a major clinical significance and may have profound health benefits on a number of diseases including cancer, which represents a major cause of mortality and morbidity in our societies.

In this sense, we also need to take inconsideration the role of immunological balance Th1/Th2 on a genetically predisposed perspective, so that several functional single nucleotide polymorphisms (SNP) located in critical transcription positions may affect the high and low production of inflammation mediators, and these molecular differences are all together responsible of individual difference in exacerbation or fast resolution of inflammatory events (Lio et al. 2003, Forte et al. 2006).

1.5 Aims of the study and outline of this thesis

Aging is a post-maturational process that, because of a diminished homeostasis and increased organism vulnerability, causes a reduction of the response to environmental stimuli. The progressive decrease in physiological capacity and the reduced ability to respond to stresses lead to increased susceptibility and vulnerability to disease. Thus, mortality due to all causes increases exponentially with aging.

Aging involves all the cells, tissues, organs, and organisms and is modulated by external factors.

Ageing is accompanied by chronic low-grade inflammation state clearly showed by 2–4-fold increase in serum levels of inflammatory mediators such as cytokines and acute phase proteins in aged population, which act as predictors of mortality independent on pre-existing morbidity.

More interestingly, studies of gene expression have demonstrated that in the aged several genes show an increase or a decrease in expression. Usually the genes over expressed are active in stress conditions, such as inflammation (Weindruch, R. *et al.* 2002; Troen, B.R. 2003; Wick, G. *et al.* 2003; Franceschi, C. *et al.* 2000).

Thus inflammaging is a mechanism of aging *per se,* and this could constitute the common driving force of most age-related pathologies.such as atherosclerosis, cardiovascular diseases, type 2 diabetes, metabolic syndrome, sarcopenia, osteoporosis, cognitive decline and frailty (De Martinis M. et al. 2005; Bucci L. et al 2009).

Centenarians are equipped to reach the extreme limits of human life span and, most importantly, to show relatively good health, being able to perform their routine daily life and to escape fatal age-related diseases. Thus, they are the best example of extreme longevity, representing selected people in which the appearance of major age-related diseases, such as cancer, and cardiovascular diseases among others, has been consistently delayed or escaped.

In this scenario, multiple cytokines play a critical role in orchestrating and perpetuating inflammation in age-related disease pathogenesis and several specific cytokines and chemokines inhibitors are now in development as future therapy for these diseases (Barnes et al. 2003).

Tight relation between successful ageing and inflammation is, nowadays, largely claimed in literature and confirmed by the recognized inflammatory pathogenesis of main age-related diseases as Atherosclerosis, Alzheimer Disease, cardiovascular diseases, cancer etc.

Therefore, improvement in acknowledgments of individual genetic contribute and molecular mechanisms occurring in inflammatory response are essential in future therapeutic approach to favorite successful ageing.

Aim of the researches reported in this thesis was to contribute on the understanding genetic predisposing background role in onset of some age-related disease, in particular, cardiovascular diseases, as Acute Ischemic Stroke, Thoracic Aortic Aneurysm, Takotsubo Cardiomyopathy, Myocardial infarction and Sporadic Colon Cancer thath have a common soil in methabolic syndrome in the light of data acquired on genetic of longevity of Sicilian centenarians and of nonagenarians as a model of successful ageing.

Here are reported data from association studies that have produced informative results on inflamm-ageing obtained studying age-advanced population, centenarians and patients affected of different cardiovascular diseases.

In particular, **Chapter 2** is devoted to a collection of our data gathered for over 10 years in Sicilian centenarians and recently summarized in a review (Balistreri C.R. et al. 2012). Our group examined the role of their genetic background and immune system on longevity. We have discussed the role of an over-expression of anti-inflammatory sequence variants of immune/inflammatory genes in longevity. We investigated in Sicilian population, SNPs or genetic variants of TLR4, CCR5, Cox2, 5-Lo genes observing the association of gender and longevity. Furthermore, we analyzed allelic and genotypic frequencies of such polymorphisms located on IFN-g and IL10 genes.

In **Chapter 3** are reported data from an our study that suggested the possibility to use cytokine profile as biomarker of successful ageing, by evaluating through Luminex technology cytokine serum levels in Sicilian nonagenarians and control subjects (aged between 30 and 50 years old) (Palmeri M. et al. 2012).

In particular, we analyzed a panel of 17 cytokines, comprehensive of haematopoietic factors T helper 1 (Th1), Th2, inflammation regulatory cytokines, and chemokines, as**:** IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1b

(MIP-1b), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF).

In **Chapter 4** are reported data from an our study investigating the role of some cytokine polymorphisms, conditioning individual inflammatory response in a typical cardiovascular event: the acute ischemic stroke (Tuttolomondo A. et al 2012).

In particular, in patients affected by acute ischemic stroke, we evaluated the role of SNPs of some pro-inflammatory/anti-inflammatory and coagulation/fibrinolytic genes. We analyzed a functional TNF-α polymorphism (-308G/A), -1082/819 haplotypes of IL-10 gene, IL-1RN exon 2 VNR polymorphism, alleles at the 174 nucleotide (174G/C) of IL-6 gene, PAI-1675 5G/4G polymorphism and alleles at the 7351 nucleotide (7351C/T) of tPA gene was undertaken in the patient groups and in the control subjects.

To understand better the genetic basis of complex diseases like ischemic stroke and to delineate a possible stroke risk profile in subjects with cerebrovascular risk factors.

Chapter 5, is dedicated to the examination of the role of the genetic background in susceptibility to acute myocardial infarction (AMI) in young men (Vaccarino L. et al. 2013).

In this study, we analyzed the frequencies of polymorphisms 20210G/A of FII and 308G/A of the promoter region of TNF-α in a group of Sicilian patients aged <46 years affected by AMI and evaluated the effect of these polymorphisms on the blood levels of myocardial tissue damage and clotting markers, to evaluate the possibility to use typing of these polymorphisms in association with selected haematochemical parameters in prognostic evaluation of these patients.

Young adults are a relatively small proportion of patients who experience AMI. They have some distinct characteristics compared to older patients as young people are more likely to have normal coronary arteries; so they are an example of unsuccessful aging.

In our previous reports, we have observed a significant distribution of pro/anti-inflammatory genes among male controls, male centenarians and male patients affected by acute myocardial infarction (AMI).

The frequencies of the studied pro inflammatory alleles were significantly lower in centenarians and higher in AMI patients, i.e. the number of centenarians with a pro-inflammatory allele was lower than the number of AMI patients with the polymorphism.

Conversely the frequencies of anti-inflammatory alleles were significantly lower in AMI patients and higher in oldest old, in both cases age-related controls presented intermediate frequency values (Balistreri et al., 2004; Caruso et al., 2004;Lio et al., 2004; Listı` et al., 2005; Listı` et al., 2006a,b; Candoreet al., 2006d; Grimaldi et al., 2006).

These results strengthen the hypothesis that genetic background protecting against cardiovascular disease is a relevant component of the longevity trait at least in men where these studies have been performed (Candore et al., 2006a,d; Caruso et al., 2005), thus in successful ageing: genetic background of male centenarians is protective against coronary heart disease.

Furthermore, In **Chapters 6** and **7** it was described another cardiovascular disease that characterizes aging: the Thoracic aortic aneurysm (TAA), a progressive disorder involving gradual dilation of ascending and/or descending thoracic aorta with dissection or rupture as complications.

In particular, in **Chapter 6** it were researched phenotypes associated with the risk of aorta rupture and dissection in aged S-TAA individuals (Balistreri C.R. et al. 2014).

Thus, in this study, were assessed histopathological and immunohistochemical analyses in aorta specimens from 100 S-TAA patients with median age of 62.95 – 11.44 years and age and gender-matched controls.

Our major goal was to identify a biomarker of rupture and/or dissection in aged individuals that is useful both for applying different surgical approaches and providing appropriate surgical indications.

In **Chapter 7** it was evaluated the role of functional variants located in some cytokine genes to understand better the sporadic TAA susceptibility factors and prevention (Vaccarino L. et al. 2014).

Our interest has been focused on investigating the role of genetic variants of transforming growth factor-β (TGF-β) pathways in TAA risk.

We analyzed allelic and genotypic frequencies of these polymorphisms located on encoding TGF-βisoforms and receptors genes, as: rs1800471 TGF-β1 cod25; rs900 TGF-β2 3'UTR; rs334348 TGF-βR1 3'UTR; rs334349 TGF-βR1 3'UTR and rs4522809 TGF-βR2 Intron +3919.

In addition, other cytokines, including IL-10,an anti-inflammatory cytokine able to modulate activity of TGF-β pathways. orchestrate TAA pathophysiology. Thus, we ana-

lyzed also, the role of some common single nucleotide polymorphisms (SNPs) of genes encoding IL-10 and receptor in sporadic TAA, as: rs1800896 IL-10 (−1082 G>A); rs1800871 IL-10 (−819 C>T); rs1800872 IL-10 (−592 C>A); rs3024496 IL10 (Not defined C>T) and rs283467 IL-10RB (Codon 47 A>G).

Their balance determines the ultimate fate of the aortic wall as healing atherosclerosis or aneurysm formation.

Chapter 8 is dedicated to Takotsubo cardiomyopathy (TTC) is an increasingly reported clinical syndrome that mimics acute myocardial infarction without obstructive coronary artery disease and it characterized by transient systolic dysfunction of the apical and/or midsegments of the left ventricle (Novo G. et al. 2014).

We analyzed the role of a SNP rs17098707 (L41Q) of the G-protein-coupled receptor kinase 5 (GRK5) in TTC patients and control.

The purpose of this study was to assess the genetic susceptibility to TTC, to favoure a better understanding of the pathogenesis of this peculiar syndrome, that could allow the development of more appropriated preventive strategies and tailored treatment.

Finally, in **Chapter 9** it was evaluated the role of functional variants located in some cytokine genes to understand better the controversial mechanisms, by which cytokines underlie neoplastic transformation and progression. These studies are supported by strong evidences that both tumor and free stromal cells are able to produce cytokines that seem to affect the complex phenomena occurring at the tumour–host interface, thus leading to tumour.

This study (**Genetic determined expression of pro and anti inflammatory mediators influence the clinical history and response to chemotherapy in patients with Sporadic Colorectal cancer (CRC)**, Forte GI et al. Submitted) firstly, focused on the genetic role of polymorphisms located on crucial pro-inflammatory and anti-inflammatory cytokine genes: -174G/C_rs1800795 (IL-6); +874A/T_rs2430561 (INF-γ); -592A/C_rs1800872, -819C/T_rs1800871, -1082G/A_ rs1800896 (IL-10); R25P_rs1800471 (TGF-β), able to direct the immune system response, in the disease susceptibility. Secondly, we were interested to assess the immunological systemic movements caused both by the host-tumor interaction and by the chemotherapy treatment, in the sporadic colorectal cancer. In particular, the chemotherapy effect was evaluated in the long term, waiting at least 20 days from the drug administration.

To this aim, we evaluated the expression level changes of some pro-inflammatory (IL-6, INF-γ, Cyclooxygenase (COX)-2), and anti-inflammatory (IL-10, TGF-β) type crucial cytokines produced by peripheral blood mononuclear cells (PMBC) in a group of patients having sporadic colorectal carcinoma across the period of their clinical management.

CHAPTER 2

Genetics of longevity. Data from the studies on Sicilian centenarians

Carmela R Balistreri1, Giuseppina Candore, Giulia Accardi, Manuela Bova, Silvio Buffa, Matteo Bulati, Giusi I Forte, Florinda Listì, Adriana Martorana, Marisa Palmeri, MariaValeria Pellicanò, Loredana Vaccarino, Letizia Scola, Domenico Lio and Giuseppina Colonna-Romano.

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Genetics of longevity. Data from the studies on Sicilian centenarians

Carmela R Ballistreri[1*], Giuseppina Candore[1], Giulia Accardi[1], Manuela Bova[1], Silvio Buffa[1], Matteo Bulati[1], Giusi I Forte[1], Florinda Listì[1], Adriana Mattorana[1], Marisa Palmeri[1], MariaValeria Pellicanò[1,2], Loredana Vaccarino[1], Letizia Scola[1], Domenico Lio[1] and Giuseppina Colonna-Romano[1]

Abstract

The demographic and social changes of the past decades have determined improvements in public health and longevity. So, the number of centenarians is increasing as a worldwide phenomenon. Scientists have focused their attention on centenarians as optimal model to address the biological mechanisms of "successful and unsuccessful ageing". They are equipped to reach the extreme limits of human life span and, most importantly, to show relatively good health, being able to perform their routine daily life and to escape fatal age-related diseases, such as cardiovascular diseases and cancer. Thus, particular attention has been centered on their genetic background and immune system. In this review, we report our data gathered for over 10 years in Sicilian centenarians. Based on results obtained, we suggest longevity as the result of an optimal performance of immune system and an over-expression of anti-inflammatory sequence variants of immune/inflammatory genes. However, as well known, genetic, epigenetic, stochastic and environmental factors seem to have a crucial role in ageing and longevity. Epigenetics is associated with ageing, as demonstrated in many studies. In particular, ageing is associated with a global loss of methylation state. Thus, the aim of future studies will be to analyze the weight of epigenetic changes in ageing and longevity.

Keywords: Immune system, Genetics, Pro/anti-inflammatory polymorphisms, Epigenomics

Introduction

Data from centenarian offspring

As well known, life expectancy is a familial trait and longevity is determined by different factors. In particular, the environmental milieu and genetic background play a central role. As demonstrated by many epidemiological studies, family members of long-lived subjects have a significant survival advantage compared to general population. In this context, the study of centenarian offspring (CO), a group of healthy elderly people with a familiar history of longevity, might help gerontologists to better identify the correlation between genetic profile and hope of a healthy ageing. Previous studies have reported that CO, like their centenarian parents, have genetic and immune system advantages, which reflect a minor risk to develop major age-related diseases, such as cardiovascular diseases, hypertension or diabetes mellitus as well as cancer [1,2]. The lower cardiovascular disease risk in CO suggests the probability that CO have some protective factors against atherosclerosis, such as a good lipid profile. Male CO have higher plasma HDL-C levels and lower plasma LDL-C levels. Since lipid profile is directly correlated to atherosclerotic cardiovascular diseases, this metabolic feature could preserve CO both to develop these diseases and, as consequence, to reach a healthy ageing and longer survival [3]. Furthermore, Rose et al. [4] reported that centenarians and CO show significantly higher levels of heteroplasmy in mtDNA control region than controls, a favorable condition for longevity.

In these last years, some researchers have speculated about the distinctive immunological profile of offspring enriched for longevity respect to the immunological features of coeval elderly. The cytomegalovirus (CMV) is one of the most common viruses that affect elderly people. Many evidences have shown that CMV infection

* Correspondence: carmelaritaballistreri@unipa.it
[1]Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, Palermo 90134, Italy
Full list of author information is available at the end of the article

may influence the T cell subset distribution, having an essential role in immunosenescence [5-7]. CMV infection is strongly related to both a reduction of $CD8^+CD45^+CCR7^+CD27^+CD28^+$ naive T cells and to a contemporarily increase of $CD8^+CD45RA^+CCR7^-CD27^-CD28^-$ late differentiated effector memory and CD45RA-re-expressing T cells. These parameters are considered typical of immunosenescence in elderly. Recently, it has been demonstrated that CMV-seropositive offspring of long-lived people don't show the age-associated decrease of naive T cells. On the other hand, memory T cell subsets above described do not increase in offspring of long-lived families, differently from that observed in age-matched controls [8]. It has been also demonstrated that CMV-seropositive offspring of long-lived people have reduced levels of CD8+ T cells expressing CD57 and KLRG1, sometimes referred as "marker of senescence", when compared to their CMV-infected age-matched controls. The reduction of effector memory T cells lacking the expression of CD27 and CD28 and expressing CD57 and KLRG1, observed in CMV-infected offspring could explain their high proliferative response against CMV. The CMV-seropositive offspring have also shown significantly lower CRP levels compared to their CMV-seropositive age-matched controls that could be related to a lower pro-inflammatory status [8].

During ageing, B cell compartment also shows significant modifications in numbers and functions [9-12]. In fact, advanced age is per se a condition characterized by lack of B clonotypic immune response to new extracellular pathogens. In any event, data are suggesting that the loss of naive B cells could represent a hallmark of immunosenescence [13]. On the other hand, a B cell population lacking of both IgD and CD27 resulted increased in healthy elderly [14]. We have suggested that this IgD^-CD27^- B cell subset is a population of memory B cells lacking CD27, a typical memory marker, likely considered a late memory exhausted B cell subset (Table 1) [14-16]. This population resulted also increased in active Lupus patients [17], in healthy subjects challenged with respiratory syncitial virus [18], and in HIV patients [19]. CO don't show the typical naive/memory B cell shift observed in elderly. Although a decreased B cell count was observed in CO and their age-matched controls, it has been demonstrated that naive B cells (IgD^+CD27^-) were more abundant and DN B cells (IgD^-CD27^-) were significantly decreased, as looked similarly in young people [20]. This B cells distribution in CO could suggest that antigenic load or inflammatory environment play a central role in exhaustion of the B cell branch. It is well documented that the quality and the size of the humoral immune response declines with age [15,21-26]. This change is characterized by lower antibody responses and decreased production of high affinity antibodies. The evaluation of IgM secreted in CO serum shows that the values are within the range of the levels observed in young subjects [20]. In this way, CO could have a bigger advantage to fight against new infections and appropriately respond to vaccinations, giving them a selective advantage for longevity in healthiness.

Table 1 Main modifications of B cells and B cells products in elderly human observed in our laboratory

B cells or B cells products	Changes	References
Total B cells (percentage)	↓	[9]
$CD19^+CD5^+$ B1 cells (percentage and absolute number)	↓	[10]
IgG, IgA	↑	[11]
IgM, IgD	↓	[11]
IgE	=	[11]
Autoantibodies	↑	[12]
Naive (IgD^+CD27^-)	↓	[13]
DN (IgD^-CD27^-)	↑	[14-16]

In conclusion, individuals genetically enriched for longevity possess immune different signatures respect to those of the general population (Table 2). This suggests the idea of the "familiar youth" of the immune system. In addition, the lower pro-inflammatory status in CMV-infected offspring of long-lived people might represent an optimal advantage for healthy longevity and against mortality associated to major age-related diseases.

Gender and longevity

A characteristic enigma of longevity is the gender and the social phenomenon of "*feminization of old age*". The demographic and social changes of the past decades, responsible for longevity and the improvements in public health, have created new and often very dissimilar realties for women and men. People are all aware that they differ in their anatomy and physiology, but also in more complex traits, such as lifespan (in Italy, 78.8 years for men and 84.1 years for women, respectively) and mortality [27-29]. No conclusive explanation for these new differences is actually demonstrated. An intricate interaction between environmental, social structural, behavioural (i.e. the complex pattern of roles and values that define what is thought as *masculine* and *feminine*) and genetic factors have been suggested as the more probable reason [30-32].

From a genetic prospective, our suggestion based on the studies in Sicilian population supports a female-specific gene-longevity association, by emphasizing the paradoxical role of socio-cultural habits in female longevity [33]. This concerns the HFE gene, the most telomeric HLA class I gene, codifying for a class I α chain, the HFE protein, which seemingly no longer participates

Table 2 Cellular and humoral immune modification in offspring from longevity families compared to their AM controls

T and B cell Phenotypes and Products	Changes	References
Naïve T cells ($CD3^+CD8^+CD45RA^+CCR7^+CD27^+CD28^+$)	Increase	[8]
Late differentiated effector memory T cells ($CD3^+CD8^+CD45RA^-CCR7^-CD27^-CD28^-$)	Decrease	[8]
TEMRA ($CD3^+CD8^+CD45RA^+CCR7^-CD27^-CD28^-$)	Decrease	[8]
Naïve B cells (IgD^+CD27^-)	Increase	[10]
Double Negative B cells ($IgG^+/IgA^+IgD^-CD27^-$)	Decrease	[10]
Serum IgM	Increase	[10]

in immunity. It has lost its ability to bind peptides due to a definitive closure of the antigen binding cleft that prevents peptide binding and presentation. The HFE protein, expressed on crypt enterocytes of the duodenum, regulates the iron uptake by intestinal cells, having acquired the ability to form complex with the receptor for iron-binding transferring. Mutations in HFE gene are associated with hereditary hemochromatosis, a disorder caused by excessive iron uptake [34,35]. Three common mutations, C282Y, H63D and S65C, have been identified in HFE gene. In particular, the C282Y mutation (a cysteine-to-tyrosine mutation at amino acid 282) destroys its ability to make up a heterodimer with β2-microglobulin. The defective HFE protein fails to associate to the transferring receptor, and the complex cannot be transported to the surface of the duodenal crypt cells. As a consequence, in homozygous people, two to three times the normal amount of iron is absorbed from food by the intestine, resulting in end-organ damage and reducing lifespan. Two other mutations, H63D (a histidine to aspartate at amino acid 63) and S65C (a serine to cysteine at amino acid 65), are associated with milder forms of this disease [34,35].

An association between C282Y mutation and longevity characterizes the Sicilian population studied [33]. In particular, women carriers of C282Y mutation had a higher frequency among the oldest old compared to control women (Table 3). Thus, the C282Y mutation may confer a selective advantage in terms of longevity in Sicilian women. Considering the historical and social context in which the generation of women under study lived, our data seem to propose that the possession of iron-sparing alleles significantly increases the possibility for women to reach longevity. For instance, in Sicily, many pregnancies and an iron-poor diet, consisting mainly in grains, vegetables, and fruits, were still the rule for women born at the beginning of last century. In fact, meat was available for men but not for women; this clearly explains how genetic background also interacts with culture habits [30,31,33].

Our data, showing the relevance of C282Y for women survival to late age, allow adding another piece of evidence to the complex puzzle of genetic and environmental factors involved in control of lifespan in humans. The complex interaction of environmental, historical and genetic factors, differently characterizing the various parts of a country, i.e. Italy, likely plays an important role in determining the gender-specific probability of attaining longevity [30,31,33,36].

Role of innate immunity genes in longevity: the paradigmatic case of TLR4, CCR5, COX-2 and 5-LO genes

According to evolutionary ageing theories, most of the parameters influencing immunosenescence appear to be under genetic control [32,37,38]. An example is given by the innate immune system, involved in neutralizing infectious agents [39]. It plays a beneficial role until the time of reproduction and parental care. In old age, a period largely not foreseen by evolution, it can determine an opposite and detrimental effect through chronic inflammatory responses ("antagonistic pleiotropy") [38,40]. Genetic pro-/anti-inflammatory variations in innate immune response are, indeed, thought to influence the susceptibility of age-related human diseases, by altering host response to environmental and endogenous stress [41]. Thus, they are able to determine a negative or positive control of inflammation, by affecting both interactions between host and microbes and survival of the individual and attainment of longevity. Furthermore, they appear both to be responsible, at least in large part, for different men and women strategies to achieve longevity, and to contribute to the preferential sex dimorphism of the age-related diseases [30,31,33].

From our investigations in Sicilian population, TLR4, CCR5, Cox2, 5-Lo genes can be considered good examples. They provide an ideal model to understand the different implications of their genetic variants in the risk of age-related diseases, i.e. atherosclerosis and prostate cancer (PC), and reciprocally in increased chance to attain longevity.

TLR4 gene (number accession of GenBank: NM-138554.1) codifies the best understood TLR member involved in recognition of LPS, the prototypic TLR4 ligand, and other exogenous and endogenous (i.e. HSPs, hyaluronic acid, β-defensin-2, ox-LDL, fibronectin and amyloid peptide) ligands. TLR4 activation implies a downstream signaling mediated by several intracellular adaptor molecules and the consequent activation of transcription

Table 3 Data from our investigations in Sicilian population

Gene	Alleles of genetic variants	Centenarians	Young controls (< 55 years)		P
		N = 35 females	N = 106 females		
HFE	C282	47 (84%)	132 (0%)		8.3×10^{-5} [33]
	282Y	9 (16%)	0 (0%)		
Gene	**Alleles of SNPs or genetic variants**	**Centenarians**	**Young controls (< 55 years)**	**MI patients (< 55 years)**	**P**
		N = 55 males	N = 127 males	N = 105 males	
TLR4	+896A	94 (85.4%)	239 (94.1%)	205 (97.6%)	< 0.001 [49]
	+896 G	16 (0.46%)	15 (5.9%)	5 (2.4%)	
		N = 123 males	N = 136 males	N = 133 males	
CCR5	WT	221(89.8%)	252 (92.6%)	263 (98.8%)	0.00006 [48]
	Δ32	25 (0.02%)	20 (7.4%)	3 (1.2%)	
		N = 96 males	N = 170 males	N = 140 males	
Cox-2	-765 G	122 (63.5%)	240 (70.6%)	232(82.8%)	0.0000007 [47]
	-765 C	70(36.5%)	100(29.4%)	48(17.2%)	
5-Lo	-1708 G	180 (93.7%)	302(88.8%)	224(80%)	0.00003 [47]
	-1708A	12(6.3%)	38(11.2%)	56(20%)	0.001
	21 C	176(91.7%)	299(88%)	225(80.4%)	
	21 T	16(8.3%)	41(12%)	55(19.6%)	
Genes	**Alleles of SNPs or genetic variants**	**Centenarians**	**Young controls (< 55 years)**	**PC patients (< 55 years)**	**P**
		N = 55 males	N = 125 males	N = 50 males	
TLR4	+896A	94 (85%)	235 (94%)	99 (99%)	0.001 [54]
	+896 G	16 (15%)	15 (6%)	1 (1%)	
Cox-2	-765 G	67 (61%)	176 (70%)	77 (77%)	0.05
5-Lo	-765 C	43 (39%)	74 (30%)	23(23%)	0.0007
	-1708 G	104 (95%)	223 (89%)	77 (77%)	
	-1708A	6 (5%)	27 (11%)	23 (23%)	
		N = 53 males		N = 50 males	
CCR5	WT	95 (89.6%)		97 (97%)	0.03 [53]
	Δ32	11(10.4%)		3(3%)	
Gene	**Alleles of SNPs or genetic variants**	**Centenarians**	**Young controls (< 55 years)**	**BF patients (30-60 years)**	**P**
		N = 42 females	N = 42 females	N = 42 females	
TLR4	+896A	81 (96.4%)	78 (92.9%)	76 (90.4%)	0.003 [55]
	+ 896 G	3 (3.6%)	6 (7.1%)	8 (9.6%)	

factors, such as NF-kB. This determines the production of different pro/anti-inflammatory mediators. These lasts, such as IL-10, are produced by the parallel activation of anti-inflammatory pathways to limit the potential tissue damage from excessive activation of the innate immune system [42]. SNPs seem to modulate both TLR4 activity and function. In human, only two SNPs, +896A/G (Asp299Gly; rs4986790) and +1196 C/T (Thr399Ile; rs4986791), have a frequency > 5%. They induce a blunted response to LPS, as first suggested by Arbour et al., and are phenotypically associated to changes in the production of cytokines, principally those carrying the Asp299Gly mutation [43-45]. Accordingly, recent literature data suggest the ability of this SNP to modulate the risk of major age-related diseases [42].

The CCR5 gene (number accession of GenBank: NM-00579) codifies for a G protein-coupled chemokine receptor, which regulates trafficking and effector functions of memory/effector Th1 cells, macrophages, NK cells and immature dendritic cells. CCR5 and its ligands are important molecules in viral pathogenesis. Recent evidence has also demonstrated the role of CCR5 in a variety of human diseases, ranging from infectious and inflammatory age-related diseases to cancer. A notable variant of CCR5 gene is a 32 bp (Δ32) deletion, which causes a frame shift mutation in exon 4 (CCR5Δ32; rs333) and determines stop protein maturation and loss of expression of functional CCR5 receptor [46]. Accordingly, it seems to have a protective role against CVD and other age-related diseases, such as PC. It, indeed,

determines a slower progression of atherogenesis or cancerogenesis as a consequence of an attenuated inflammatory response.

COX-2 gene maps in the 1q25 chromosome and codifies for the Cox-2 enzyme involved in the conversion of arachidonic acid to prostaglandins. Polymorphisms regulate its expression and hence prostanoid biosynthesis. In particular, it has been identified a guanine to cytosine substitution at position -765 G/C, located within a putative binding site for the transcription factor Sp1, associated to a reduction in the risk of clinical cardiovascular events. COX-2 is expressed at low levels in most tissues, but its expression enhances under inflammatory stimuli and in inflammatory age-related processes, i.e. atherosclerosis, rheumatoid diseases and cancer [47].

The 5-LO gene maps in the chromosome 10q11.2 and codifies the 5-Lo enzyme involved in the synthesis of LTs. The 5-LO pathway has been associated to atherosclerosis in mouse and human histological studies. Several SNPs have been described. In particular, the - 1708 G/A, -1761 G/A and 21 C/T SNPs in promoter region and exon-1 of 5-LO gene modify the gene transcription or the putative protein [47].

An over-expression of anti-inflammatory CCR5Δ32 variant, +896 G (299Gly) TLR4 allele, -765 C Cox-2 allele, -1708 G and 21 C 5-Lo alleles characterizes male Sicilian centenarians (Table 3) [47-49]. So, male centenarians are people who seem genetically equipped for defeating major age-related diseases. They present SNPs in the immune system genome (i.e. SNPs or other genetic variations, located within the promoter regions of pro-inflammatory cytokines) which, regulating the immune-inflammatory responses, seem to be associated to longevity [30-32]. Furthermore, centenarians are characterized by marked delay or escape from age-associated diseases, responsible for the high mortality in earlier ages. In particular, it has been demonstrated that centenarian offspring have an increased likelihood of surviving to 100 years and show a reduced prevalence of age associated diseases, such as CVD, and lower prevalence of CVD risk factors [1,30-32,50] Thus, genes involved in CVD may play an opposite role in human male longevity. Our data in male Sicilian population confirm this suggestion and emphasize the role of antagonistic pleiotropy in ageing and longevity [51,52]. A high frequency of proinflammatory CCR5wt variant, +896A TLR4 allele, -765 G Cox-2 allele, 1708A and 21 T 5-Lo alleles characterizes male Sicilian patients affected by MI (Table 3) [47-49]. In a recent study, we also found a similar overexpression of these proinflammatory SNPs in male Sicilian patients affected by PC (Table 3). Opposite data were obtained in male centenarians [53,54].

In contrast, female Sicilian centenarians have a different frequency of the alleles of +896A/G TLR4 SNP than that observed in male Sicilian centenarians. In particular, female Sicilian centenarians show an over-expression of the pro-inflammatory +896A TLR4 allele respect to female patients affected by Boutonneuse fever and age-matched controls (Table 3) [55].

On the other hand, pro-inflammatory responses are evolutionary programmed to resist fatal infections. Thus, it is not surprising that the genetic background of people that survive to an advanced age may be protective against infections [55].

Based on our data, we suggest that Sicilian men and women may follow different trajectories to reach longevity. For men it might be more important to control atherogenesis and cancerogenesis, whereas for women it might be more important to control infectious diseases [30,31].

In order to confirm our suggestions on the biological effects of +896A/G TLR4 SNP and its role in the pathophysiology of age-related diseases studied (i.e. MI and PC) and longevity, we recently assessed the levels of IL-6, TNF-α, IL-10 and eicosanoids in LPS-stimulated whole blood samples from 50 young healthy Sicilians, screened for the presence of this SNP. Both pro-inflammatory cytokines and eicosanoids were significantly lower in carriers bearing the +896 G TLR4 allele, whereas the anti-inflammatory IL-10 values were higher [56]. This suggests the ability of the +896 G TLR4 allele to mediate a better control of inflammatory responses induced by chronic stimuli, so likely decreasing the effects of atherogenetic damage and prostate carcinogens.

On the basis of data reported herein, some suggestions can be drawn. First, pathogen load, by interacting with the host genotype, determines the type and intensity of inflammatory responses, according to the pro-inflammatory status and tissue injury, implicated in the patho-physiology of major age-related diseases. Second, adequate control of inflammatory response might reduce the risk of these diseases, and, reciprocally, might increase the chance of extended survival in an environment with reduced pathogen load. Accordingly, a higher frequency of the anti-inflammatory +896 G TLR4 allele has been observed in centenarians [49].

Cytokine profile: a biomarker for successful ageing

Cytokines are considered key players in maintaining lymphocyte homeostasis [57,58]. Their function is not limited to induce response after an immune insult, but they can modulate the nature of response (cytotoxic, humoral, cell mediated, inflammatory or allergic) or, in contrast, they may cause non-responsiveness and active immune suppression [58]. Furthermore, sequence variations in several cytokine genes, such as IFN-γ and IL-10 genes, have been demonstrated to be associated with successful ageing and longevity [58]. On the other hand, individual

changes in type and intensity of immune response affecting life span expectancy and health ageing seem to have a genetic component. A well-preserved immune function characterizing the successful ageing has been found in centenarians [38]. Recent evidence suggests that centenarians seem to be genetically equipped gene polymorphism for overcame the major age-related diseases and polymorphisms in immune system genes involved in regulation of immune responses have been found associated to longevity. In particular, associations between both cytokine gene polymorphisms and longevity, and differential gender longevity in males and females, and reciprocally to age-related diseases have been demonstrated [38,58,59].

Our data in Sicilian population confirm these associations and suggest that differences in the genetic regulation of immune inflammatory processes might explain the reason why some people but not others develop age-related diseases and why some develop a greater inflammatory response than others. In particular, this suggestion seems to be suitable for some SNPs in IFN-γ and IL-10 genes (Table 4) [60-63].

IFN-γ gene codifies for a cytokine involved in defense against viruses and intracellular pathogens, and in induction of immune mediated inflammatory responses. Its production is genetically regulated. A variable length CA repeat sequence in the first intron of IFN-γ gene has been described to be associated with high IFN-γ production. Furthermore, a SNP, T to A (+874 T/A), at 59 end of the CA repeat region has been described and T presence has been related to high-producing microsatellite allele 2. This SNP coincides with a putative NF-κB binding site, which might have functional consequences for transcription of IFN-γ gene. Thus, this SNP might directly influence IFN-γ production levels associated to CA microsatellite marker [60].

IL-10 gene codifies for IL-10 cytokine. IL-10 is produced by macrophages, T and B cells. It is one of the major immune-regulatory cytokines, usually considered to mediate potent down-regulation of inflammatory responses. IL-10 production, independently on interaction with other cytokine gene products, is generally controlled by several polymorphic elements in the 5' flanking region of IL-10 gene. Multiple SNPs have been identified in human IL-10 5' flanking region and some of these (i.e. -592, -819, -1082) combine with microsatellite alleles to form haplotype associated with differential IL-10 production. These three SNPs in the IL-10 proximal gene region (considered potential targets for transcription regulating factors) might be involved in genetic control of IL-10 production, even if contrasting literature data have been reported. In particular, the homozygous -1082GG genotype seems to be associated with higher IL-10 production respect to G/A heterozygous and AA homozygous genotypes. Furthermore, this SNP seems to be functionally relevant. It has been demonstrated that -1082 A carriers (low producers) seem likely develop a major number of chronic inflammatory diseases [61-63].

Our results demonstrated an increase of subjects carrying the -1082 G IL-10 allele in centenarian men [61-63]. This allele is associated to significantly increased IL-10 production. Conversely, we observed that the frequency of -1082A allele, associated to low IL-10 production, was significantly higher in MI patients (Table 4) [63]. Thus,

Table 4 Cytokine data from our studies in Sicilian population

Gene	Genotypes	Centenarians	Young controls (< 55 years)		P
		N = 31 males	N = 161 males		
IL-10	-1082GG	18 (58%)	55 (34%)		< 0.025 [61]
	-1083GA	9 (29%)	88 (55%)		
	-1082AA	4 (13%)	18 (11%)		
		N = 72 males	N = 115 males		
IL-10	-1082GG	33 (46%)	32(28%)		0.019 [62]
	-1083GA	34(47%)	64(56%)		
	-1082AA	5(7%)	19(16%)		
		Centenarians	Young controls (< 55 years)	MI patients (< 55 years)	P
		N = 52 males	N = 110 males	N = 90 males	
IL-10	-1082GG	25 (48.1%)	26(23.6%)	17 (18.9%)	0.003 [63]
	-1083GA	23 (44.2%)	56 (50.9%)	29 (32.2%)	
	-1082AA	6(11.5%)	28 (25.5%)	44 (48.9%)	
Genes	**Alleles of SNP**	**Centenarians**	**Young controls (< 55 years)**		P
		N = 142 females	N = 90 females		
IFN-γ	+ 874 T	102 (35.9%)	85 (47.2%)		0.02 [60]
	+ 874A	182 (64.1%)	95 (52.8%)		

high IL-10 production seems to be protective vs. MI and a possible biomarker for longevity. People with exceptional longevity have genetic factors (i.e. protective factors for CVD) that modulate ageing processes [63]. This supports the opinion that a genetic background protective against CVD is a component of longevity. On the other hand, our immune system has evolved to control pathogens and pro-inflammatory responses are likely programmed by evolution to resist fatal infections. From this prospective, low IL-10 production is correlated with increased resistance to pathogens. In older ages not evolutionally programmed, increased IL-10 levels might better control inflammatory responses induced by chronic vessel damage and reduce the risk for atherogenetic complications. These conditions might permit to achieve exceptional ages in an environmental with a reduced pathogen load [63].

In contrast, female Sicilian centenarians are characterized by an over-expression of +874 INF-γ allele (Table 4) [60]. The INF-γ production is also influenced by hormonal control fundamentally mediated by 17β extradiol. Hormonal regulation of this cytokine has been suggested to modulate, in part, the ability of estrogens to potentiate many types of immune responses and to influence the disproportionate susceptibility of women for immune-inflammatory diseases. Thus, gene variants representing genetic advantage for one gender might not be reciprocally relevant for the other gender in terms of successful or unsuccessful ageing [60].

The data from Sicilian investigation add another piece to complex puzzle of genetic and environmental factors involved in the control of life span expectancy in humans. Studies on cytokine gene SNPs may promise to individuate a complex network of trans-inactive genes able to influence the type and strength of immune responses to environmental stressors, and as final result, conditioning individual life expectancy [60-63]. On the other hand, we recently suggested the possibility to use cytokine profile as biomarker of successful ageing, by evaluating through Lumines technology cytokine serum levels in 44 Sicilian nonagenarians and 79 control subjects (aged between 30 and 50 years old) [64]. IFN-γ and IL-2 levels are unmodified, suggesting a substantial maintenance of relevant T functions. In addition, a significant increase of IL-12 serum levels was observed. This condition might be associated with the increase of NK cell function with ageing. Furthermore, an increase of IL-13 and a reduction of IL-4 were found. Thus, the maintenance of some effector's mechanisms of immune-response characterizes advanced ages. From a general point of view, our data firstly confirm the age-related remodeling of cytokine network. Furthermore, they underline the presence of unchanged levels of some crucial cytokines useful in preserving key immune function in long-living persons [64].

Future perspectives

The ageing process and longevity are multi-factorial events. Genetic, epigenetic, stochastic and environmental factors seem to have a crucial role in ageing and longevity. Epigenetic is associated to ageing, as shown in the major number of studies. In particular, ageing is associated to a global loss of methylation state [65]. In addition, tissue-dependent age-related hypermetylation of specific DNA regions have been observed. Thus, it can be concluded that epigenetic age-related modification are stochastic and no linked to specific DNA region, while epigenetic changes linked to specific environmental stimuli are limited in specific DNA region [66,67]. These observations have led to address the research on epigenomics and its implication in ageing and longevity.

Epigenomics is the systematic study of the global gene expression changes due to epigenetic processes, but not to DNA base sequence changes. Epigenetic processes consist in heritable modification that result in a selective gene expression or repression and consequently in phenotype changes [68]. These changes include nucleosome positioning, post-translation histone modifications, action of small RNAs, DNA replication timing, heterochromatinization and DNA methylation [69]. This last one consists in the addition of a methyl group (-CH3) in the carbon 5 of cytosines, particularly in the CpG dinucleotide. This condition particularly concerns the CpG islands (CpGIs), located at the regulatory site of gene promoter regions. Methylation rate is associated to transcriptional regulation. In particular, gene silencing is associated to increase of -CH3 groups on DNA, conversely hypometylation of CGIs is associated to an open chromatin state resulting in gene expression [70].

Although the association between ageing and epigenetic is a real evidence, processes involved are not clear. Certainly, the nutrition affects epigenetic modifications. Nutrients can be active on specific sites. For example, vitamin B12, vitamin B6, riboflavin, methionine, choline and betaine, well known as folates, regulate levels of S-adenosylmethionine and S-adenosylhomocysteine, donor of -CH3 group and methyltransferase inhibitor respectively [71]. Curcumin, resveratrol, polyphenols and flavonoids, phytoestrogen, and lycopene are also considered key nutritional factors both for regulation of enzyme involved in acetylation and deacetylation mechanism and for one-carbon metabolism [71,72]. A diet rich in vegetables and fruit, such as Mediterranean diet, may contain these nutrients. Sicilian centenarians are characterized to observe this kind of diet, as we reported [73]. Since genetic and environmental factors contribute to longevity, it may suggest that epigenetic events associated to the modifications diet-induced are very important for successful ageing processes. Furthermore, several literature data reported a possible link between epigenetic and several age-related diseases, such as cancer, metabolic

syndrome, diabetes and neurodegenerative disorders. Stable propagation of gene expression from cell to cell during disease pathogenesis is regulated by epigenetic mechanisms. For example, during the diabetes onset epigenetic changes act on insulin and insulin metabolism regulating the gene coding [74]. In particular, a recent study has demonstrated that human insulin gene and mouse insulin 2 gene expression are under control of epigenetic changes in CpGIs. Insulin non expressing cells are, indeed, methylated in the promoter region of insulin coding gene, while insulin expressing cells are completely demethylated in the same site resulting in insulin gene expression [75]. Another study on monozygotic twin has demonstrated that insulin resistance is also under control of DNA methylation [76]. Alterations in insulin pathway are known to be involved in metabolic disease, such as metabolic syndrome, insulin resistance and type 2 diabetes. Recent data also support the existence of a correlation between these alterations and Alzheimer's disease.

In the light of these observations, the purpose of our future studies will be to evaluate the weight of epigenetic changes in ageing and longevity, using centenarians as super-controls.

Abbreviations

AD: Alzheimer's disease; BF: Bustonmese fever; CCR5: CC chemokine receptor 5; COX-2: Cyclo-oxygenase 2; CRP: C reactive protein; CVD: Cardiovascular disease; HSPs: Heat-shock proteins; INF-γ: Interferon- γ; IL-6: Interleukin-6; IL-10: Interleukin-10; 5-LO: 5-lipoxygenase; LPS: Lipopolysaccharide; LTs: Leukotrienes; MI: Myocardial infarction; oxLDL: Oxidized-Low Density Lipoproteins; PC: Prostate cancer; PGs: Prostaglandins; SNPs: Single nucleotide polymorphisms; TLR4: Toll-like-receptor-4; TNF-α: Tumor necrosis factor-α.

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Author details

[1]Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, Palermo 90134, Italy. [2]Department of Internal Medicine II, Center for Medical Research, University of Tübingen, Tübingen Ageing and Tumor Immunology group, Waldhörnlestr. 22, Tübingen 72072, Germany.

Authors' contributions

CRB, GA, SB, MB, AM and GCR wrote the first draft. Subsequent drafts were written by CRB, who had the overall supervision of the review processing. All authors edited the paper and approved its final version.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 3

Cytokine serum profile in a group of Sicilian nonagenarians.

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Marisa Palmeri [a], Gabriella Misiano [a], Mariano Malaguarnera [b], Giusi Irma Forte [a], Loredana Vaccarino [a], Salvatore Milano [a], Letizia Scola [a], Calogero Caruso [a], Massimo Motta [b], Domenico Maugeri [b] & Domenico Lio [a]

[a] Department of Biopathology and Medical and Forensic Biotechnology, University of Palermo, Palermo, Italy

[b] Department of Aging, Neurological and Urological Science, University of Catania, Catania, Italy

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CYTOKINE SERUM PROFILE IN A GROUP OF SICILIAN NONAGENARIANS

Marisa Palmeri,[1] Gabriella Misiano,[1] Mariano Malaguarnera,[2] Giusi Irma Forte,[1] Loredana Vaccarino,[1] Salvatore Milano,[1] Letizia Scola,[1] Calogero Caruso,[1] Massimo Motta,[2] Domenico Maugeri,[2] and Domenico Lio[1]

[1]*Department of Biopathology and Medical and Forensic Biotechnology, University of Palermo, Palermo, Italy*
[2]*Department of Aging, Neurological and Urological Science, University of Catania, Catania, Italy*

☐ *The aim of our study was to evaluate the possibility of using multiplex analysis of the cytokine profile as a marker for successful aging by comparing cytokine plasmatic levels of a group of Sicilian nonagenarians with those of young controls. We analyzed a panel of 17 cytokines, comprehensive of haematopoietic factors T helper 1 (Th1), Th2, inflammation regulatory cytokines, and chemokines. The assay was carried out using the Luminex system. Interleukin (IL)-6 levels ($p = 0.01$) were increased in nonagenarians, whereas no modifications of other proinflammatory cytokines and chemokines were observed. Interferon-gamma (IFNγ) and IL-2 levels are unmodified, suggesting a substantial maintenance of relevant T cell functions. In addition, a significant increase of IL-12 serum levels in nonagenarians versus young controls that might be related to the increase of natural killer (NK) cell functions characterizing aging processes was observed. The analysis of Th2 cytokines show an increase of IL-13 and a reduction of IL-4 levels mirroring the maintenance of some effector's mechanisms of the immunoresponse in advanced ages. Our results suggest that the multiplex analysis of cytokine levels might be useful in defining a successful aging profile.*

Keywords circulating cytokine levels, immunoassay, Luminex, serum profile, successful aging

INTRODUCTION

Centenarians provide the best example of successful aging. They are people who have escaped major age-related diseases and have reached

Address correspondence to Giusi Irma Forte, PhD, Department of Biopathology and Medical and Forensic Biotechnology, University of Palermo, Corso Tukory 211, Palermo 90134, Italy. E-mail: giusiforte@unipa.it

the extreme limit of human life in good clinical condition.[1] For example, laboratory parameters of centenarians indicate that they are characterized by reduced levels of blood glucose, transaminases, cholesterol, and platelets with respect to older subjects with an age range between 65 and 85 years.[2] In most of the cases, histories of centenarians reveal them to be free of cancer, dementia, diabetes, and cardiovascular diseases, which is surely due to a successful interaction between environmental and genetic factors.

Advancing age is correlated to an increase of inflammatory response, which is believed to be a direct consequence of the continuous attrition caused by antigenic load during the life-span—a condition that is commonly called by the authors "inflamm-aging,"[3] also sustained by the immune system remodeling, which physiologically occurs during aging ("immuno-senescence"). It is a slow but inexorable process leading to an immune system that shows peculiar features, predisposing older people to a different kind of reaction to injuries in comparison to young individuals.[4]

In particular, the dedicated immune system tissues, such as as thymus, bone marrow, spleen, and lymph nodes, undergo involution or they show regressive phenomena. The number of germinal centers is reduced, and there is a progressive loss of cell-mediated immunity, generally recognizable by a tendency toward the switch from Th1 vs. Th2 type response.[5] Moreover, in the aging process, and even in centenarians, there is a redistribution of monocytes, neutrophils, B and T subsets, and a quite normal number of T lymphocytes, even if these cells mostly show a memory phenotype, whereas a progressive reduction of virgin cells is observable, which makes the old individuals unable to respond to antigens not previously encountered.[6]

Several studies have largely demonstrated an important role of genetic background in the achievement of advanced age. A group of genes that has often been tested for association with successful aging is that which influences inflammation and immune responses. Among these, there are genes coding for interleukins. Our group has demonstrated that particular cytokine polymorphisms, especially located on the functional promoter sequence of important cytokines genes such as interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), and IL-10, may influence the susceptibility to age-associated diseases or may alternatively contribute to the genetic background associated with longevity.[7–15] These data highlight the role of cytokine production in determining the successful or unsuccessful aging phenotype. In this view, it seems of some interest to check the possibility of identifying a serum cytokine profile that might characterize successful aging. To reach this result, a technology that allows a contemporaneous and highly precise determination of a large number of cytokines should be applied.

In this article, we report data evaluation by Luminex technology of the blood levels of 17 pro- and anti-inflammatory cytokines and chemokynes, which are crucial in the orchestration of the immune response, in order to identify the circulating cytokine profile of subjects >90 years old.

MATERIALS AND METHODS

Subjects Recruitments

In our study, two groups of subjects were tested. In particular, we analyzed sera from 44 Sicilian ultra-nonagenarians (age >90 years) and 79 control subjects consisting of a group of healthy young Sicilian individuals, aged between 30 and 50 years old. None of ultra-nonagenarian subjects recruited for the study show any major age-related diseases or severe cognitive impairment (dementia or neoplastic, cardiovascular, or infectious disease), nor did they receive any drugs that influence immune functions at the time of the study. Their age was verified by archival records at the City Hall and/or church registries, verifying the concordance between reported age and personal chronologies (age of marriage and of military service for men, age of first and last pregnancy for women, age of children, etc.). The Sicilian ethnicity of all the participants at the study was established by confirming that all four grandparents were born in Sicily, in order to gain a certain guarantee of a homogeneous population, as immigration and intermarriage have historically been rare at the beginning of the last century.[14] Written informed consent for enrolling in the study and for personal data management was obtained from all subjects according to Italian laws.

Evaluation of Cytokine Blood Level Assay

Each subject underwent a fasting blood sampling. The blood samples were collected in lithium heparin additioned vacutainer tubes, immediately centrifuged at 1800 rpm for 15 min to isolate cell free plasmas that were immediately stored at −80°C.

Immediately before the cytokine assay, thawed samples were centrifuged at 12,000 rpm for 5–10 min to allow precipitation of any lipids excess that may interfere with subsequent analysis.

The samples were tested for a panel of 17 cytokines and chemokines [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), granulocyte-macrophage

colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF)] using Bio-plex kit (BioRad, Milan, Italy) and following the manufacturer's instructions. The assay was carried out using the Luminex system (BioRad, München, Germany), based on the measurement of fluorescent signals released by a suspension of microspheres, bringing immobilized multiplex cytokine specific antibodies in 96-well plates. The combination of a fluorimetric signal of microspheres with that released by a secondary antibody allows us to measure cytokine concentration–related signals converted by a processor. Briefly, 50 µL of samples diluted 1:4 in a dilution buffer were incubated at room temperature in the presence of beads conjugated with specific antibodies for the different cytokines. After a wash to remove the excess of not bound serum components, an incubation with the biotin conjugated secondary antibodies was performed. Finally, after another washing step and the streptavidin-PE complex addition, the fluorimetric signal was detected by using the Luminex plate reader. The assay was performed using an eight-point standard curve for every cytokine. Samples were analyzed on a Luminex 100 device (BioRad), and the data were evaluated using the Bio-Plex Manager software (BioRad). Standards, internal controls, and samples were reported as means of duplicate measurements.

Statistics

All the data are shown as mean concentrations (pg/ml) ± standard error (SE). Differences in cytokine levels among nonagenarians and healthy controls were assessed by the Mann–Whitney test. Differences were considered significant when a p value <0.05 was obtained.

RESULTS AND DISCUSSION

As we already described in the Introduction, aging is characterized by a remodeling process of immune system, where several functions are reduced, whereas others remain unchanged or are increased.[15] A few notable instances are, surely, a persistence of a low-grade chronic inflammatory status and a consistent increase of activated cells that progressively fill the immunological space,[4] considered as the compartment occupied by the immune cell subsets and a reduced capability to cope with new immunological stimulations. In this scenario the reshaping of cytokine production network seems to play a central role.

In this article, we focus on the evaluation of the circulating cytokine profile of a group of healthy nonagenarians in an attempt to describe a plasmatic cytokine profile that might characterize successful ageing. To

reach this goal the highly sensitive multiplex Luminex technology is applied to minimize differences in cytokine evaluation due to differences of performances among methodologies useful to measure a single cytokine at a time.

The selected panel of cytokines measured included the following: (a) hematopoietic cytokines; (b) proinflammatory and anti-inflammatory cytokines and chemokines; (c) Th1, Th2, and Th17 cytokines.

As reported in Table 1, the levels of the three hematopoietic cytokines analyzed (IL-7, G-CSF, and GM-CSF) are not significantly different among young and nonagenarians groups. However, it seems relevant that IL-7, implied in lymphopoiesis, is reduced in nonagenarians with a difference with young subjects near the statistically significant threshold. As reported by Pawelec et al.[6] dysregulated hematopoiesis is seen in older individuals, raising the possibility that this could contribute to altered immune function in aged persons. Actually, data from some experimental models seems to suggest that a reduced production of IL-7 might be implied in

TABLE 1 Plasmatic Cytokine Concentration Expressed as Mean (pg/mL) ± Standard Error

Cytokines	Young Controls (79)	Nonagenarians (44)	P Value
IL-7	1.60 ± 0.10	1.0 ± 0.30	0.06
G-CSF	0.55 ± 0.15	0.30 ± 0.08	0.14
GM-CSF	5.11 ± 1.06	11.50 ± 4.41	0.16
IL-2	11.05 ± 1.49	9.30 ± 2.05	0.11
IL-12(p70)	**2.88 ± 0.6**	**7.20 ± 1.53**	**0.01**
IFN-g	10.22 ± 5.93	8.24 ± 2.69	0.27
IL-4	**0.55 ± 0.08**	**0.35 ± 0.05**	**0.03**
IL-5	0.65 ± 0.09	0.66 ± 0.06	0.76
IL-13	**0.77 ± 0.05**	**1.23 ± 0.19**	**0.02**
IL-1b	11.89 ± 4.02	11.24 ± 5.40	0.26
IL-6	**5,16 ± 3.61**	**11.18 ± 2.53**	**0.01**
TNF-a	5.80 ± 1.99	7.72 ± 1.61	0.81
IL-10	0.44 ± 0.04	0.75 ± 0.20	0.11
IL-17	7.36 ± 1.34	17.10 ± 5.32	0.09
IL-8	31.08 ± 84.9	86.10 ± 5.89	0.41
MCP-1	79.90 ± 17.06	180.80 ± 29.57	0.14
MIP-1b	125.70 ± 10.71	189.90 ± 7.54	0.47

Differences in cytokine levels among nonagenarians and healthy controls were assessed by the Mann–Whitney test.

progressive reduction of the number of naive lymphocytes associated with aging.[16]

One of the major characteristics of senescence is the constant presence of a low-grade inflammatory status characterizing aging. This subclinical inflammation produces a persistent immune system activation, resulting in continuous low-grade tissue damage, as well as in the reduction of the normal immune system response to new antigens caused by a net consuming of naive cells.[3,4,17] As reported in Table 1, nonagenarians show a significant increased level of IL-6 (p value = 0,01), which seems to suggest that an increased IL-6 level might be detected also in a successful aging phenotype to confirm the age-dependent pro-inflammatory imbalance. Moreover, increased levels of IL-6 could also be related to the increased level of IL-17 (Table 1), even if the result did not reach statistical significance (p = 0.09), as IL-6 acts to enhance the Th17 lymphocytes' activity by stimulating the production of IL-17, which is involved in inflammation and in the amplification of the inflammatory response. However, the concentrations of other proinflammatory and anti-inflammatory cytokines and chemokines crucial in the balance of inflammation (IL-1-beta, TNF-a, IL-8, MCP-1, MIP-1b, and IL-10), are not significantly different between the two groups of subjects, and this allows us to speculate that the cytokine profile of the studied nonagenarians is characterized by a very low grade of proinflammatory cytokine signature. In addition, the slight increase of antinflammatory IL-10 observed is in agreement with our previous description of the genetic background owned by centenarians, as we previously found among these subjects a significant increase of the frequency of carriers possessing the allele related to higher IL-10 production.[10,12]

IFN-γ and IL-2 levels are not modified in nonagenarians with respect to the controls. As reported by different groups, Th1 and IL-2 cytokine production is generally decreased in older subjects,[6] even if in selected healthy old subjects (SENIEUR protocol) these cytokines seem to be normally secreted.[6] Our data obtained in healthy nonagenarians seem to suggest that the preservation of IFN-γ and IL-2 production might be one of the components of the successful aging phenotype. In particular the maintenance of an equilibrated concentration is favorable to prevent an excessive or prolonged Th1 response. Concerning IL-2 secretion maintenance in the oldest subjects, in our previous study, no modifications of genotypic and allelic frequencies of the IL-2 functional polymorphism −330 T/G were demonstrated.[18]

In our study we found a statistically significant increase in serum levels of IL-12 in nonagenarians in comparison with controls (p value = 0.01) (Table 1). Conflicting data on IL-12 production in old subjects have been published.[6] Both increase and reduction in IL-12 secretion have been

reported in animal experimental models, as well as after in vitro stimulation of human peripheral blood cells.[6] Our data, which were obtained by evaluating IL-12 serum levels, might be related to the increase of monocytes and NK cells, which characterize aging processes, mirrored by a very well-preserved cytotoxic activity. Indeed, a well-preserved NK cell activity can be considered a factor of longevity.

It is reported that advanced-aged individuals are characterized by high plasma levels of immunoglobulins.[19] In particular IgG, IgA, and IgE levels are increased. Analysis of Th2 cytokines shows similar IL-5 levels among nonagenarians and young controls. Instead the analysis of other Th2 cytokines shows divergent results. Actually, a significant reduction of IL-4 levels ($p = 0.04$) is accompanied by a significant increase of IL-13 levels in nonagenarians versus controls ($p = 0.02$). In a recent article, we focused on B cells in the aged by studying the expression of some surface markers. In particular, in older people and in centenarians, there was an increase of CD27 + B cells with a decrease of CD27 − B lymphocytes. CD27 is considered a marker of primed memory B cells. The decrement of virgin CD27 − B lymphocytes cells and the concurrent increase of memory CD27 + B lymphocytes seem to have an impact on the antibody repertoire of older individuals.[19–21] In this view, our data might be related to the different role played by IL-4 and IL-13 in the Th2 switch and B cell response maintenance in aging. Actually the Th2 switch, where IL-4 has a major key role, is already strongly reduced in advanced aged individuals, where there is a higher ratio between differentiated memory cells and virgin naïve ones, whereas, IL13 is mainly produced by primed Th2 cells.[22] So, the contemporaneous circulating IL-4 reduction and IL-13 increase detected in nonagenarians might mirror Th2 committed cells related to increasing age. Our results taken together confirm that, although the cytokine profile is modified by aging, we were able to show that long-living persons maintain unchanged levels of some crucial cytokines useful in preserving crucial immune-system function, and this might contribute to a person reaching such an advanced age.

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CHAPTER 4

Single nucleotide polymorphisms (SNPs) of pro-inflammatory/anti-inflammatory and thrombotic/fibrinolytic genes in patients with acute ischemic stroke in relation to TOAST subtype.

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Single nucleotide polymorphisms (SNPs) of pro-inflammatory/anti-inflammatory and thrombotic/fibrinolytic genes in patients with acute ischemic stroke in relation to TOAST subtype

Antonino Tuttolomondo [a,*], Domenico Di Raimondo [a,1], Giusi Irma Forte [b,1], Alessandra Casuccio [c], Loredana Vaccarino [b], Letizia Scola [b], Rosaria Pecoraro [a], Antonia Serio [a], Giuseppe Clemente [a], Valentina Arnao [c], Marisa Palmeri [b], Gabriella Misiano [b], Domenico Lio [b], Antonio Pinto [a], Giuseppe Licata [a]

[a] Dipartimento Biomedico di Medicina Interna e Specialistica, Università degli Studi di Palermo, Italy
[b] Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Università degli Studi di Palermo, Italy
[c] Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Università degli Studi di Palermo, Italy

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ABSTRACT

Background: The genetic basis of complex diseases like ischemic stroke probably consists of several predisposing risk factors, such as genes involved in inflammation and thrombotic pathways. On this basis the aim of our study was to evaluate the role of SNPs (single nucleotide polymorphisms) of some pro-inflammatory/anti-inflammatory and coagulation/fibrinolytic genes in patients with acute ischemic stroke.
Methods: The study population consisted of 144 consecutive Caucasian adult patients who were hospitalized in the Internal Medicine Department at the University of Palermo between November 2006 and January 2008, and who met inclusion criteria. The cases were patients admitted with a diagnosis of acute ischemic stroke, and age-matched (±3 years) control subjects: patients admitted to our Internal Medicine Department for any cause other than acute cardiovascular and cerebrovascular events and for routine checkup examinations.
Methods: Molecular analysis of alleles at the −308 nucleotide (−308G/A) of TNF-α gene, −1082/−819 haplotypes of IL-10 gene, IL-1RN exon 2 VNR polymorphism, alleles at the −174 nucleotide (−174G/C) of IL-6 gene, PAI-1675 5G/4G polymorphism, alleles at the −7351 nucleotide (−7351C/T) of tPA gene was undertaken in both patient groups.
Results: We analyzed 96 subjects with acute ischemic stroke and 48 control subjects. We observed a significantly higher frequency of IL-10 1082 AA genotype in stroke patients with a significant risk trend. We also reported a higher frequency in stroke subjects with a significant risk trend of the TPA 7351-CT genotype and of IL-1RN-VNTR 86 bp 2/2 genotype. Moreover, we observed a significant relationship with TOAST subtype only with regard to CC TPA genotype and 1/1 IL-1 VNTR 86 bp and lacunar strokes.
Conclusions: Ischemic stroke is a common multifactor disease, which is affected by a number of genetic mutations and environmental factors. Our findings showing a relationship between pro-inflammatory/anti-inflammatory and thrombotic/fibrinolytic genes SNPs and ischemic stroke may contribute to delineate a possible stroke risk profile in subjects with cerebrovascular risk factors.

1. Introduction

Ischemic stroke is a common multifactor disease, which is affected by a number of genetic mutations and environmental factors. The genetic basis of multifactor diseases like ischemic stroke probably consists of several predisposing risk factors that can interact with environmental factors to produce the disease phenotype. Recent data suggest that inflammatory reactions are involved in the pathogenesis of cerebral ischemia [1]. Nevertheless not only inflammation but also coagulation and fibrinolytic pathways have been involved in stroke pathogenesis.

Several frequent polymorphisms have been identified in the gene encoding TNF-α [2]. Among them, a G to A substitution located at position −308 in the promoter region was suggested as affecting the rate of transcription of the TNF-α gene, the less common A allele (TNF2) being associated with higher constitutive and

* Corresponding author. Tel.: +39 09 16552 128; fax: +39 09 16552 285.
E-mail address: lotuttolo@unipa.it (A. Tuttolomondo).
[1] These authors contributed equally to this work.

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inducible levels of transcription [3–6]. Only a few studies related to the connection of the TNF-a polymorphisms and stroke were published earlier [7–9].

While TNF-α is generally pro-inflammatory, IL-10 limits and terminates inflammatory reactions. Interleukin 10 is a potent suppressor of TNF-α, IL-1β, IL-1 [10].

Heritable differences are reported in IL-10 production, and several polymorphic sequences have been identified in the IL-10 gene promoter [11–13].

Three common single nucleotide polymorphisms (SNPs) in the IL-10 gene promoter (−1082 G/A, −819 C/T, −592 C/A) show strong linkage disequilibrium and form three common haplotypes, designated as [ATA] [ACC] and [GCC] haplotypes. The [ATA] haplotype has been associated with decreased synthesis of IL-10 [14] and is frequently associated with pathological conditions [14].

The IL-1 RA, a naturally occurring antagonist of IL-1 activity, appears to play a major role in controlling the pro-inflammatory activities of IL-1β in vivo. A variable number of 86 bp tandem repeat (VNTR) polymorphism described in intron 2 of the IL-1 receptor antagonist (IL-1RA) gene has been studied as a predisposing factor for atherosclerosis and other aging associated diseases [15].

Impaired fibrinolytic function secondary to elevated plasminogen activator inhibitor-1 (PAI-1) levels has been implicated in ischemic stroke. Polymorphisms in the plasminogen activator inhibitor-1 (PAI-1) gene, as the 4G allele of the PAI-1 4G/5G promoter polymorphism, associated with higher levels of PAI-1, and tissue-type plasminogen activator (tPA) levels, have been implicated in stroke pathogenesis [16]. High levels of plasminogen activator inhibitor type 1 (PAI-1) have been implicated as a risk factor for cardiovascular disease [17], but its precise role remains controversial.

On this basis our study is devoted to the contemporary evaluation of the role of gene polymorphisms involved in pro-inflammatory, anti-inflammatory and coagulation pathways in patients with acute ischemic stroke, with the goal of delineating a possible genetic risk profile useful in prevention and therapy strategies.

2. Materials and methods

2.1. Patient selection

The study population consisted of 1400 consecutive Caucasian adult patients who were hospitalized in the Internal Medicine Department at the University of Palermo between November 2006 and January 2008, and who met inclusion criteria. The cases were patients admitted with a diagnosis of acute ischemic stroke, and age-matched (±3 years) control subjects: patients admitted to our Internal Medicine Department for any cause other than acute cardiovascular and cerebrovascular events and for routine checkup examinations.

Inclusion criteria: Stroke was defined by focal neurological signs or symptoms thought to be of vascular origin that persisted for >24 h, confirmed by brain CT and/or MRI in baseline conditions and brain CT with contrast medium after 48–72 h [7].

Cardiovascular risk factors were evaluated for both cases and controls on the basis of the criteria shown below. Hypercholesterolemia was defined as the presence of total cholesterol blood levels ⩾ 200 mg/dL. Hypertension was defined as present if subjects had been previously diagnosed according the World Health Organization/International Society of Hypertension guidelines and were routinely receiving antihypertensive therapy. Patients were defined as type 2 diabetics if they had known diabetes treated by diet, oral hypoglycemic drugs or insulin before stroke.

Previous coronary artery disease was determined on the basis of a history of physician-diagnosed angina, myocardial infarction, or any previous revascularization procedure assessed by a questionnaire.

Previous cerebrovascular disease (TIA/ischemic stroke) was assessed by history, specific neurological examination performed by specialists, and hospital or radiological (brain computed tomography or brain magnetic resonance) records of definite previous stroke.

Subjects were classified as having previous peripheral artery disease (PAD) when they had a history of ABI <0.9 and/or of intermittens claudicatio or of critical limb ischemia or when they had undergone a peripheral arterial bypass or amputation.

The study protocol was approved by the local ethics committee, and all participants gave written informed consent.

The type of acute ischemic stroke was classified according to the TOAST classification [18]: (1) Large Artery AtheroSclerosis (LAAS); (2) CardioEmbolic Infarct (CEI); (3) LACunar infarct (LAC); (4) Stroke of Other Determined Etiology (ODE); (5) Stroke of UnDetermined Etiology (UDE).

Large Artery AtheroSclerosis (LAAS): These patients have clinical and brain imaging findings of either significant (>50%) stenosis or occlusion of a major brain artery or branch cortical artery, presumably due to atherosclerosis. Clinical findings include those of cerebral cortical impairment (aphasia, neglect, restricted motor involvement, etc.) or brain stem or cerebellar dysfunction. Cortical or cerebellar lesions and brain stem or subcortical hemispheric infarcts greater than 1.5 cm in diameter on CT or MRI are considered to be of potential large-artery atherosclerotic origin. Supporting evidence by duplex imaging or arteriography of a stenosis of greater than 50% of an appropriate intracranial or extracranial artery is needed. Diagnostic studies should exclude potential sources of cardiogenic embolism.

CardioEmbolic Infarcts (CEI): This category includes patients with arterial occlusions presumably due to an embolus arising in the heart. Cardiac sources are divided into high-risk and medium-risk groups based on the evidence of their relative propensities for embolism. At least one cardiac source for an embolus must be identified for a possible or probable diagnosis of cardioembolic stroke. Clinical and brain imaging findings are similar to those described for large-artery atherosclerosis. Evidence of a previous TIA or stroke in more than one vascular territory or systemic embolism supports a clinical diagnosis of cardiogenic stroke. Potential large-artery atherosclerotic sources of thrombosis or embolism should be eliminated. A stroke in a patient with a medium-risk cardiac source of embolism and no other cause of stroke is classified as a possible cardioembolic stroke.

LACunar Infarct (LAC): The patient should have one of the traditional clinical lacunar syndromes and should not have evidence of cerebral cortical dysfunction. A history of diabetes mellitus or hypertension supports the clinical diagnosis. The patient should also have a normal CT/MRI examination or a relevant brain stem or subcortical hemispheric lesion with a diameter of less than 1.5 cm demonstrated.

Stroke of Other Determined Etiology (ODE): This category includes patients with rare causes of stroke, such as non-atherosclerotic vasculopathies, hypercoagulable states, or hematological disorders. Patients in this group should have clinical and CT or MRI findings of an acute ischemic stroke, regardless of the size or location. Diagnostic studies such as blood tests or arteriography should reveal one of these unusual causes of stroke. Cardiac sources of embolism and large-artery atherosclerosis should be excluded by other studies.

Stroke of UnDetermined Etiology (UDE): In several instances, the cause of a stroke cannot be determined with any degree of confidence. Some patients will have no likely etiology determined despite an extensive evaluation. In others, no cause is found but the evaluation was cursory.

All the ischemic stroke patients underwent: medical history with recording of potential stroke risk factors, blood and

The TPA 7351 CT genotype was more frequent in stroke subjects in comparison of control subjects ($p = 0.019$), the CC has a significantly higher frequence ($p = 0.002$) in controls vs. stroke subjects, whereas no significant difference in TT genotype IL-10 1082 GG genotype frequence between stroke subjects and controls was observed (see Table 2).

IL-6 GC genotype was significantly more frequent in controls ($p = 0.028$), whereas no significant difference with regard of CC and GG genotypes between stroke patients and controls (see Table 2).

Regarding IL-1RN-VNTR 86 bp alleles we observed no significant difference between stroke patients and controls with regard of frequency of 1/1, 1, 2 and 1/3 genotypes, whereas the 2/2 genotype was significantly more frequent in stroke subjects ($p = 0.017$) vs. controls (see Table 2).

According to TOAST classification, subjects with acute ischemic stroke were classified: 37 LAAS, 34 lacunar and 25 cardioembolic (CEI).

For each polymorphism, genotype frequencies according to each TOAST subtypes and odds ratios are given in Table 3.

Concerning TNF-α promoter polymorphism at position −308 we observed no significant difference in frequency of GG, GA and AA genotypes between stroke subtypes (see Table 3).

Regarding polymorphism of IL-10 gene in position 1082, no significant difference with regard of frequency of GG, GA and AA genotypes between stroke subtypes, analogously regarding the IL-10 819 CT polymorphism, no significant difference with regard of frequency of CC, CT TT genotypes was observed between stroke subtypes (see Table 3).

Regarding PAI-1675 5G/4G polymorphism no significant difference with regard of frequency of 5G/5G, 5G/4G and 4G/4G genotypes was observed between stroke subtypes (see Table 3).

TPA CC genotype was significantly more frequent ($p = 0.028$) in subjects with lacunar subtype of stroke, whereas no significative difference was observed in frequency of CT genotype and TT genotype between stroke subtypes (see Table 3).

Regarding IL-6 polymorphism in position −174, no significant difference was observed in frequency of CC, CG and GG genotype was observed between stroke subtypes (see Table 3).

IL-1RN-VNTR 86 bp 1/1 genotype was significantly more frequent in subjects with lacunar subtype of stroke ($p = 0.05$), whereas with regard of frequency of 1/2, 1/3 and 2/2 genotypes no significant difference was observed between stroke subtypes (see Table 3).

4. Discussion

In our stroke patients and in controls we evaluated single nucleotide polymorphisms (SNPs) of some candidate genes involved in inflammatory and thrombotic/fibrinolytic mechanisms.

Our study shows a significant association between ischemic stroke and IL-10 −1082 AA and TPA-7351 CT genotypes.

The IL-10 GCC haplotype (G at position −1082, C at position −819, C at −592) is associated with high IL-10 secretion, while the ACC and ATA haplotypes are associated with intermediate and low IL-10 secretion, respectively [26,27,29].

Trompet et al. showed that the IL-10 −1082 AA genotype was associated with an increased risk of incident stroke. [28]. Moreover, van Exel et al. [30] demonstrated that elderly subjects with low IL-10 production capacity have an increased risk of incident stroke. The agreement of in vivo and in vitro data allows us to suggest a potential neuroprotective role of IL-10 against cerebral ischemia and on this basis it's possible to explain our finding concerning an association between stroke and IL-10 – 1082 AA genotype associated with low plasma levels of IL-10.

Nevertheless the majority of ischemic strokes occur because of thrombotic or thromboembolic occlusions; it would therefore be simplistic not to assess the possible role of polymorphisms regard-

Table 3
Proinflammatory/anti-inflammatory and thrombotic/fibrinolytic genotypes polymorphism prevalence in subjects with acute ischemic stroke in relation of TOAST subtype.

	LAAS (n.: 37)	lacunar (n: 34)	Cardioembolic (n: 25)	p	HR (95% CI)
Occurrence of the TNF-α −308 G/A genotype in patients with different subtypes of stroke according TOAST classification					
GG	30 (81.1%)	33 (97.1%)	20 (80.0%)	0.079	
GA	7 (18.9%)	1 (2.9%)	4 (16.0%)	0.104	
AA	0 (0%)	0 (0%)	1 (4.0%)	0.258	
Occurrence of the IL-10 Aft. 1082 AG genotype with different subtypes of stroke according TOAST classification					
GG	10 (27.0%)	9 (26.5%)	5 (20.0%)	0.797	
GA	4 (10.8%)	4 (11.8%)	6 (24.0%)	0.258	
AA	23 (62.2%)	21 (61.8%)	14 (56.0%)	0.870	
Occurrence of the IL-10 Aft. 819 CT genotypes with different subtypes of stroke according TOAST classification					
CC	23 (62.2%)	22 (64.7%)	18 (72%)	0.718	
CT	5 (13.5%)	4 (11.8%)	5 (20%)	0.657	
TT	9 (24.3%)	8 (23.5%)	2 (8%)	0.226	
Occurrence of the of PAI-1675 5G/4G genotype in patients with different subtypes of stroke according TOAST classification					
5G/5G	27 (72.9%)	26 (76.5%)	15 (60.0%)	0.363	
5G/4G	10 (27.0%)	8 (23.5%)	9 (36.0%)	0.564	
4G/4G	0 (0%)	0 (0%)	1 (4.0%)	0.258	
Occurrence of the TPA genotype with different subtypes of stroke according TOAST classification					
CC	2 (5.5%)	10 (29.4%)	5 (20.0%)	0.028	8.0 (1.2–52.6), p = 0.031
CT	27 (72.9%)	19 (55.9%)	17 (68.0%)	0.304	
TT	8 (21.6%)	5 (14.7%)	3 (12.0%)	0.565	
Occurrence of the IL-6 −74 GG genotype with different subtypes of stroke according TOAST classification					
CC	3 (8.1%)	4 (11.8%)	3 (12.0%)	0.841	
CG	16 (43.2%)	16 (47.0%)	14 (56.0%)	0.610	
GG	18 (48.6%)	14 (41.2%)	8 (32.0%)	0.426	
Occurrence of the IL -1 VNTR 86 bp genotype with different subtypes of stroke according TOAST classification					
1/1	14 (37.8%)	22 (64.7%)	10 (40.0%)	0.05	3.92 (1.2–12.5), p = 0.021
1/2	15 (40.5%)	6 (17.6%)	7 (28.0%)	0.104	
1/3	0 (0%)	0 (0%)	2 (8.0%)	0.054	
2/2	8 (21.6%)	6 (17.6%)	6 (24.0%)	0.828	

ing candidate genes involved in clotting mechanisms and in particular those involved in the balance between fibrinolysis and thrombosis such as tPA/PAI1 system.

Recently, a single nucleotide polymorphism located at position −7351 within the enhancer region of the TPA gene was identified and shown to be strongly correlated with endothelial TPA release rates [42]. The polymorphism was also found to have functional importance as it occurred within an Sp1 binding site, a factor promoting DNA transcription. Possession of a thymidine (T) allele was shown to inhibit Sp1 binding and was associated with less than half the TPA release observed in those homozygous for the cytosine (C) allele [41]. A recent study also reported an increased risk of ischemic stroke for the TT genotype [43].

Among genetic variants at the PAI-1 locus, the −1675-4G/5G SNP in the promoter has been demonstrated to be functional with a higher transcriptional activity for the 4G allele [36]. The 4G4G genotype has been associated with increased risk of MI [38]. The possible connection to stroke has received less attention, and published studies [40,41] and also our findings do not reveal a clear association.

We also reported a higher frequency of IL-1 VNTR 86 bp 2/2 genotype in stroke subjects compared to controls with a significant hazard ratio (OR = 6.05; $p = 0.017$) at regression analysis.

The penta-allelic polymorphic site in intron 2 of the IL-1RN gene consisting in a variable number tandem repeats (VNTR, 86 bp repeats) has been extensively investigated in relation to a variety of pathological conditions [33,34], but no study evaluated the possible association between genotypes of IL-1 VNTR 86 bp and ischemic stroke. So our finding could appear original and could offer a possible explanation of the association of the IL-1RN2 allele with ischemic stroke owing to an overall increase of IL-1 activity due to a less effective IL-1ra inhibitory activity [34,35] associated with this genotype [37,38].

In our stroke patients regarding PAI 5G/4G polymorphism in position −1675, we showed a higher frequency of 5G/4G genotypes, whereas regarding TPA 7351 CT polymorphism, we reported a higher frequency of CT genotype in stroke subjects vs. control subjects not confirming previous data regarding an association between TT genotype and ischemic stroke [39,44].

Although the common 4G/5G polymorphism in the promoter of the PAI-1 gene was suggested to be a risk factor for some of the thrombotic disorders, its significance in the development of thrombosis is still controversial. Previous studies reported that 4G/4G genotype is associated with a higher risk of thrombosis in vessels of internal organs especially in the portal veins [45].

A deletion/insertion polymorphism (4G or 5G) in the promoter of the plasminogen activator inhibitor type 1 gene has been suggested to be involved in regulation of the synthesis of the inhibitor, the 4G allele being associated with enhanced gene expression. A relationship between 4G/5G polymorphism and PAI-1 levels was found in patients with cardiovascular and metabolic diseases, but not in healthy subjects. In patients with deep vein thrombosis the 4G polymorphism of PAI-1 gene promoter may influence the expression of PAI-1 and it should be taken into consideration as a facilitating condition for pathological fibrinolysis together with other environmental and genetic factors [46]. Whether this has any significance in regard to the pathogenesis of arterial thrombosis remains to be proven.

Furthermore, in patients association between the 4G/5G genotypes and PAI activity was observed, with the highest PAI activity values in the 4G/4G genotype, intermediate in the 4G/5G genotype and the lowest in the 5G/5G genotype [47].

So our findings about higher frequence of 5G/5G and 4G/5G genotypes appear not easily explainable, maybe this finding could be due to a minor role of fibrinolysis in acute ischemic stroke and so to a spurious association.

The etiology of ischemic stroke affects prognosis, outcome, and management. We recently compared plasma levels of immuno-inflammatory variables in patients with acute ischemic stroke in relation to TOAST subtype showing that cardioembolic stroke has higher levels of immuno-inflammatory variables in the acute phase compared to other TOAST subtypes whereas lacunar stroke has the lowest plasma levels [48,49].

Analyzing the relationship between inflammatory and thrombotic-fibrinolytic genotypes in relation to diagnostic subtype cerebral ischemia is therefore an interesting perspective to better characterize the relationship between candidate genes from the standpoint of pathogenesis and cerebral ischemia. Only one study has examined this relationship [50] so our findings regarding the contemporary evaluation of the distribution of anti-inflammatory and thrombotic-fibrinolytic genotypes in relation to diagnostic subtype of cerebral ischemia may be useful.

We reported a significant relationship with TOAST subtypes only regarding TPA 7351 CC genotype and 1/1 IL-1 VNTR 86 bp genotype and lacunar TOAST subtype vs. LAAS subtype with a significant risk trend for both. Regarding TPA 7351 CC polymorphism, TPA homozygous subjects for the cytosine (C) allele [41] compared to subjects with a thymidine (T) allele have higher plasma levels of TPA and this finding could contribute to defining the pathogenetic background of lacunar strokes, that we reported as characterized by a minor degree of immuno-inflammatory activation of the acute phase [42,43]. This aspect could be confirmed by our finding concerning and higher frequency of 1/1 IL-1 VNTR 86 bp genotype in lacunar stroke due to the fact that this genotype is associated with higher IL-1ra inhibitory activity resulting in a high degree of anti-inflammation [31,32].

Inflammation of the wall of these penetrating arteries and impairment of thrombotic/fibrinolytic balance may explain the significant association between these genotypes and lacunar type of ischemic stroke, although subsequent studies might further reveal the pathogenetic basis of genetic predisposition of lacunar and other subtypes of stroke.

A genetic model of stroke pathogenesis is expected to be multifactor, involving several genetic polymorphisms that each confer small increases in risk; on this basis we evaluated several aspects of possible stroke pathogenesis involving both inflammation and thrombosis, evaluating the possible contribution of several inflammatory/anti-inflammatory genotypes and thrombotic/fibrinolytic. Nevertheless, we observed significant differences with regard of prevalence of some cerebrovascular risk factor such as hypertension, cholesterol blood levels that could partially explain stroke risk in these patients, but this is probably due to the multifactorial nature of cerebrovascular risk encompassing genetic and acquired factors.

A possible limitation of our study is that not all genotype distribution was in Hardy–Weinberg equilibrium (IL-10 1082AG and 819CT, IL-1 VNTR 86 bp, TPA7351 C/T). Nevertheless, despite the absence of a Hardy–Weinberg equilibrium for these polymorphisms we do not believe that this resulted in a major distortion of our findings owing to the characteristics of subjects enrolled as controls that probably do not represent a good sample of overall population because they are hospitalized patients without ischemic stroke but with other atherosclerotic co-morbidities. Moreover, a recent study investigating plasma PAI-1 and 4G/5G genotypes reported the absence of a Hardy–Weinberg equilibrium for the 4G/5G polymorphism [50].

Another possible limitation is the fact that the relatively small number of cases and controls and the performance of multiple subgroup analyses may increase the likelihood of spurious results.

In conclusion, our present study has shown a significant association between some inflammatory (IL-1 VNTR 86bp 2/2 genotype), and anti-inflammatory genotypes (IL-10 −1082 AA) and a pro-

coagulation tests, 12-lead ECG, 24 h electrocardiography monitoring, trans-thoracic echocardiography, carotid ultrasound, brain CT or MRI at admission (repeated between the third and the seventh days of stroke onset).

Neurological deficit score on admission was evaluated by Scandinavian Stroke Scale (SSS). SSS assesses neurological deficit through an evaluation of consciousness level, eye movement, strength in arms, hands, and legs, orientation, language, facial weakness and gait, giving rise to a score ranging from 58 (absence of deficit) to 0 (death).

2.2. Laboratory methods

Analysis of some gene polymorphisms involved in pro-inflammatory, anti-inflammatory and coagulation pathway was undertaken for patients with acute ischemic stroke and for control subjects.

2.2.1. Molecular analysis of alleles at the −308 nucleotide (−308G/A) of TNF-α gene

Genomic DNA was extracted from EDTA anticoagulated blood samples by using the salting out method [19]. The −308G/A polymorphism (rs1800629) of TNF-α was identified using a modification of PCR-Restriction Fragment Length Polymorphism assay as previously described [20]. Briefly, 0.5 μM of forward and reverse primers (5′AGG CAA TAG GTT TTG AGG GCC AT3′ and 5′GGC GGG GAT TTG GAA AGT T3′) were mixed to 5–10 ng of DNA template, with a final concentration of 0.2 U Taq DNA polymerase (Perkin Elmer BioSystem, Rome, Italy), 200 M of each deoxynucleotide and 1X reaction buffer. PCR was performed for 35 cycles at 94 °C, 58 °C and 72 °C for 35 s, respectively. Restriction enzyme digestion with NcoI (M-Medical, Milan, Italy) of the PCR-amplified product (159 bp) and subsequent electrophoresis on a 2–5% agarose gel discriminated between the two alleles; −308A showed two fragments of 146 and 13 bp, while −308G was undigested and resulted in a single band of 159 bp. Heterozygous individuals were detected by the presence of all the three fragments.

2.2.2. Haplotype molecular analysis of alleles at the −1082 nucleotide of IL-10 gene

Three different biallelic polymorphisms, rs1800896 (−1087C/A), rs1800871 (−824C/T) and rs1800872 (−597C/A), of IL-10 gene were identified using a −1082/−819/−592 haplotype specific typing method, as previously described [21]. Briefly, 12 couples of 3′ and 5′ allele specific sequence primer pairs were separately mixed in a 13 μl total volume containing 5–10 ng of DNA template, 2.00 mM $MgCl_2$, 9.8 mM ammonium sulfate, 39.6 mM TRIS–HCl 200 μM deoxyribonucleotide triphosphates (dNTPs), and 0.2 U Taq-DNA polymerase (Perkin Elmer BioSystem, Rome, Italy). Cycling was performed at 96 °C for 1 min followed by 5 cycles at 96 °C for 25 s, 70 °C for 45 s, and 72 °C for 45 s, 20 cycles at 96 °C for 25 s, 65 °C for 50 s, and 72 °C for 45 s, and finally 5 cycles at 96 °C for 25 s, 55 °C for 60 s, and 72 °C for 120 s. PCR products, potentially containing the two −592/−819/ or −592/−1082, or −819/−1082 possible allele combinations, were detected by electrophoresis on 2% agarose. Molecular analysis of PAI-1 4G/5G promoter polymorphism was done. The DNA was amplified by polymerase chain reaction (PCR) using 17-mer allele specific oligonucleotides; for the 5G allele (5′GTC TGG ACA CGT GGG GG3′) and for the 4G allele (5′GTC TGG ACA CGT GGG GA3′), in separate reactions with a common downstream primer 5′TGC AGC CAG CCA CGT GAT TGT CTA3′, and a fourth primer (5′AAG CTT TTA CCA TGG TAA CCC CTC GT3′), located upstream of the polymorphic region, was used as an internal control for verification of DNA amplification as described by Naran et al. [22].

2.2.3. Molecular analysis of alleles at the −7351 nucleotide (−7351C/T) of tPA gene

tPA-7351C/T polymorphism was amplified using PCR. Digestion with BanI restriction endonuclease produced 1 of 2 characteristic sets of fragments, depending on the presence of a C or a T allele at the SNP. The restriction fragments were separated on a 2% agarose gel [23].

2.2.4. Molecular analysis of alleles at the −174 nucleotide (−174 C/G) of IL-6 gene

Amplification of the −174C/G locus was performed as previously described [24]. Briefly, PCR amplification was followed by an overnight restriction digest of 15 μl PCR products with NIa III enzyme (M-Medical, Milan, Italy). The presence of a cytosine (C allele) at nucleotide −174 was revealed by the presence of the NIa III cutting site. The two alleles were detected by electrophoresis analysis in a 2% agarose gel stained with ethidium bromide.

2.2.5. Molecular analysis of IL-1RN exon 2 VNR polymorphism

The IL-1RN exon 2 polymorphism characterized by a variable number of 86 bp repeats, was analyzed as previously reported [25]. Conditions used were as follows: 95 °C for 5 min, then 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and, finally, 72 °C for 5 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Allele 1 (4 repeats) was 410 bp, allele 2 (2 repeats) was 240 bp, allele 3 (5 repeats) was 500 bp, allele 4 (3 repeats) was 325 bp, and allele 5 (6 repeats) was 595 bp in length.

2.3. Statistical analysis

The sample size of 144 patients (96 cases vs. 48 controls) was required to detect an effect with 80% power ($\alpha = 0.05$). Power calculation was generated assuming an additive model to detect the effect of any polymorphism having a frequency ≥ 0.2, associated with a Genotype Relative Risk (GRR) ≥ 2.0.

Fisher's exact test was used to assess intergroup significance between categorical variables, and Student's *t* test was used to determine differences between continuous variables. Mantel–Haenszel chi square test and Fisher's exact test were used to test differences in genotype frequencies between groups. Logistic regression was performed to estimate odds ratios (OR) and 95% confidence intervals (CI) for the effect of genotypes on risk of acute ischemic stroke. Statistical analyses were performed using SPSS for Windows, version 13.0; statistical significance was set at $p \leq 0.05$.

3. Results

We evaluated 96 subjects with acute ischemic stroke and 48 control subjects. Relevant characteristics of the cases and controls and odds ratios are given in Table 1.

For each polymorphism, genotype frequencies were determined by gene counting and odds ratios are given in Table 2.

The genotype distribution was in Hardy–Weinberg equilibrium for TNFα-308GA, PAI 1 675 5G/4G, IL 6 174 C/G genotypes and it was not in equilibrium for and 819CT, IL-1 VNTR 86 bp, TPA7351 C/T genotypes.

Regarding the TNF-α promoter polymorphism at position −308 we observed, between patients with acute ischemic stroke and control subjects, no significative difference with regard of frequency of GG, GA and AA genotypes.

With regard of IL-10 1082AG genotypes in stroke patients in comparison with controls we observed a higher frequence of AA genotype ($p = 0.05$), GA genotype was more frequent ($p = 0.007$) in controls, whereas no significant difference in GG genotype frequence between stroke subjects and controls was observed.

Table 1
General characteristics of stroke patients and controls.

Variable	Stroke pts (n: 96)	Controls (n: 48)	p
Age (years)	71.9 ± 9.75	71.4 ± 7.45	0.73
M/F (n)	45/51	16/32	0.121
Diabetes (n/%)	52 (54.16)	23 (47.91)	0–515
Hypertension (n/%)	64 (66.7)	18 (37.5)	0.001
Atrial fibrillation (n/%)	31 (32.29)	19 (39.58)	0.360
Glucose blood levels (mg/dl)	135.5 ± 68.8	125.79 ± 44.04	0.365
Cholesterol blood levels (mg/dl)	186.27 ± 41.33	143.2 ± 26.39	<0.0005
LDL cholesterol blood levels (mg/dl)	100.04 ± 35.14	91.83 ± 38.25	0.191
Triglycerids blood levels (mg/dl)	131.93 ± 61.97	161.4 ± 103.3	0.166
White blood cells (per mm^3)	13 391.5 ± 7497.6	/	
Neutrophils (%)	8481.3 ± 9180.9	/	
SSS	30.0 ± 14.0	/	
NIHSS	16.7 ± 11.5	/	
rankin score at discharge			
I	12 (12.5)	–	
II	30 (31.25)	–	
III	26 (27.08)	–	
IV	14 (14.58)	–	
V	14 (14.58)	–	
Death (n/%)	12 (12.5%)		
CAD (n/%)	36 (37.5)	16 (33.33)	0.624
Previous TIA (n/%)	48 (50.0)	–	0.014
Previous stroke (n/%)	43 (44.79)	–	0.012
Microalbuminuria (n/%)	46 (47.91)	13 (27.08)	0.018
Carotid plaque (n/%)	71 (73.95)	9 (18.75)	0.009
LVH (n/%)	45 (46.87)	13 (27.08)	0.022
Previous brain infarct at neuroimage (n/%)	57 (59.37)	–	0.001
LAAS (n/%)	37 (38.54)	–	–
Lacunar (n/%)	34 (35.41)	–	–
Cardioembolic (n/%)	25 (26.05)	–	–

Table 2
Proinflammatory/anti-inflammatory and thrombotic/fibrinolytic genotypes polymorphism prevalence in subjects with acute ischemic.

	Stroke subjects (n: 96)	Controls (n: 48)	p-Value	HR (95% CI)
Comparison of frequency of TNF-α −308 GA genotype in patients with ischemic stroke and in controls				
GG	83 (86.4%)	35 (72.9%)	0.047	2.17 (0.87–5.39), $p = 0.014$
GA	12 (12.5%)	11 (22.9%)	0.171	
AA	1 (1.1%)	2 (4.2%)	0.257	
Comparison of frequency of IL-10 1082 AG and 819 CT genotypes in patients with ischemic stroke and in controls				
GG	24 (25.0%)	11 (22.9%)	0.945	2.64 (0.97–7.23), $p = 0.057$
GA	14 (14.6%)	17 (35.4%)	0.007	
AA	58 (60.4%)	20 (41.7%)	0.013	3.52 (1.47–8.41), $p = 0.005$
Comparison of frequency of IL-10 819 CT genotypes in patients with ischemic stroke and in controls				
CC	63 (65.6%)	26 (54.2%)	0.813	2.94 (1.26–6.83), $p = 0.012$
CT	14 (14.6%)	17 (35.4%)	0.004	
TT	19 (19.8%)	5 (10.4%)	0.156	4.61 (1.37–15.51), $p = 0.013$
Comparison of frequency of PAI 1675 5G/4G genotype in patients with ischemic stroke and in controls				
5G/5G	68 (70.8%)	48 (100%)	0.0001	
5G/4G	27 (28.1%)	0 (0%)	0.0001	
4G/4G	1 (1.1%)	0 (0%)	1.0	
Comparison of frequency of TPA 7351 C/T genotypes in patients with ischemic stroke and in controls				
CT	63 (65.6%)	21 (43.7%)	0.019	3.70 (1.65–8.31), $p = 0.001$
CC	17 (17.7%)	21 (43.7%)	0.0016	
TT	16 (16.7%)	6 (12.6%)	0.682	3.29 (1.05–10.25), $p = 0.040$
Comparison of frequency of IL-6–174GG genotype in patients with ischemic stroke and in controls				
GC	46 (47.9%)	33 (68.7%)	0.028	
CC	10 (10.4%)	1 (2.1%)	0.300	
GG	40 (41.7%)	14 (29.2%)	0.201	
Comparison of IL-1 VNTR 86 bp alleles in patients with ischemic stroke and controls				
1/1	46 (47.9%)	23 (47.9%)	0.859	
1/2	28 (29.2%)	21 (43.7%)	0.120	
1/3	2 (2.1%)	2 (4.2%)	0.600	
2/2	20 (20.8%)	2 (4.2%)	0.017	7.50 (1.57–35.68), $p = 0.011$

Regarding IL-10 polymorphism at position –819, the CT genotype was significantly more frequent ($p = 0.007$) in controls, whereas no significative difference was observed with regard of TT and CC genotypes between stroke subjects and controls (see Table 2).

The PAI 5G/5G genotype was more significantly frequent in controls ($p = 0.0005$), 5G/4G has a significantly higher frequency in stroke subjects ($p = 0.0001$) whereas no significative difference was observed between stroke patients and controls with regard of 4G/4G genotype.

ing candidate genes involved in clotting mechanisms and in particular those involved in the balance between fibrinolysis and thrombosis such as tPA/PAI1 system.

Recently, a single nucleotide polymorphism located at position −7351 within the enhancer region of the TPA gene was identified and shown to be strongly correlated with endothelial TPA release rates [42]. The polymorphism was also found to have functional importance as it occurred within an Sp1 binding site, a factor promoting DNA transcription. Possession of a thymidine (T) allele was shown to inhibit Sp1 binding and was associated with less than half the TPA release observed in those homozygous for the cytosine (C) allele [41]. A recent study also reported an increased risk of ischemic stroke for the TT genotype [43].

Among genetic variants at the PAI-1 locus, the −1675-4G/5G SNP in the promoter has been demonstrated to be functional with a higher transcriptional activity for the 4G allele [36]. The 4G/4G genotype has been associated with increased risk of MI [38]. The possible connection to stroke has received less attention, and published studies [40,41] and also our findings do not reveal a clear association.

We also reported a higher frequency of IL-1 VNTR 86 bp 2/2 genotype in stroke subjects compared to controls with a significant hazard ratio (OR = 6.05; $p = 0.017$) at regression analysis.

The penta-allelic polymorphic site in intron 2 of the IL-1RN gene consisting in a variable number tandem repeats (VNTR, 86 bp repeats) has been extensively investigated in relation to a variety of pathological conditions [33,34], but no study evaluated the possible association between genotypes of IL-1 VNTR 86 bp and ischemic stroke. So our finding could appear original and could offer a possible explanation of the association of the IL-1RN2 allele with ischemic stroke owing to an overall increase of IL-1 activity due to a less effective IL-1ra inhibitory activity [34,35] associated with this genotype [37,38].

In our stroke patients regarding PAI 5G/4G polymorphism in position −1675, we showed a higher frequency of 5G/4G genotypes, whereas regarding TPA 7351 CT polymorphism, we reported a higher frequency of CT genotype in stroke subjects vs. control subjects not confirming previous data regarding an association between TT genotype and ischemic stroke [39,44].

Although the common 4G/5G polymorphism in the promoter of the PAI-1 gene was suggested to be a risk factor for some of the thrombotic disorders, its significance in the development of thrombosis is still controversial. Previous studies reported that 4G/4G genotype is associated with a higher risk of thrombosis in vessels of internal organs especially in the portal veins [45].

A deletion/insertion polymorphism (4G or 5G) in the promoter of the plasminogen activator inhibitor type 1 gene has been suggested to be involved in regulation of the synthesis of the inhibitor, the 4G allele being associated with enhanced gene expression. A relationship between 4G/5G polymorphism and PAI-1 levels was found in patients with cardiovascular and metabolic diseases, but not in healthy subjects. In patients with deep vein thrombosis the 4G polymorphism of PAI-1 gene promoter may influence the expression of PAI-1 and it should be taken into consideration as a facilitating condition for pathological fibrinolysis together with other environmental and genetic factors [46]. Whether this has any significance in regard to the pathogenesis of arterial thrombosis remains to be proven.

Furthermore, in patients association between the 4G/5G genotypes and PAI activity was observed, with the highest PAI activity values in the 4G/4G genotype, intermediate in the 4G/5G genotype and the lowest in the 5G/5G genotype [47].

So our findings about higher frequence of 5G/5G and 4G/5G genotypes appear not easily explainable, maybe this finding could be due to a minor role of fibrinolysis in acute ischemic stroke and so to a spurious association.

The etiology of ischemic stroke affects prognosis, outcome, and management. We recently compared plasma levels of immuno-inflammatory variables in patients with acute ischemic stroke in relation to TOAST subtype showing that cardioembolic stroke has higher levels of immuno-inflammatory variables in the acute phase compared to other TOAST subtypes whereas lacunar stroke has the lowest plasma levels [48,49].

Analyzing the relationship between inflammatory and thrombotic-fibrinolytic genotypes in relation to diagnostic subtype cerebral ischemia is therefore an interesting perspective to better characterize the relationship between candidate genes from the standpoint of pathogenesis and cerebral ischemia. Only one study has examined this relationship [50] so our findings regarding the contemporary evaluation of the distribution of anti-inflammatory and thrombotic-fibrinolytic genotypes in relation to diagnostic subtype of cerebral ischemia may be useful.

We reported a significant relationship with TOAST subtypes only regarding TPA 7351 CC genotype and 1/1 IL-1 VNTR 86 bp genotype and lacunar TOAST subtype vs. LAAS subtype with a significant risk trend for both. Regarding TPA 7351 CC polymorphism, TPA homozygous subjects for the cytosine (C) allele [41] compared to subjects with a thymidine (T) allele have higher plasma levels of TPA and this finding could contribute to defining the pathogenetic background of lacunar strokes, that we reported as characterized by a minor degree of immuno-inflammatory activation of the acute phase [42,43]. This aspect could be confirmed by our finding concerning and higher frequency of 1/1 IL-1 VNTR 86 bp genotype in lacunar stroke due to the fact that this genotype is associated with higher IL-1ra inhibitory activity resulting in a high degree of anti-inflammation [31,32].

Inflammation of the wall of these penetrating arteries and impairment of thrombotic/fibrinolytic balance may explain the significant association between these genotypes and lacunar type of ischemic stroke, although subsequent studies might further reveal the pathogenetic basis of genetic predisposition of lacunar and other subtypes of stroke.

A genetic model of stroke pathogenesis is expected to be multifactor, involving several genetic polymorphisms that each confer small increases in risk; on this basis we evaluated several aspects of possible stroke pathogenesis involving both inflammation and thrombosis, evaluating the possible contribution of several inflammatory/anti-inflammatory genotypes and thrombotic/fibrinolytic. Nevertheless, we observed significant differences with regard of prevalence of some cerebrovascular risk factor such as hypertension, cholesterol blood levels that could partially explain stroke risk in these patients, but this is probably due to the multifactorial nature of cerebrovascular risk encompassing genetic and acquired factors.

A possible limitation of our study is that not all genotype distribution was in Hardy–Weinberg equilibrium (IL-10 1082AG and 819CT, IL-1 VNTR 86 bp, TPA7351 C/T). Nevertheless, despite the absence of a Hardy–Weinberg equilibrium for these polymorphisms we do not believe that this resulted in a major distortion of our findings owing to the characteristics of subjects enrolled as controls that probably do not represent a good sample of overall population because they are hospitalized patients without ischemic stroke but with other atherosclerotic co-morbidities. Moreover, a recent study investigating plasma PAI-1 and 4G/5G genotypes reported the absence of a Hardy–Weinberg equilibrium for the 4G/5G polymorphism [50].

Another possible limitation is the fact that the relatively small number of cases and controls and the performance of multiple subgroup analyses may increase the likelihood of spurious results.

In conclusion, our present study has shown a significant association between some inflammatory (IL-1 VNTR 86bp 2/2 genotype), and anti-inflammatory genotypes (IL-10 −1082 AA) and a pro-

coagulant genotype such as TPA 7351 CT and PAI-1 1675 5G/4G and ischemic stroke. Moreover our study has shown a significant relationship with TOAST subtype regarding only CC TPA genotype and 1/1 IL-1 VNTR 86 bp and lacunar strokes.

Taking into account results of the present study might be clinically relevant to develop a new risk chart and statistical algorithm that comprise genetic risk factors and their interaction with other clinical and epidemiological factors.

Conflict of interest statement

No funding to disclose.
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CHAPTER 5

Myocardial infarction marker levels are influenced by prothrombin and tumor necrosis factor-α gene polymorphisms in young patients.

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Myocardial infarction marker levels are influenced by prothrombin and tumor necrosis factor-α gene polymorphisms in young patients

Loredana Vaccarino [a,*], Silvana Vitale [b], Marco Caruso [c], Marisa Palmeri [a], Letizia Scola [a], Manuela Bova [a], Calogero Caruso [a], Maria Fatima Massenti [b], Francesco Vitale [b], Salvatore Novo [c], Domenico Lio [a], Giusi Irma Forte [a]

[a] Department of Biopathology and Medical and Forensic Biotechnology, University of Palermo, Italy
[b] Department of Health Promotion Sciences, University of Palermo, Palermo, Italy
[c] Department of Internal Medicine and Cardiovascular Diseases, University of Palermo, Palermo, Italy

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ABSTRACT

Polymorphisms of genes encoding key factors for the control and activation of inflammatory response and coagulation cascade regulation may play a role in genetic susceptibility to acute myocardial infarction (AMI). This study sought to analyze the effect of TNF −308G/A and pro-thrombin (FII) 20210G/A polymorphisms on the laboratory parameters of young patients affected by AMI. Results indicated that TNF −308A positive genotype frequencies were increased in these patients and that a genetically determined higher production of TNF-α is associated in young subjects to a more severe cardiac damage as depicted by higher levels of troponin, Creatine kinase-MB Isoenzyme (mCK-MB) and a significant increased plasma fibrinogen levels. Similar and probably additive effects on might have a genetically determined increased production of pro-thrombin even if no significant differences in genotype frequencies of pro-thrombin (FII) 20210G/A polymorphisms were observed in this study. All together these results, indicating the relationship among genetically determined TNFs and FII production and increased levels of tissue damage markers of AMI, suggest that a complex genetic background, might be involved in susceptibility to AMI in young men influencing the extension and severity of the disease.

1. Introduction

Acute myocardial infarction (AMI) the leading cause of mortality and morbidity in industrialized countries is a common outcome of coronary atherosclerosis. As other multifactorial disease, AMI probably involves many different gene variants that might interact to result in an additive or a synergistic co-effect. Young adults are a relatively small proportion of patients who experience AMI [1]. They have some distinct characteristics compared to older patients as young people are more likely to have normal coronary arteries [2]. Although the exact pathogenetic mechanism of AMI in young patients remains unknown it has been proposed that thrombus formation due to atherosclerotic plaque rupture or erosion is, always, the main mechanism [3].

The importance of genetic factors seems to be particularly relevant in younger subjects, where it is assumed that the genetic background influences the susceptibility of these subjects to environmental risk factors for AMI identified in the general population. In particular a prominent role of genetic factors in the onset of this disease has been documented in twins and family based studies [4].

Recently a number of candidate genes and chromosomal loci have been identified to be associated with the susceptibility to myocardial infarction and a majority of these genes have been implicated in the processes of inflammation [5,6]. The single nucleotide polymorphisms (SNPs) present in the genes CD14 (−159 C/T), TNFα (−308 G/A), IL-1a (−889 C/T), IL-6 (−174 G/C), PSMA6 (−8 C/G), and PDE4D (SNP83 T/C, respectively), were found to be associated with increased risk of cardiovascular diseases in different populations [7–12]. In addition previous studies have shown that polymorphisms of genes encoding key factors for the control and

Abbreviations: AMI, acute myocardial infarction; aPTT, time (in seconds) of activated partial thromboplastin; AST, aspartate aminotransferase; CHD, coronary heart disease; CTnI, cardiac troponin I isoenzyme; FII, factor II (pro-thrombin); FV, factor V Leiden; HLA, human leukocyte antigen; IL-6, interleukin-6; IL-10, interleukin-10; INR, international normalized ratio; mCK-MB, creatine kinase-MB; PLTs, platelets; PT, prothrombin activity; SNPs, single nucleotide polymorphisms; TNF-α, tumor necrosis factor-α; UTR, upstream untranslated region.

* Corresponding author. Address: Department of Biopathology and Medical and Forensic Biotechnology, University of Palermo, Corso Tukory 211, 90134 Palermo, Italy. Fax: +39 0916555913.
E-mail addresses: loredana.vaccarino@unipa.it, loredanavaccarino@libero.it (L. Vaccarino).

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activation of inflammatory response may play a role in genetic susceptibility to AMI in young men [13,14].

In particular the pleiotropic pro-inflammatory cytokine tumor necrosis factor (TNF)-alpha, lying at the telomeric end of the class III region of the human leukocyte antigen (HLA) [15–18]. Play a central role in the inflammatory responses with multiple biologic activities.

Wilson and his colleagues [19] first reported the bi-allelic polymorphism within the 5′ genomic region of the TNF-α gene promoter at position −308 (−308G → A; rs1800629). Carrying the A allele enhances transcriptional activity and is reported to be associated with higher levels of circulating TNF-α [20,21]. Serum levels of TNF-α is elevated in coronary heart disease (CHD) patients and may modify the risk for developing coronary events since it affects endothelial cell hemostatic function [22] and in a recent metanalysis [23] it has been demonstrated that this polymorphism might be a risk factor for coronary heart disease.

Moreover congenital or acquired mutations located on genes coding for antithrombin III, protein C, protein S, factor V leiden (FV) and prothrombin (FII), normally associated to the occurring of deep venous thrombosis, were also inconstantly found in patients with arterial thrombosis. Recent observations stressed the hypothesis that mutations of FV and FII may be risk factors for acute myocardial infarction [24–28].

The prothrombin gene is organized in 14 exons, separated by 13 introns with the 5′ upstream untranslated region (UTR) and the 3′-UTR which may play regulatory roles in gene expression [29,30]. One genetic variation in the 3′-UTR region of the prothrombin gene is the G to A transition at nucleotide position 20210, at or near the cleavage site of the mRNA precursor to which poly A is added. This was termed as the factor II G 20210A mutation. The prevalence of carriers of factor II 20210G/A substitution (rs1799963) in healthy Northern Europeans is 1.7% whereas in Southern Europeans the prevalence is nearly double (3%) [31].

In 1996, Poort et al. [29] reported that the 20210G/A single nucleotide polymorphism (SNP) is associated with an increased risk of venous thrombosis. Several studies confirmed this initial observation both in venous thrombosis and arterial disease [30,32].

In the present study, we analyze the frequencies of polymorphisms 20210G/A of FII and −308G/A of the promoter region of TNF-α in a group of Sicilian patients aged <46 years affected by AMI and evaluate the effect of these polymorphisms on the blood levels of myocardial tissue damage and clotting markers, to evaluate the possibility to use typing of these polymorphisms in association with selected haematochemical parameters in prognostic evaluation of these patients.

2. Materials and methods

2.1. Subjects

In this study, we analyzed two cohorts of men affected by acute myocardial infarction (AMI) and unrelated controls matched for age and sex. The cohort comprised 60 young male patients (age range 23–46 years) affected by acute myocardial infarction, consecutively admitted to the Cardiac Unit of Palermo University Hospital in the last year, and 130 healthy age and gender matched controls, all living in western Sicily. The diagnosis of acute myocardial infarction was based on standard laboratory (troponin I greater than decision limit, 3.0 ng/mL) [33] findings, typical electrocardiographic changes and confirmed by echocardiography and coronary angiography. According to the Helsinki declaration and to local ethical committee recommendations for the observational studies, written informed consent for enrolling in the study and for personal data management was obtained from all the subjects. Blood samples from patients were collected 48 h from symptoms, transported and processed promptly according to pre-analytical recommendations [34].

2.2. Biochemical and hematological analyses

Cardiac markers were examined by automatic analyzers in the fresh blood sample collected 48 h after myocardial infarction. The activity of aspartate aminotransferase (AST) and levels of CK-MB isoenzyme (CK-MB mass, mCK-MB) and Troponin I were quantified by routine chemical and immunochemichal clinical laboratory methods (Biochemical automatic analyzer: Modular P and E, Roche, Basel, Switzerland); haemochromecytometric test was performed by analyzer Sismex 9500, coagulation tests: prothrombin activity (PT), time of activated partial thromboplastin (aPTT), fibrinogen Clauss by automatic analyzer Futura Advance.

2.3. DNA genotyping

Genomic DNA was isolated by a standard method using proteinase K digestion followed by a standard salting-out technique [16].

2.3.1. Molecular analysis of alleles at the −308 nucleotide (−308G → A) of TNF-α gene

The −308G/A polymorphism (rs1800629) of TNF-α was identified using a modification of PCR-Restriction Fragment Length Polymorphism assay described by Galbraith and Pandey [35]. Briefly, 0.5 μM of forward and reverse primers (5′AGG CAA TAG GTT TTG AGG GCC AT 3′ and 5′GGC GGG CAT TTG GAA ACTT 3′) were mixed to 5–10 ng of DNA template, with a final concentration of 0.2 U Taq DNA polymerase (Perkin Elmer BioSystem, Rome, Italy), 200 μM of each deoxynucleotide and 1X reaction buffer. PCR was performed for 35 cycles at 94 °C, 58 °C and 72 °C for 35 s, respectively. Restriction enzyme digestion with NcoI (M-Medical, Milan, Italy) of the PCR amplified product (159 bp) and subsequent electrophoresis on a 2–5% agarose gel discriminated between the two alleles; −308A showed two fragments of 146 bp and 13 bp, while −308G was undigested and resulted in a single band of 159 bp. Heterozygous individuals were detected by the presence of all the three fragments.

2.3.2. Molecular analysis of alleles at the 20210 nucleotide (20210G → A) of prothrombin (factor II) gene

The identification of the alleles in the polymorphic site of Factor II was obtained by Real Time PCR using TaqMan Pre-Developed Assay Reagents for Allelic Discrimination essays optimized by Applied Biosystems on a 7300 Real Time PCR System as previously described [36]. Briefly, 10 ng of DNA for each sample were used in a PCR reaction, containing 1X optimized master mix and 1X specific primers/probes mix assay, according to manufactory protocol in a final volume of 25 μl. Two identical probes, except for the central nucleotide that specifically recognizes the single nucleotide polymorphism (SNP) were used, each one labeled at the 5′ extremity with different dyes (for the wild-type allele the fluorochrome FAM, and the fluorochrome VIC for probe specific for minor allele) and at the 3′ extremity with a quencher dye, that in this case was the Minor Groove Binder (MGB) dye. Then, the amplification was performed in 7300 Real-Time ABI Prism PCR System (Applied Biosystems, USA), using a standard amplification protocol (1 cycle of 2′ at 50 °C; 1 cycle of 10′ at 95 °C and 40 cycles of 15″ at 95 °C plus 1′ at 60 °C), and the results available in the report sheet of 7300 System SDS v1.3 Software. Finally, samples were graphically grouped in 3 genotypic clusters, easily recognizable in the Allelic Discrimination plot on the basis of the two probe's fluorescence intensity emissions, whereas the uncertain cases were also evalu-

ated for the grow up of the fluorescence emission curve of each dye on the component's sheet.

2.4. Statistical analysis

The Hardy–Weinberg equilibrium was confirmed by Pearson's test (goodness of fit between the observed and expected genotype (3 × 2 tables) and allele (2 × 2 tables) frequencies). Fisher's exact tests were performed to calculate significant different genotype or allelic distributions between patients with acute myocardial infarction and healthy controls. Odds ratio and 95% of confidence intervals were calculated with Woolf's approximation to quantify the risk in carriers of minor allelic variants. Differences in quantitative and qualitative data were analyzed using formal statistical tests (ANOVA followed by *t*-Student test). Differences were considered significant when $p < 0.05$.

3. Results

In previous studies we have demonstrated that proinflammatory gene variants determine an increased individual's risk for myocardial infarction [14]. Table 1 shows the analysis of genotypic frequencies of the single nucleotide polymorphisms of TNF (−308G/A) an of the prothrombin (factor II, FII 20210G/A) among the 60 male patients and 130 healthy controls. Both patient' and control' genetic frequencies fit the Hardy–Weinberg equilibrium for both the two SNPs. We observed a significant increase of frequency of genotypes positive for the minor allele (A) of −308G/A SNP ($p = 0.0018$), that are represented with a percentage of 36.7% among patients, against 14.6% among the healthy controls ($p = 0.0048$, odds ratio 4.00; 95% confidence interval: 1.51–10.56). In addition a not significant increase of the percentage of subject bearing the 20210A allele of the factor II gene (8.3% vs 6.9%).

As well known AMI is diagnosed using some serum specific markers as Cardiac troponin I (CTnI) and Creatine kinase-MB Isoenzyme protein (mCK-MB). Table 2 reports data on the effect of TNF −308G/A and FII 20210G/A polymorphisms on the levels of these haematochemical markers, on the levels of Aspartate Transpeptidases (AST) measurement, nowadays considered obsolete for this purpose and leukocytes. Stratifying haematochemical data according the two SNP genotypes the higher levels of CTnI were observed in subject bearing the TNF-α −308A or the FII 20210A alleles.

On the other hand subjects positive for −308A shows the higher levels of mCK-MB whereas the FII 20210A ones shows the higher levels of leucocytes. The analysis of the effect of −308G/A TNFα and 20210G/A factor II genotypes on coagulation parameters, showed a significant increasing of plasma fibrinogen levels and of circulating platelets concentration in young men with acute myocardial infarction homozygous or heterozygous for −308A allele TNFα (Table 3). A similar pattern of fibrinogen concentration was observed in subjects bearing Factor II GA or AA genotypes.

4. Discussion

Signs of a systemic inflammatory response such as fever, leucocytosis and elevated acute phase reactants are frequently observed in patients with AMI and CHD. In patients with extensive myocardial infarction a pronounced inflammatory response may further complicate the clinical course [17]. After acute myocardial infarction, systemic inflammatory response is associated with the increases in plasma cytokines, such as TNF-α, interleukin-6 (IL-6) and IL-10, in myocardium and blood. TNF-α as a pro-inflammatory cytokines and can cause severe damage to cardiomyocytes and suppress cardiac function [36].

In addition excessive thrombin generation has been described in individuals at high risk of fatal CHD [37]. It seems biologically plausible that the higher prothrombin levels related to the 20210A variant may also confer an increased risk of arterial disease.

Results reported in Table 1 confirm reports from previous researches [13,14] on the role of TNF −308A allele in AMI and suggest that this gene variant might be an AMI risk factor for young men but do not allow to confirm the association between FII 20210A allele and AMI. To date, actually, studies attempting to answer this question have yielded conflicting results. In some reports, being a carrier of the mutation was associated with an increased risk of acute myocardial infarction (AMI) [38,39]. Nevertheless, prospective studies failed to establish any association between the 20210A allele and AMI [40].

As reported in Table 1 the cumulative frequencies of genotypes positive for Prothrombin 20210A allele in our patients and control populations are almost doubled respect the published frequencies for the South Europe. These differences are probably due to the patient and control populations sampling (young men). So our subjects cannot be considered representative of the general population. On the other hand the North to South gradient in distribution of genotypes in Caucasians was established at 50°N latitude [8]. Sicily is at very South of this distribution so one could speculate that the frequency of Prothrombin 20210A allele might be further increased in our population. In all cases a different and larger population sample is necessary to determine Prothrombin 20210G/A genotype and allele frequencies in general Sicilian population.

Our data indicate that TNF −308G/A and FII 20210G/A polymorphisms impinge upon the levels of haematochemical markers associated to acute myocardial infarction. As well known the cardiac form of Cardiac troponin I (CTnI) levels are routinely measured for diagnosing acute myocardial infarction. Cardiac troponin measurements also provide information concerning prognosis and the effect of early intervention in patients with acute coronary syndromes. Similarly measurement of concentration of Creatine kinase-MB Isoenzyme protein (mCK-MB) represent an important marker of degree of myocardial damage immediately after AMI [41]. On the other hand Cardiac tissue necrosis induces an

Table 1
−308G/A TNF and 20210G/A FII genotype frequencies in 60 young patients affected by acute myocardial infarction (AMI) and 130 health sex and age matched controls.

Genotypes		AMI	%	Healthy controls	%	O.R.	95%C.I.	p
TNF SNP −308G/A	GG	38	63.4	111	85.4	0.29	0.14–0.62	0.0018
	AG	20	33.3	17	13.1	3.43	1.58–7.45	0.0026
	AA	2	3.3	2	1.5	1.86	0.26–13.57	n.s.
	AG/AA	22	36.7	19	14.6	4.00	1.51–10.56	0.0048
Factor II SNP 20210G/A	GG	55	91.7	121	93.1	0.44	0.11–1.84	n.s.
	AG	4	6.6	7	5.4	1.26	0.35–4.46	n.s.
	AA	1	1.7	2	1.5	1.08	0.10–12.21	n.s.
	AG/AA	5	8.3	9	6.9	1.22	0.39–3.82	n.s.

Table 2
Evaluation of blood data (Mean ± D.S.) of 60 young patients affected by acute myocardial infarction (AMI) according to tumor necrosis factor α (TNFα) −308G/A and prothrombin (factor II) 20210G/A genotypes.

Genotypes	CTnI	CK-MBm	AST	WBC	NEUT
TNF GG (38)	12.1 ± 5.4	23.5 ± 9.2	53.9 ± 43.8	9.2 ± 1.6	6.05 ± 1.7
TNF GA/AA (22)	20.8 ± 7.8	39.7 ± 11.3	61.7 ± 30.8	9.2 ± 5.2	6.9 ± 5.1
p	<0.0001	<0.0001	n.s.	n.s.	n.s.
Factor II GG (55)	11.2 ± 3.8	26.5 ± 8.7	44.33 ± 21.9	8.7 ± 4.8	5.7 ± 2.0
Factor II GA/AA (5)	19.9 ± 6.7	31.4 ± 9.2	75.9 ± 41.1	13.7 ± 3.2	9.33 ± 4.4
p	0.0456	n.s.	n.s.	0.0244	n.s.

Table 3
Evaluation of haematic coagulation marker profile (Mean ± D.S.) of 60 young patients affected by acute myocardial infarction (AMI) according to tumor necrosis factor α (TNFα) −308G/A and to prothrombin (factor II) 20210G/A genotypes.

Genotypes	INR	APTT	Fibrinogen mg/dL	PLT × 10^3/L
TNF GG (38)	1.01 ± 0.07	23.1 ± 10.1	364.5 ± 41.1	181.1 ± 46.2
TNF GA/AA (22)	1.03 ± 0.16	23.8 ± 12.2	418.15 ± 113.0	275.0 ± 46.9
p	n.s	n.s	0.0110	<0.0001
Factor II GG (55)	0.89 ± 0.10	23.1 ± 15.8	315.7 ± 39.7	205.0 ± 56.3
Factor II GA/AA (5)	1.00 ± 0.23	23.0 ± 25.3	412.7 ± 95.2	250.0 ± 61.1
p	n.s.	n.s.	0.0017	n.s

increased inflammatory response mirrored by a not specific increase of circulating leucocytes due to neutrophil mobilization. Inflammatory response is an important feature of acute coronary syndromes and myocardial infarction. In AMI signs of inflammation are well known and elevated levels of acute phase reactants have been shown to be associated with a worse short- and long-term prognosis [17,42]. Our data strongly suggest that a genetically determined higher production of TNF-α is associated in young subjects to a more severe cardiac damage in AMI as depicted by higher levels of troponin and mCK-MB.

A similar and probably additive effect might have a genetically increased production of prothrombin. On the other hand considering the small number of patients (5 subjects) positive for the 20210A allele and the relative variability of results obtained in this small group of subjects the definition of weight of this genetic variant on haematochemichal marker necessitate further studies on larger group of subjects.

Fibrinogen is the major coagulation protein in blood by mass, the precursor of fibrin, and an important determinant of blood viscosity and platelet aggregation [24,43]. Our data indicate that subjects bearing proinflammatory or procoagulant genotypes have the high plasma fibrinogen. As well known starting from the seminal paper by Hashmi et al. [44], high fibrinogen levels are predictor of coronary artery lesions, more recently it has been reported that plasma fibrinogen level may predict critical coronary artery stenosis in young adults with myocardial infarction [45]. In this view our data seem to suggest that the typing of the studied SNP of TNF-α and of FII associated to plasma fibrinogen measurement might be an useful tool to the prognostic evaluation of IMA in young subjects.

5. Conclusions

All together the results of the present study, indicating the relationship among genetically determined TNFα and FII production and increased levels of tissue damage markers of AMI as well central molecules for clotting processes, suggest that a complex genetic background, might be involved in susceptibility to AMI in young men and in the extension and severity of the disease.

In conclusion our data might prompt an approach to defining individual risk profiles that can be applied to healthy subjects to predict intrinsic risk of AMI with different ages at onset. Such risk profiles, when better established in a larger cohort of patients, can be used to trigger further diagnostic procedures and early therapeutic interventions aimed at preventing or significantly delaying the clinical manifestations of cardiovascular disease.

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CHAPTER 6

Identification of three particular morphological phenotypes in sporadic thoracic aortic aneurysm: phenotype III as sporadic thoracic aortic aneurysm biomarker in aged individuals.

Balistreri CR, Maresi E, Pisano C, Di Maggio FM, **Vaccarino L**, Caruso C, Lio D, Ruvolo G, Candore G.

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Identification of Three Particular Morphological Phenotypes in Sporadic Thoracic Aortic Aneurysm: Phenotype III As Sporadic Thoracic Aortic Aneurysm Biomarker in Aged Individuals

Carmela Rita Balistreri,[1,*] Emiliano Maresi,[2,*] Calogera Pisano,[3] Federica Maria Di Maggio,[1] Loredana Vaccarino,[1] Calogero Caruso,[1] Domenico Lio,[1] Giovanni Ruvolo,[3] Giuseppina Candore[1]

Abstract

Aging has a striking impact on the heart and the vascular system, and particularly on the large elastic arteries (*i.e.*, aorta), resulting in a multitude of changes at different structural and functional levels. As result, medial degeneration (MD) occurs. A characteristic example of MD is sporadic thoracic aortic aneurysm (S-TAA), whose patho-physiological mechanisms remain unclear. In this study, typical MD morphological phenotypes were researched in S-TAA cases and control aorta specimens using histopathological and mainly immunohistochemical analyses. Three phenotypes (I, II, and III) were detected, but the phenotype III was observed. Elevated cystic MD, plurifocal medial apoptosis, and increased metalloproteinase-9 amount characterize it. In addition, it was significantly correlated with the severity of elastic fragmentation, hypertension, and smoking, and particularly with advancing age. Thus, phenotype III might represent the typical MD phenotype associated with S-TAA in old people that have a major risk of aorta rupture and dissection independently on aneurysm diameter. This might permit the assumption that phenotype III with its typical histological abnormalities is an optimal biomarker of rupture and/or dissection in aged individuals and is useful both for applying different surgical approaches and providing appropriate surgical indications.

Introduction

AGING HAS A STRIKING IMPACT on heart and vascular system, particularly on the large elastic arteries (*i.e.*, aorta), determining a multitude of changes at different structural and functional levels.[1–3] As result, medial degeneration (MD) occurs.[4] At the macroscopic level, this pathological entity induces weakening of the aorta wall and a progressive stiffening.[4,5] At the microscopic level, MD is characterized by endothelial dysfunction, increased oxidative stress, inflammatory reaction, inflammatory cell infiltration in the aortic wall, and apoptosis of vascular smooth muscle cells (VSMCs), followed by degeneration of aortic media, elastin fracture, and degradation.[4,5] In turn, this results in aortic dilatation and aneurysm, and an increased risk of the onset of complications, *i.e.*, aortic dissection and rupture.[4] A characteristic model of MD is sporadic thoracic aortic aneurysm (S-TAA).[4] It is becoming a serious health risk because of growing enhancement of underlying diseases, *i.e.*, hypertension and aging, in Western populations.[6–10] The aged population seems to experience a increased incidence of S-TAA with advancing of years, as recently reported by epidemiological studies performed in geographic regions with stable populations with little out- or in-migration, such as in Minnesota and Sweden.[9,10] Hypertension is a widely prevalent and important risk factor for cardiovascular diseases, including S-TAA.[6] On the other hand, new guidelines have emphasized hypertension as the commonest cause of preventable death and as being significantly increased in aged population of developed countries.[6]

In contrast to the extensive study of syndromic and familial TAAs, the elucidation on the genetic, molecular, and cellular mechanisms of sporadic TAA forms has recently begun.[4] On the basis of these observations, the eventual presence of typical MD morphological phenotypes was

[1]Immunosenescence Group, Department of Department of Patho-biology and Medical and Forensic Biotechnologies, [2]Department of Pathologic Anatomy, and [3]Unit of Cardiac Surgery, Department of Surgery and Oncology, University of Palermo, Palermo Italy.
*These authors contributed equally to this study.

studied in aorta sporadic TAA samples through histopathological and immunohistochemical analyses. Our major goal was to identify one phenotype associated mainly with the risk of aorta rupture and dissection in old people.

Materials and Methods

Aorta specimens from 100 S-TAA patients (97 men and 13 women, whose median age was 62.95 ± 11.44 years) and 30 control individuals (20 men and 10 women, mean age 62.9 ± 11.57) died of causes unrelated to aortic disease and no sepsis, confirmed by autopsy, were collected. To perform histopathological and immunohistochemical analyses, patient and control aortas were fixed in 10% neutral buffered formalin for 24 hr, and then processed for routine paraffin embedding. For examinations by microscopy, multiple histological sections from each sample were prepared and stained with Hematoxylin & Eosin, Weigert-van Gieson, and Alcian-Periodic Acid Schiff (PAS). TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) testing and immuno-histochemical assessment for the evaluation of metalloproteinase-9 (MMP-9) levels and medial apoptosis were also performed, using procedures described in Supplementary Data (Supplementary Data are available at www.liebertonline.com/rej/).

The aortic wall was mainly evaluated for following histological features: (1) Fibrosis (defined as an increase in interstitial collagen); (2) medio-necrosis (defined as a focal loss of smooth muscle cell nuclei in the media); (3) cystic medial necrosis (defined as mucoid material accumulation); (4) focal or medial plurifocal apoptosis; (5) elastic fragmentation (defined as focal fragmentation of elastic lamellae in the media); (6) amounts of MMP-9 (see Figs. S1 and S2).

Histo-pathological abnormalities of aortic wall were graded and defined according to the definitions and grading systems used by Matthias Bechtel and colleagues[11] and also described in our recent studies (see Figs. S1 and S2). They were defined as follows. Cystic medial change: Grade I, minute cystic cavities holding a basophilic ground substance that occupy the total width of one lamellar unit; grade II, cystic cavities holding a basophilic ground substance that occupy the total width of two or more contiguous lamellar units; and grade III, large cystic cavities, holding a basophilic ground substance, that occupy the total width of aortic media (Fig. S1). Elastic fragmentation: Grade I, up to five foci of elastic fibers interruption in two-four neighboring lamellar units in one microscopic field of 100×magnification; grade II, more than five foci of elastic fibers interruption in two-four neighboring lamellar units; grade III, elastic fibers interruptions occupy the total width of aortic media with disarray of smooth muscle cells (Fig. S1). Fibrosis: Grade I, an increase of collagen fibers in an area less than one-third of the total medial thickness; grade II, an increase of collagen fibers occupying between one-third and two-thirds of the medial thickness; grade III, an increase of collagen fibers that occupy more than two-thirds of the medial thickness (Fig. S1). Medial necrosis: Grade I, focal loss of smooth muscle cells nuclei in an area less than one-third the thickness of the media; grade II, focal loss of smooth muscle cells nuclei that occupy between one-third and two-thirds the medial thickness; grade III, focal loss of smooth muscle cells nuclei that occupy more than two-thirds the medial thickness (Fig. S1). Atherosclerotic aneurysms: We have defined atherosclerotic aneurysms as those that showed macroscopic intimal calcific plaques or microscopic intimal fibrotic thickness that is one-third that of the aortic media thickness.

Statistical analysis

Correlations were assessed using Spearman rank correlation. A $p<0.05$ was considered statistically significant.

Results

Interestingly, 73 case aorta tissues were with the typical MD and without atherosclerotic lesions. In these tissues, three phenotypes (I, II, and III) were detected having a different quantitative relationship of cystic MD, fibrosis, apoptosis, and amount of MMP-9 (see Figs. S1 and S2). They were described as follows: Phenotype I, cystic medial degeneration balanced by a substitutive fibrosis, in absence of medial apoptosis and with a low MMP-9 concentration; phenotype II, higher cystic medial degeneration than substitutive fibrosis, with focal medial apoptosis, and with mainly a modest MMP-9 amount; phenotype III, elevated cystic medial degeneration, without substitutive fibrosis, with plurifocal medial apoptosis, and with an elevated MMP-9 concentration.

Among these, phenotype III was mainly (63 vs. 73) observed in case aortas. It showed elevated cystic MD, plurifocal medial apoptosis, and increased MMP-9 amount (see Fig. 1A and B). In addition, phenotype III was significantly correlated with the severity of elastic fragmentation, hypertension, smoking, and particularly with advancing age ($r=0.497$, $p=0.0001$; $r=0.267$, $p=0.03$; $r=0.342$, $p=0.006$; $r=0.567$, $p=0.0001$, respectively; by non-parametrical Spearman correlation test; data not shown). In contrast, no correlation was observed with aorta diameter (data not shown). Thus, phenotype III might represent the typical MD phenotype associated with major risk of aorta rupture and dissection independently on aneurysm diameter.

Discussion

Aortic disease is currently a large pathology concern because it is common particularly in advancing age and can lead to fatal outcomes.[12] A variety of conditions affect the aorta with progressing years; the most common are aneurysm, dissection, occlusion owing to atherosclerosis, and general stiffening.[12] These conditions are characterized by micro-structural changes that frequently are the result of vascular aging.[1–3] Indeed, aging determines the loss of molecular fidelity at cellular, tissue, and organ levels, including the heart and vascular system, followed by a progressive entropy, which renders patients more easily vulnerable to internal and external stressors, frailty, disability, and disease.[13] In the thoracic aorta, age-related modifications give rise to a pathological entity, MD, having catalyst and accelerator effects for the onset of S-TAA.[4] Thus, S-TAA risk increases with biological aging. Little has been known until now about the histopathological MD-related phenotypes associated with a major risk for S-TAA and its complications, especially in old people.[4,14–16] This led us to perform histopathological studies to identify phenotypes of

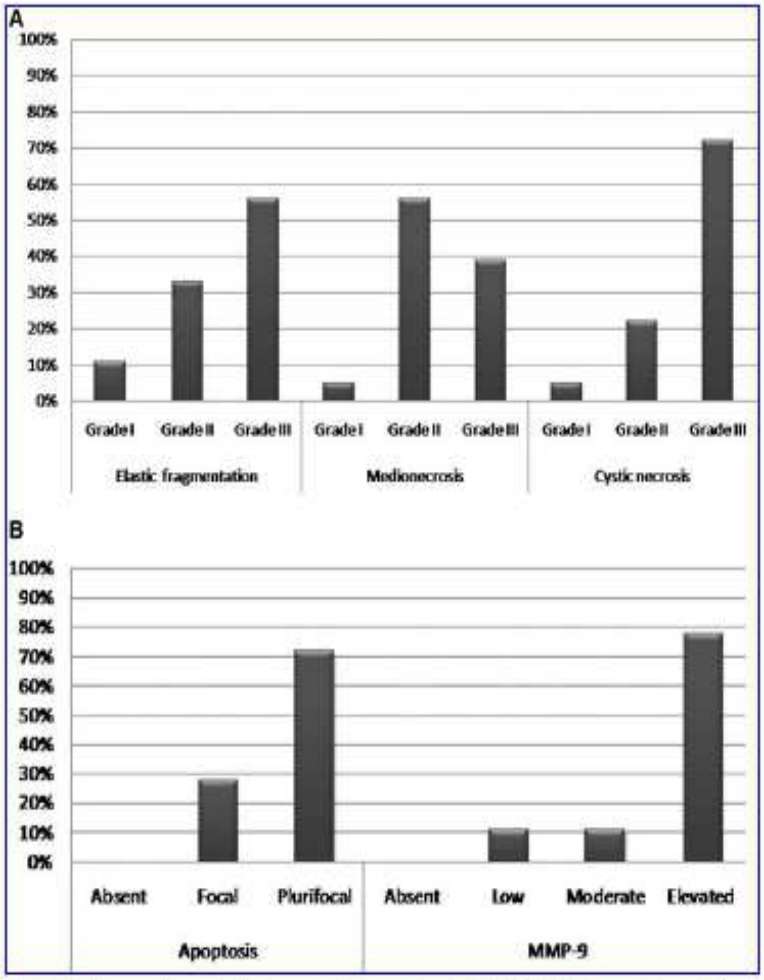

FIG. 1. (A and B) Aorta wall abnormalities characterizing phenotype III. (A) Elastic fragmentation, medio-necrosis, and cystic necrosis essentially of grades II and III were found. (B) Focal and plurifocal apoptosis were also found in all tissue aorta samples from patients versus controls. In addition, low, moderate, and elevated metalloproteinase-9 (MMP-9) amounts were also observed.

S-TAA, which, more than others, evolve into dissection or rupture.[7,18]

In a previous study, we detected a morphological identity of medial lesions that might be assumed as precursor and consequently as optimal biomarker of type A dissection (TAD), independently of aneurysm diameter or valvular disorder.[17] On the other hand, we observed that the severity of aortic media degeneration in TAD cases is not related to the diameter of the aneurysm.[17] Other research groups frequently found the onset of TAD in patients with a normal aortic diameter.[17,18] These promising findings encouraged us to find phenotypes associated with the risk of aorta rupture and dissection in aged S-TAA individuals. Thus, in the present study, histopathological and immunohistochemical analyses were assessed in aorta specimens from 100 S-TAA patients with median age of 62.95±11.44 years and age- and gender-matched controls. Interestingly, we observed that a large number of aorta samples showed a morphological identity, defined as phenotype III and characterized by elevated cystic MD, plurifocal medial apoptosis, and increased MMP-9 levels. This phenotype appears to be significantly correlated with the severity of elastic fragmentation. In addition, it shows a relative absence of a reparative fibrosis, and consequently it might mainly predispose the

S-TAA patients to aortic rupture. These data seem to be in agreement with the emerging literature data suggesting the key involvement of an up-regulation of metalloproteinase and apoptosis correlated with inflammatory age-related processes and genetic factors in the pathophysiology of S-TAA.[18] On the other hand, we recently suggested that inflammation producing MMPs, cytokines, and death mediators seem to be the shared pathological mechanism for TAD. In addition, significant associations between single-nucleotide polymorphisms in inflammatory genes and the TAD risk were also reported in our study.[18]

Furthermore, phenotype III seems to be the result of three major risk factors of S-TAA, including hypertension, smoking, and advancing age. Indeed, a significant positive correlation was, observed between phenotype III and these risk factors. On the other hand, in a recent study we demonstrated that smoking, hypertension, and age were the exclusive risk S-TAA factors significantly associated with the reduction of the mean leukocyte telomere length.[19] Their association seems plausible with the biological effects that they mediate on the aortic wall. In contrast, no correlation was observed with aortic diameter.

Together our results, although obtained from a relatively large number of individuals, might indicate that phenotype III, with its typical histological abnormalities, is an optimal biomarker of rupture and/or dissection in aged individuals and would be useful both for applying different surgical approaches and providing appropriate surgical indications. On the other hand, an increase of S-TAA incidence in the aged population has been recently observed by epidemiological studies.[7,9,10] In addition, this might be useful because S-TAA is clinically and predominantly a silent ailment until rupture or dissection occurs and is insidious in its onset and progression. Furthermore, until now its diagnosis is also exclusively based on imaging technologies, and no blood tests exist. Certainly, our findings require continued research on the genetic, cellular, and molecular mechanisms involved in the development of this phenotype. It and combined efforts might lead to further elucidation about the role of S-TAA phenotype III, especially in old people.

A major advance would be to be translate these findings into individualized and effective pharmacological treatments oriented toward molecular and genetic mechanisms, allowing for tailored medical and surgical approaches to this very serious condition.[20] Accordingly, recently Castellano and colleagues emphasized that we are ignoring this disease, which is growing in incidence and characterized by a different location and etiology with respect to abdominal aorta aneurysm (AAA). These aortic diseases need to be considered as distinct entities and not hastily grouped together. Thus, the aortic diameter and therapies used for AAA care are not suitable for S-TAA, as recently evidenced by new guidelines for S-TAA.[21] Clinical trials for S-TAA are required before medical therapies, such as β-blocken, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, statins, or macrolide antibiotics, can be recommended. On the other hand, we have proposed in our recent studies that a surgical approach for patients with S-TAA should consider not only the diameter of the aortic aneurysm portion but also the histological features and the genetic risk profile.[17,18,22]

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Carmela Rita Balistreri
Department of Pathobiology and Medical and Forensic Biotechnologies
University of Palermo
Corso Tukory 211
Palermo, 90134
Italy

E-mail: carmelarita.balistreri@unipa.it

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CHAPTER 7

Role of TGF-β pathway polymorphisms in sporadic thoracic aortic aneurysm: rs900 TGF-β2 is a marker of differential gender susceptibility.

Scola L, Di Maggio FM, **Vaccarino L**, Bova M, Forte GI, Pisano C, Candore G, Colonna-Romano G, Lio D, Ruvolo G, Balistreri CR.

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Research Article

Role of TGF-β Pathway Polymorphisms in Sporadic Thoracic Aortic Aneurysm: rs900 TGF-β2 Is a Marker of Differential Gender Susceptibility

Letizia Scola,[1] Federica M. Di Maggio,[1] Loredana Vaccarino,[1] Manuela Bova,[1] Giusy I. Forte,[1] Calogera Pisano,[2] Giuseppina Candore,[1] Giuseppina Colonna-Romano,[1] Domenico Lio,[1] Giovanni Ruvolo,[2] and Carmela R. Balistreri[1]

[1] *Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Corso Tukory 211, 90134 Palermo, Italy*
[2] *Unit of Cardiac Surgery, Department of Surgery and Oncology, University of Palermo, Palermo, Italy*

Correspondence should be addressed to Carmela R. Balistreri; carmelarita.balistreri@unipa.it

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Thoracic aortic aneurysm (TAA) is a progressive disorder involving gradual dilation of ascending and/or descending thoracic aorta with dissection or rupture as complications. It occurs as sporadic or defined syndromes/familial forms.Genetic, molecular and cellular mechanims of sporadic TAA forms are poorly characterized and known. Thus, our interest has been focused on investigating the role of genetic variants of transforming growth factor-β (TGF-β) pathways in TAA risk. On the other hand, no data on the role of genetic variants of TGF-β pathway in sporadic TAA exist until now. In addition, other cytokines, including IL-10, orchestrate TAA pathophysiology. Their balance determines the ultimate fate of the aortic wall as healing atherosclerosis or aneurysm formation. Thus, in this paper it was analyzed the role of ten polymorphisms of genes encoding TGF-β isoforms and receptors, and IL-10 in sporadic TAA. Our study included cases affected by sporadic TAA and two control groups. The most relevant finding obtained allows us to propose that rs900 TGF-β2 SNP is associated with sporadic TAA in women. This might open new perspectives for the analysis of sporadic TAA susceptibility factors and prevention.

1. Introduction

Thoracic aortic aneurysm (TAA) is a pathological widening of aorta resulting from degeneration of the extracellular matrix and loss of smooth muscle cells in the tunica media. TAA has different etiological causes, including monogenic syndromes (such as Marfan and Loeys-Dietz syndromes), bicuspid aortic valve (BAV) disease, and idiopathic causes [1, 2].

The pathogenesis of TAA in monogenic syndromes has been extensively studied [3]. The attested evidence, indeed, suggests the deregulation of transforming growth factor-β (TGF-β) signaling characterized by its enhanced function and damaged TGF-β receptors as their common and typical feature [4, 5].

The TGF-β family is constituted by TGF-β1, TGF-β2, and TGF-β3 members, which are pleiotropic secreted cytokines having a broad spectrum of biologic functions. Among these, the TGF-β1 has numerous cellular functions, including cell growth, cell proliferation, cell differentiation, and apoptosis. In humans, TGF-β gene product's effects can stimulate or inhibite cell growth depending cellular and tissue targets. TGF-β1 can modulate cell differentiation and proliferation in auto- or paracrine manner [6]. In vascular smooth muscle cells, TGF-β can upregulate fibronectin and connective tissue growth factor expression via activation of small mothers against decapentaplegic (Smad) proteins [7]. As a consequence, it can promote the deposition of components of extracellular matrix (ECM) [8]. Furthermore, its action depends on the interaction with specific receptors, such as TGF-β receptor (TGF-βR) I and TGF-βRII, glycoproteins of 55 kDa and 70 kDa, respectively, with core polypeptides of 500–570 amino acids [9].

TGF-β actually is considered as a crucial player in vascular remodelling, able to alter both structure and ECM composition. In Marfan syndrome, the fibrillin-1 gene mutations seem to influence the bioavailability of active TGF-β. In addition, mutations in the TGF-β receptors also impair the signalling cascade in other Marfan syndrome related disorders, including Loeys-Dietz syndrome, familial TAAs, and aortic dissection. Furthermore, mutations in Notch gene homolog 1 (NOTCH1) and Notch1 pathway, mainly identified in TAA patients with BAV, seem to influence TGF-β crosstalk [10].

In contrast, molecular and genetics mechanisms of the nonfamilial TAA forms, representing the major number of cases of TAAs, remain largely unknown [11]. Different roles of TGF-β pathways in tissue remodelling mechanisms have been reported in both sporadic thoracic and abdominal aneurysms [8]. In particular, both loss and gain of functional TGF-β signalling have been described as predisposing factors for both sporadic TAA development and dissection. The paradoxical effect of TGF-β leading to enhanced connective matrix degradation through metalloproteinase activation has been principally observed in the nonsyndromic cases of familial TAAs and dissection [4]. In addition, TGFBR1 and TGFBR2 losses induced by functional mutations have been associated with both familial syndromic and nonsyndromic TAAs [12–14]. This altered condition of TGF-β signalling has been demonstrated to induce unusually the activation of TGF-β mediated connective matrix degradation [4].

Furthermore, vascular remodelling, characterising both sporadic thoracic and abdominal aneurysm, seems prevalently to be the result not only of TGF-β pathways, but also of upregulation of multiple cytokines, including interleukin-10 (IL-10), an anti-inflammatory cytokine able to modulate activity of TGF-β pathways. A large variety of immune and tissue aorta cells evocate the typical aorta abnormalities of thoracic and abdominal sporadic aneurysms. The balance of cellular type and resultant cytokine milieu determines the ultimate fate of the aortic wall healing, atherosclerosis, or aneurysm formation. In the complex scenario, another crucial factor is the genetics propensity [15, 16]. Polymorphisms of IL-10 gene have been associated with abdominal aneurysms, while no data exist in literature about their role in sporadic thoracic aneurysms [17–20].

Based on these observations, in this paper we sought to analyse the role of some common single nucleotide polymorphisms (SNPs) of genes encoding TGF-β isoforms and receptors, and IL-10 and receptor in sporadic TAA. On the other hand, no literature data on the role of genetic variants of TGF-β and IL-10 pathways in sporadic TAA exist until now.

2. Materials and Methods

2.1. Patient and Control Populations. Our study included 144 individuals (107 men (74.3%) and 37 (25.7%) women; mean age: 63 ± 10.7) from Western Sicily enrolled precisely from January 2004 to July 2008 at time of their admission to Cardiac Surgery Unit of Palermo University Hospital. They were affected by sporadic TAA, diagnosed through ECHO, CT, and MRI imaging technologies and with localization essentially in ascending aorta (precisely in aortic sinus and tubular portion and sometimes only in tubular portion) and in aortic bulb, or both (Table 1). Familial and syndromic forms (i.e., Marfan and Ehlers-Danlos syndromes) and autoimmune connective tissue disorders were excluded through histopathological criteria and phenotypic analyses.

Medical histories pertinent to aortic diseases were obtained from patient's medical records. Thus, demographic and clinical features, comorbidity conditions, and pharmacological treatments were collected (Table 1).

To perform genotype analyses two different control populations were also enrolled. The first included 90 unrelated patients of the same cardiac unit without TAA (56 (62%) men and 34 (38%) women; mean age: 61.08 ± 5.83 years). The second control group was represented by 168 healthy control (112 (66.7%) men and 56 (33.3%) women; mean age: 45.2 ± 7.44 years). Their demographic and clinical features ECHO imaging exclusion of aorta wall abnormalities, comorbidity conditions, and pharmacological treatments were collected (see Table 1).

Patients and controls belonged to the same ethnic group, since their parents and grandparents were born in Sicily. Healthy control age was significantly lower respect to that of the two groups of patients and hypertension characterised the 79% of all patients, opportunely treated with medications like ACE inhibitors and beta-blocker, and so forth during the follow-up and after surgical procedures (Table 1).

Our study received approval from local ethic committee and all participants gave their informed consent. Data were encoded to ensure privacy protection of patients and controls. All laboratory procedures were performed without knowledge about nature of material.

2.2. Molecular Typing. As reported in Table 2, we selected ten functional and common SNPs of IL-10 and TGF-β pathways located in the promoter region, codifying and noncodifying sequences and 3′UTR region. Information about these SNPs was acquired from dbSNP NCBI, the ENSEMBL database (http://www.ensembl.org/index.html), and the UCSC Genome Browser website (http://genome.ucsc.edu/). The allelic and genotypic frequencies of TGF-β and IL-10 SNP pathways were detected through the assays on demand developed by KBioscience Ltd. (Middlesex, UK) and based on a homogeneous Fluorescence Resonance Energy Transfer (FRET) detection and allele specific PCR (Kaspar). Briefly, two specific oligonucleotides were designed for each allele of the SNPs studied. Each one of these oligos was tailed with an 18 bp sequence distinct from each other. Taq polymerase, dNTPs, an internal standard dye (rhodamine X, Rox), and reverse primers were included. In addition, the KBioscience modified versions of Taq polymerase are unable to extend primers characterised to be mismatched at their 3′ terminal base. This property was used to discriminate the two alleles. The reaction was monitored by the fluorescence signals released by two other FRET reporter oligos included in the reaction mixes. The endpoint fluorescence emission was detected on an ABI-Prism 7300 Real-Time PCR Analyzer (Applied Biosystem, USA). The genotypes were determined using the 7300 system SDS software, versus 1.3 (Applied

Table 1: Demographic and clinical characteristics of TAA patients and control subjects.

Variables	AAT patients	Control patients	P	Control patients	P
Demographic characteristics	$N = 144$	$N = 90$		$N = 168$	
Age, mean (SD)	63.0 (10.7)	62.1 (5.8)	0.834	45.2 (7.4)	<0.0001
Males, number (%)	107 (74.3)	56 (62.2)	0.060	112 (66.7)	0.172
Body mass index, mean (SD)	27.0 (4.3)	26.9 (2.9)	0.898	25.8 (8.7)	0.133
TAA size and location					
Size (mm), mean (SD)	53.3 (8)	0 (0)		0 (0)	
Location, number (%)		0 (0)		0 (0)	
Ascending aorta	72 (50.0)				
Aortic bulb	16 (11.1)				
Ascending aorta and aortic bulb	56 (38.9)				
Medical history number (%)					
Aortic aneurysm familiarity	8 (5.6)	0 (0)		0 (0)	
Cardiovascular ischemic familiarity	53 (36.8)	24 (26.7)	0.089	0 (0)	
Smoking	65 (45.1)	46 (51.1)	0.420	67 (39.9)	0.360
Hypertension	114 (79.1)	28 (31.1)	<0.001	0 (0)	
Dislipidemy	33 (22.9)	14 (15.6)	0.158	0 (0)	
Diabetes mellitus	22 (15.3)	12 (13.3)	0.677	0 (0)	
Renal failure	4 (2.8)	0 (0)	0.168	0 (0)	
Dissection	16 (11.1)	0 (0)		0 (0)	
Aortic valve pathology, number (%)					
Normal	81 (56.2)	90 (100)		168 (100)	
Prolapse	19 (13.2)	0 (0)			
Vascular calcium fibrosis	45 (31.3)	0 (0)			
Aortic valve dysfunction, number (%)					
Normal	29 (20.1)	90 (100)		168 (100)	
Faint incontinence	26 (18.0)	0 (0)			
Moderate incontinence	30 (20.8)	0 (0)			
Severe incontinence	40 (27.1)	0 (0)			
Faint stenosis	1 (0.7)	0 (0)			
Moderate stenosis	2 (1.4)	0 (0)			
Severe stenosis	16 (11.1)	0 (0)			
Atherosclerosis coronary syndrome number (%)	49 (34.0)	0 (0)		0 (0)	
Drugs, number(%)					
None				168 (100)	
Beta-blockers	56 (38.9)	0 (0)		0 (0)	
Central-adrenergic agonists	23 (16.0)	0 (0)		0 (0)	
Sartans	29 (20.1)	0 (0)		0 (0)	
Calcium-channel blockers	42 (29.2)	0 (0)		0 (0)	
ACE inhibitors	59 (41.0)	14 (15.6)		0 (0)	
Antidiabetic drugs	17 (11.8)	12 (13.3)		0 (0)	
Antiaggregant drugs	46 (31.9)	28 (31.1)		0 (0)	
Antidyslipidemic drugs	32 (22.2)	0 (0)		0 (0)	
Diuretics	32 (22.2)	28 (31.1)		0 (0)	

Biosystems) sample by sample, on the basis of the detection of a unique (homozygous samples) or double (heterozygous samples) fluorescence signals.

2.3. Statistical Analysis. Allele and genotype frequencies were evaluated by gene count. Data were tested for goodness of fit between observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by χ^2 tests. Significant differences in homozygous and heterozygous genotype distributions among groups were calculated by using χ^2 test and appropriate tables. Multiple logistic regression models were applied using dominant (major allele homozygotes versus heterozygotes plus minor allele homozygotes) and recessive (major allele homozygotes plus heterozygotes versus

Table 2: Genes, SNPs (accession number), substitutions, localization, and position investigated in the study.

Genes	SNPs	Localization	Position	Alleles
IL-10	rs1800896	Promoter	−1082	G>A
	rs1800871	Promoter	−819	C>T
	rs1800872	Promoter	−592	C>A
	rs3024496	3′UTR	Not defined	C>T
IL-10RB	rs2834167	Codifying sequencing	Codon 47	A>G
TGF-β1	rs1800471	Codifying sequencing	Codon 25	G>C
	rs900	3′UTR	+94862	A>T
	rs334348	3′UTR	Not defined	A>G
	rs334349	3′UTR	Not defined	A>G
	rs4522809	Intron	+3919	C>T

minor allele homozygotes) models. Odds ratios (OR), 95% confidence intervals (95% C.I.), and P values were determined using SPSS (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of the Frequencies of Ten SNPs in Our Population. Literature data have evidenced the association of the IL-10 and TGF-β SNPs with sporadic TAA and other cardiovascular diseases [4, 8, 12–14, 17–20]. The analysis of genotype frequencies of all SNPs examined with respect to the expected results was executed confirming that all populations were in Hardy-Weinberg equilibrium, with the exception of rs334349 genotype distribution (Table 3) in TAA patient group. The analysis of IL-10 and IL-10RB gene SNPs does not allow finding significant differences in genotype frequencies among the three populations examined (Table 3).

Comparing genotype distributions and allele frequencies of the five TGF-β pathway SNPs selected in our study between cases and the two control groups, significant differences were observed only for TGF-β2 rs900 polymorphism (see Table 4). Its frequency was significantly different in TAA patients than to both control patients ($P = 0.047$) and healthy controls ($P = 0.0059$) (Table 4). In particular, the AA genotype of TGF-β2 rs900 SNP had a reduced frequency in the TAA patients, which contrarily showed an increased frequency of TT genotype.

These results were confirmed by logistic regression analyses of dominant and recessive models performed between TAA patient and control groups (Table 5). Interestingly, the data obtained through comparisons between both control patients and healthy controls for dominant model and comparisons with the last group for recessive model evidenced that the presence of A allele in homo- or heterozygosis seems to be significantly protective against TAA.

Since the incidence of TAA is higher in men than in women with precisely a ratio of 3 : 1 [12], we assessed the rs900 TGF-β SNP frequencies according to gender. Comparing the data, we observed significant differences in genotype distribution of the rs900 SNP in women, whereas no significant differences were detected in men (Table 6). In particular, the AA genotype was significantly decreased in women affected by TAA with respect to both women of control patient group ($P = 0.0076$) and health control group ($P = 0.0003$). The TT genotype was reciprocally significantly increased ($P = 0.0027$). Thus, altogether these data emphasize cotemporally the gender related protective role of AA genotype for TAA and the increased susceptibility for TAA in individual's carriers of TT genotype (Table 6). When we perform a logistic regression analysis adjusted for gender, the significant differences of AA genotype frequency between patients and subjects of the two control groups ($P = 0.003$) and particularly between patients and healthy controls ($P < 0.0001$) were confirmed.

Table 3: Single nucleotide polymorphism frequencies of IL-10 pathway genes in patients affected by sporadic TAA and the two groups of controls subjects*.

SNPs	Genotypes	TAA patients n (%)	Control patients n (%)	Healthy controls n (%)
rs1800896	GG	17 (11.8)	7 (7.8)	21 (12.5)
	GA	66 (45.8)	44 (48.9)	67 (39.9)
	AA	61 (42.4)	39 (43.3)	80 (47.6)
rs1800871	CC	64 (44.4)	45 (50.0)	84 (50.0)
	CT	65 (45.1)	38 (42.2)	67 (39.9)
	TT	15 (10.5)	7 (7.8)	17 (10.1)
rs1800872	CC	64 (44.4)	45 (50.0)	84 (50.0)
	CA	65 (45.1)	38 (42.2)	67 (39.9)
	AA	15 (10.5)	7 (7.8)	17 (10.1)
rs3024496	CC	16 (11.1)	5 (5.5)	19 (11.3)
	CT	66 (45.8)	52 (57.8)	69 (41.1)
	TT	62 (43.1)	33 (36.7)	80 (47.6)
rs2834167	AA	67 (46.6)	35 (38.9)	91 (54.2)
	AG	66 (45.8)	44 (48.9)	60 (35.7)
	GG	11 (7.6)	11 (12.2)	17 (10.1)

*No significant differences were found comparing TAA patient genotype frequencies with control patient and healthy control groups.

4. Discussion

Risk factors involved in developing aneurysms are similar to those for heart disease, including atherosclerosis, hypertension, smoking, advanced age, and family history. However, the lack of aneurysm-specific symptoms often renders them unnoticed until the aorta ruptures associated with significant morbidity and mortality [1, 2, 13].

TAA development proceeds as a multifactorial process influenced by both cellular and extracellular mechanisms, resulting in alterations of structure and ECM composition [1, 2]. Recent evidence underlines the deregulation of TGF-β signalling in ascending TAAs from syndromic (Marfan

Table 4: Genotype frequencies of TGF-β1 and 2 isoform and R1 and R2 receptor gene single nucleotide polymorphisms in patients affected by sporadic TAA and the two groups of controls subjects.

SNPs	Genotypes	TAA patients *n* (%)	Control patients *n* (%)	*P* value	Healthy controls *n* (%)	*P* value
rs1800471 TGF-β1 cod25	GG	121 (84.03)	77 (85.56)	0.499	138 (82.14)	0.849
	CG	22 (15.28)	11 (12.22)		28 (16.67)	
	CC	1 (0.69)	2 (2.22)		2 (1.19)	
rs900 TGF-β2 3′UTR	AA	44 (30.56)	40 (44.44)	**0.047**	77 (45.84)	**0.0059[a]**
	AT	70 (48.61)	36 (40.00)		73 (43.45)	
	TT	30 (20.83)	14 (15.56)		18 (10.71)	
rs334348 TGF-βR1 3′UTR	AA	92 (63.89)	56 (62.22)	0.898	106 (63.09)	0.0994
	AG	43 (29.86)	27 (30.00)		57 (33.93)	
	GG	9 (6.25)	7 (7.78)		5 (2.98)	
rs334349 TGF-βR1 3′UTR	GG	91 (63.20)	55 (61.11)	0.376	112 (66.67)	0.0544
	GA	39 (27.08)	30 (33.33)		51 (30.35)	
	AA	14 (9.72)	5 (5.56)		5 (2.98)	
rs4522809 TGF-βR2 Intron +3919	CC	36 (25.00)	24 (26.67)	0.959	44 (26.19)	0.1438
	CT	65 (45.14)	40 (44.44)		83 (49.41)	
	TT	43 (29.86)	26 (28.89)		41 (24.40)	

[a]The genotype distribution of rs900 TGF-β2 3′UTR SNP was significantly different in TAA patients when compared to both control patients and healthy controls. Allele frequencies: TAA patients: 0.549; control patients: 0.644; healthy subjects: 0.676.

Table 5: Multiple logistic regression analyses of dominant (major allele homozygotes versus heterozygotes plus minor allele homozygotes) and recessive (major allele homozygotes plus heterozygotes versus minor allele homozygotes) models applied to TAA patient group compared with control groups.

SNP	Model	TAA versus control patients		TAA versus healthy controls	
		OR (95% C.I.)	*P*	OR (95% C.I.)	*P*
rs1800471	Dominant	0.888 (0.452–1.858)	0.859	1.144 (0.630–2.075)	0.769
	Recessive	3.250 (0.290–36.392)	0.561	1.723 (0.154–19.210)	1.000
rs900	Dominant	0.550 (0.318–0.950)	**0.036**	0.520 (0.326–0.829)	**0.0073**
	Recessive	0.700 (0.348–1.407)	0.690	0.456 (0.242–0.859)	**0.0177**
rs334348	Dominant	1.074 (0.623–1.853)	0.889	1.035 (0.652–1.643)	0.906
	Recessive	1.265 (0.454–3.526)	0.791	0.460 (0.151–1.406)	0.181
rs334349	Dominant	1.093 (0.635–1.880)	0.782	0.858 (0.538–1.369)	0.553
	Recessive	0.546 (0.190–1.572)	0.329	0.511 (0.081–1.039)	0.0516
rs4522809	Dominant	0.917 (0.503–1.671)	0.878	0.939 (0.564–1.565)	0.897
	Recessive	0.954 (0.535–1.703)	1.000	0.758 (0.459–1.252)	0.307

syndrome, Loeys-Dietz syndrome, and Ehlers-Danlos syndrome) and not syndromic TAA patients as well as in TAAs from cases affected by familial TAAs and dissections [14].

TGF-β isoforms are produced by multiple cellular types and participate in a wide array of cellular responses including proliferation, angiogenesis, differentiation, apoptosis, inflammation, and wound healing [21]. Of the three TGF-β isoforms, their role in matrix deposition (e.g., collagen synthesis) related to fibrotic disease is particularly well known [22]. However, some recent data have also demonstrated their involvement in unconventional pathways able to determine matrix degradation [4].

Furthermore, the TGF-β isoforms exhibit both overlapping and divergent properties, as evidenced principally in embryogenetic studies. In particular, TGF-β2 knockout mice are characterized to die perinatally and display a wide range of developmental defects, including cardiovascular, pulmonary, skeletal, ocular, inner ear, and urogenital manifestations [23–25]. Haplo-insufficient TGF-β2 mice have aortic root aneurysm and biochemical evidence of increased canonical and no canonical TGF-β signalling [26]. These observations led Maleszewska and colleagues to suggest the crucial role of TGF-β2 in the vascular remodelling [27]. They particularly underlined that, in presence of a low grade chronic inflammation of the cardiac and vascular tissues, the TGF-β2, interacting with other cytokines as interleukin-1B, might modify and remodel vascular aorta tissues inducing endothelial cells forward mesenchymal transition (EndMT). EndMT represents a central mechanism in cardiac valve embryogenesis, which in pathological condition might determine cardiac fibrosis.

Table 6: Statistical analysis of TGF-β2 rs900 genotype distributions in patients affected by TAA and in the two groups of control subjects stratified according to the gender[a].

	Men			Women		
	AA	AT	TT	AA	AT	TT
TAA patients *n* (%)	36 (33.65)	54 (50.46)	17 (15.89)	8 (21.62)	16 (43.24)	13 (35.13)
Control patients *n*. (%)	22 (39.29)	25 (44.64)	9 (16.07)	18 (52.94)	11 (32.35)	5 (14.71)
Healthy controls *n* (%)	43 (38.39)	56 (50.00)	13 (11.61)	34 (60.71)	17 (30.36)	5 (8.93)
	Odd ratio significance					
	Men			Women		
	AA	AT	TT	AA	AT	TT
	TAA versus control patients					
OR	0.784	1.263	0.986	0.245	1.263	3.342
95% C.I.	0.401–1.531	0.660–2.418	0.408–2.382	0.087–0.689	0.660–2.418	0.990–10.071
P value	0.495	0.512	1.000	**0.0076**	0.512	0.059
	TAA versus healthy controls					
OR	0.814	1.019	1.438	0.178	1.019	5.525
95% C.I.	0.468–1.415	0.401–1.531	0.662–3.128	0.069–0.461	0.600–1.731	1.767–17.272
P value	0.484	1.000	0.433	**0.0003**	1.000	**0.0027**

[a]Logistic regression analysis adjusted for gender confirms that the AA genotype frequency was significantly decreased and TT genotype increased in TAA women patients compared to women of the two control groups ($P = 0.003$), in particular compared to healthy controls ($P < 0.0001$).

Actually, immunohistochemistry analysis of TAA aneurysms demonstrated that both the media and adventitia from patients with Marfan syndrome and familial TAAs, as well as from sporadic cases with or without dissections or BAV diseases, are characterised by infiltration of inflammatory cells [27]. This inflammatory condition might contribute to the pathogenesis of TAA [3, 28–35].

In the light of these observations, we assessed the role of five genetic variants of TGF-β pathways (TGF-β1 and 2 isoforms and R1 and R2 receptors) in sporadic TAA. Interestingly, the most relevant finding of the present study allows proposing that rs900 TGF-β2 SNP is associated with sporadic TAA in women. On the other hand, recent reports assigned a direct or an indirect central role to TGF-β2 and its genetic variants in the pathogenesis of both syndromic and familial TAAs [3, 21, 28–35]. In addition, it has been reported that mutations in the TGFBRII genes deregulate the TGF-β2 signalling pathway involved in TAA pathogenesis [3, 28–35]. TGF-β2 gene mutations have been found in familial TAAs and dissections associated with mild systemic features of Marfan syndrome, Loeys-Dietz syndrome and in TAA and dissection associated with mitral valve disease [3, 28–35]. In these diseases, the TGF-β2 dependent EndMT might play a role. In spite of these findings, the exact role of TGF-β2 in TAA pathogenesis is not clear. In particular, both the loss of function genetically determined and the "paradoxical" augment in the downstream TGF-β signaling pathway might be important for TTA development [14].

However, to our knowledge, no literature data exist about the role of genetic variants of TGF-β2 pathway in sporadic TAAs. Thus, this is the first report that identified a common and functional TGF-β2 SNP, the rs900 SNP, as genetic risk marker for sporadic TAA. The rs900 SNP is located at the 2814 position downstream of the TGF-β2 gene coding region. Scanning allelic rs900 sequences for UTR structural motif using an online tool (http://itbtools.ba.itb.cnr.it/utrscan) has shown that the T allele introduces a new open reading frame ATG in the 3'UTR region of TGF-β2 gene that potentially might interfere with ribosomal translation. This may allow hypothesising that the rs900 T allele may interfere with the rate of protein production.

5. Limitations and Conclusions

As reported above, TAAs occur most frequently in Caucasians than in other ethnic groups and they afflict men two to four times more frequently than women [4]. As consequence, our results, suggesting that rs900 TGF-β2 SNP might be one of genetic factors involved in the woman susceptibility for TAA, might open new perspectives for the analysis of sporadic TAA susceptibility factors and prevention. Actually these findings obtained in this relatively small study, which need certainly to be confirmed in larger populations of different genetic background, might prompt studies on gender oriented pharmacological strategies to prevent TAA development in predisposed subjects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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CHAPTER 8

G-protein-coupled receptor kinase 5 polymorphism and Takotsubo cardiomyopathy.

Novo G, Giambanco S, Guglielmo M, Arvigo L, Sutera MR, Giambanco F, Evola S, **Vaccarino L**, Bova M, Lio D, Assennato P, Novo S.

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Corresponding Author:	Salvatore Giambanco, M.D. Division of Cardiology, A.O. Policlinico "P. Giaccone", University of Palermo, Italy Palermo, PA ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Division of Cardiology, A.O. Policlinico "P. Giaccone", University of Palermo, Italy
Corresponding Author's Secondary Institution:	
First Author:	Giuseppina Novo
First Author Secondary Information:	
Order of Authors:	Giuseppina Novo
	Salvatore Giambanco, M.D.
	Marco Guglielmo
	Luisa Arvigo
	Maria Rita Sutera
	Francesco Giambanco
	Salvatore Evola
	Loredana Vaccarino
	Manuela Bova
	Domenico Lio
	Pasquale Assennato
	Salvatore Novo
Order of Authors Secondary Information:	
Manuscript Region of Origin:	ITALY
Abstract:	Background Takotsubo cardiomyopathy (TTC) is an increasingly reported clinical syndrome that mimics acute myocardial infarction without obstructive coronary artery disease and it characterized by transient systolic dysfunction of the apical and/or midsegments of the left ventricle. The syndrome mainly occurs in postmenopausal women with high adrenergic state conditions. Nowadays, the pathophysiology of TTC is not yet known and the possibility of a genetic predisposition is controversial. Aims The purpose of this study was to assess the genetic susceptibility to TTC through analysis of the L41Q polymorphism of the G-protein-coupled receptor kinase 5 (GRK5). Methods and results In a cohort of 20 patients enrolled in two tertiary Italian centers with diagnosis of TTC, accordingly to the commonly accepted Mayo Clinic criteria and in 22 healthy subjects (control) we have evaluated the polymorphism in GRK5 gene. The TTC patients had a mean age of 65±9 years and 19/20 were females. The presence of one or two L41 allels of GRK5 was significantly more frequent in TTC

	group than in control group (40% vs 8%, p=0.0372). Conclusions In our study we have found a significant difference in the frequency of GRK5 polymorphism between TTC patients and controls, supporting a genetic predisposition to this cardiac syndrome.

G-protein-coupled receptor kinase 5 polymorphism and Takotsubo cardiomyopathy

Giuseppina Novo[a,c], Salvatore Giambanco[a], Marco Guglielmo[a], Luisa Arvigo[a], Maria Rita Sutera[a], Francesco Giambanco[b], Salvatore Evola[a], Loredana Vaccarino[c], Manuela Bova[c], Domenico Lio[c], Pasquale Assennato[a], Salvatore Novo[a]

[a]Division of Cardiology, A.O. Policlinico "P. Giaccone", University of Palermo, Italy
[b]Division of Cardiology "G.F. Ingrassia" Hospital, Palermo, Italy
[c]Department of Biopathology and Medical and Forensic Biotechnology, University of Palermo, Italy

ABSTRACT

Background Takotsubo cardiomyopathy (TTC) is an increasingly reported clinical syndrome that mimics acute myocardial infarction without obstructive coronary artery disease and it characterized by transient systolic dysfunction of the apical and/or midsegments of the left ventricle. The syndrome mainly occurs in postmenopausal women with high adrenergic state conditions. Nowadays, the pathophysiology of TTC is not yet known and the possibility of a genetic predisposition is controversial.

Aims The purpose of this study was to assess the genetic susceptibility to TTC through analysis of the L41Q polymorphism of the G-protein-coupled receptor kinase 5 (GRK5).

Methods and results In a cohort of 20 patients enrolled in two tertiary Italian centers with diagnosis of TTC, accordingly to the commonly accepted Mayo Clinic criteria and in 22 healthy subjects (control) we have evaluated the polymorphism in GRK5 gene. The TTC patients had a mean age of 65±9 years and 19/20 were females. The presence of one or two L41 allels of GRK5 was significantly more frequent in TTC group than in control group (40% vs 8%, p=0.0372).

Conclusions In our study we have found a significant difference in the frequency of GRK5 polymorphism between TTC patients and controls, supporting a genetic predisposition to this cardiac syndrome.

Keywords: Takotsubo cardiomyopathy; GRK5 gene; polymorphism; genotype.

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Introduction

Takotsubo cardiomyopathy (TTC), also called "stress cardiomyopathy" or "transient left ventricular apical ballooning syndrome", is an infrequent heart syndrome, accounting for at least 2% of acute coronary syndrome (ACS) cases[1]. It has received considerable interest from the scientific community around the world, and in recent years it has been confirmed by the continuous and significant increase of publications related to this cardiomyopathy[2-5]. TTC is characterized by transient left ventricular dysfunction, symptoms and ECG changes mimicking acute myocardial infarction, and modest elevation in cardiac troponin, but in the absence of obstructive coronary disease or angiographic evidence of acute plaque rupture[6]. Although the syndrome has usually a benign prognosis and a complete recovery occurs in two to three weeks[7], it occasionally can result in acute complications including hemodynamic instability, atrial and ventricular arrhythmias[8], heart failure, and cardiogenic shock in a minority of patients[9,10]. The risk of in-hospital mortality is low (1% to3%)[11-13]. TTC just over 20 years ago was first described by Japanese investigators[6], and subsequently was also recognized by American and European populations[14-17]. It occurs almost exclusively in women especially in post-menopausal period, and the onset of acute event is often preceded by emotional and/or physical stress[18]. Classically, left ventriculography shows "apical ballooning" pattern with hyperkinesia of the basal segments[19]. Recently, new variants have been described with different wall motion anomalies

of the left ventricle (LV) such as "midventricular ballooning" and sparing or hyperkinesia of the basal and apical segments, or an "inverted Takotsubo" pattern with akinesia of the basal portions and hyperkinesia of the apex[20,21]. The right ventricle (RV) involvement is getting increasing recognition and it is associated with a worse prognosis[22].

To date, the pathophysiology of TTC is still unclear[23], but the fact that in TTC patients, plasma catecholamines are elevated 2 to 3 times higher than in acute myocardial infarction (AMI), has led to one of the most convincing hypothesis that it is the result of a transient direct toxic effect of catecholamines on the myocardium[24]. Several studies have provided evidence that high cathecolamine levels could be responsible for an increase in the expression and enzymatic activity of G-protein-coupled receptor kinases (GRKs). In physiological conditions, the intracellular signalling, following catecholamine binding to the receptor, is mediated by βARs, activating myocytes by coupling to the Gα subunit of the Gs protein complex. At the same time, catecholamines promote activation of G-protein-coupled receptor kinase (GRK) that induces phosphorylation of βARs; which promotes β-arrestin binding, and G-protein uncoupling to shut-off signalling. This regulation system is called "βARs desensitization"[25,26]. The up-regulation of GRKs could be somewhat connected to the desensitization and down-regulation of the β-adrenoceptors (βAR) in the failing heart, suggesting that genetic GRK variants might modify outcomes in cardiac failure and ischemia[27-29]. Of the seven human

GRKs, GRK2 and GRK5 predominate in myocardium[29], even if GRK2 seems to be the most relevant isoform at the cardiovascular level[30-33]. Recently, several studies evaluating genetic polymorphisms potentially involved in the pathogenesis of TTC have been published, however, the findings have been contradictory[34]. The aim of our study is to further investigate the genetic susceptibility to TTC through analysis of the L41Q polymorphism of the G-protein-coupled receptor kinase 5 (GRK5), one of the protein kinases most expressed in the heart and stress-induced acute ventricular dysfunction.

Methods

From May 2007 to April 2013 we have enrolled consecutive patients with TTC from 2 Italian tertiary hospitals. TTC was diagnosed according to the diagnostic criteria of Mayo Clinic[35], including: (1) Transient hypokinesis, akinesis or dyskinesis of the left ventricular mid segments with or without apical involvement. The regional wall motion abnormalities typically extend beyond a single epicardial coronary distribution; (2) Absence of obstructive coronary disease or angiographic evidence of acute plaque rupture; (3) New electrocardiographic (ECG) abnormalities (either ST-segment elevation and/or T-wave inversion) or modest elevation in cardiac troponin; (4) Absence of pheochromocytoma and myocarditis.

Peripheral blood samples in EDTA have been collected from each patient of the study group and control group (healthy subjects). DNA has been extracted using the "salting out technique" and the polymorphism rs17098707 for GRK5 was identified using Polymerase Chain Reaction (PCR) followed by a Restriction Fragment Lenght Polymorphism (RFLP) analysis as previously described. Written informed consent was given by each subject. Statistical analysis of data was performed using the Statgraphics software. Continuous data were reported as mean ± standard deviation and categorical data were expressed in number and percentage. The differences between the groups were analyzed using the Chi Square test for categorical variables while the association between multiple variables using the multivariate linear regression model. A value of $p < 0.05$ was considered statistically significant.

Results

Of the 43 consecutive TTC patients overall recruited, 20 gave consent for genetic analysis. The main clinical features of the study population are shown in Table 1. The analysis of polymorphism rs17098707 A/T L41Q for GRK5 was performed in 20 TTC patients and 22 healthy subjects were used as control group (Table 2). In the TTC group the "wild-type" genotype (AA) was found in 12 patients (60%), while the "variant" condition was found in 5 patients (25%) in the heterozygosity (AT) and the homozygosity state (TT) in 3 patients (15%). In the

control group the expression of the AA genotype was observed in almost all of the analyzed population (92%) since the presence of polymorphism for GRK5 was detected only in 1 subject (4%) in heterozygosity (AT) and in 1 subject (4%) in homozygosity state (TT). Both groups seem to be in "Hardy-Weinberg equilibrium". The distribution of genotypes between the two populations appears significantly different (p=0.0372) with an increased frequency of genotypes positive for the T allele in TTC patients. The analysis of the significance of the frequency distribution of genotypes between patients and control group shows that homozygous genotype AA has a high probability of being protective of TTC (OR=0.150; 95% CI=0.027 - 0.827) and the difference in distribution between the two populations is significant (p=0.0296). The AT (heterozygous) and TT (homozygous) genotypes do not seem to have a clear predisposing effect for TTC and the difference in the genotype distribution between both groups is not significant (Table 3). About the frequency distribution of the alleles between patients and controls (Table 4) it has been found that the T allele, mainly represented in the TTC group (p=0.0174), seems to be one of the genetic risk factors for Takotsubo syndrome (RR=4.033; 95% CI=1.211 - 13.431).

Discussion

The stimuli promoting the imbalance of the autonomic system and triggering TTC are multiple, and the real challenge for cardiologists is not their determination, but rather to understand why only some individuals develop stress cardiomyopathy. The identification of a genetic susceptibility in TTC, favouring a better understanding of the pathogenesis of this peculiar syndrome, could allow the development of more appropriated preventive strategies and tailored treatment.

The adrenal-cardiac axis has been increasingly identified as an important contributory factor to the pathogenesis of TTC[36,37]. The catecholamines, produced by the adrenal glands, are released in response to sympathetic nervous system stimulation, and increase cardiac function. However, when catecholamines levels remain chronically elevated, βARs desensitization occurs by a process which involves proteins including GRKs and beta-arrestins, both of which have been associated with cardiac dysfunction[31]. In detail, the βARs are phosphorylated by GRKs, translocated, and subsequently internalized after binding to beta-arrestins. Beta-arrestin 1 prevents the inhibition of catecholamine release from the adrenal glands, enhances the secretion of aldosterone, and may contribute to the pathogenesis of TTC. Furthermore, it inhibits the inotropic effects of cardiac β1ARs resulting in decreased cardiac function[37]. In addition, increased cardiac GRK2 levels have been described in chronic heart failure and are associated with elevated sympathetic nervous system activity. As recently proposed by Santulli et al.[31],

GRK2 could be predictive of ventricular remodeling after myocardial infarction and could facilitate the tailoring of appropriate therapy for high-risk patients. Fusco et al.[32] reported that ischemia causes acute cellular and mitochondrial accumulation of GRK2, and induces mitochondria biogenesis after ischemia/reperfusion, indicating a protective effect of GRK2 for mitochondria after acute stress. On the other hand, it has been reported that GRK5-mediated βAR desensitization could provide adaptive, beneficial effects during early ventricular decompensation, and prior to frank failure[29]. Moreover, GRKs may reflect other pathophysiologic processes. Indeed, it has been demonstrated the ability of the amino terminus of GRK5 (GRK5-NT) to reduce myocardial transcription factor NF-κB activity and left cardiac hypertrophy[33]. In recent years, several experimental and clinical studies analyzing polymorphisms potentially involved in the pathogenesis of TTC have been published. However, the data reported by various studies are rather controversial[34]. Given the known higher frequency of some polymorphism in the African-American than Caucasian population, racial differences may partly justify this discrepancy[38-40]. Specifically, the genetic variants most extensively investigated have been those affecting cardiac adrenergic receptor (AR) subtypes (α, β1, and β2), Gs-protein alpha subunit (GNAS), and GRK5.

Zaroff and colleagues[41] have shown an association between polymorphisms of the genes encoding the β1 adrenergic receptor (ADRB1 *Arg389Gly* and *Ser49Gly*), β2 (ADRB2 *Gly16Arg*, *Gln27Glu*, and *Thr164Ile*) and α2c (ADRA2C

del322-325) and cardiac dysfunction attributed to the release of catecholamines after subarachnoid hemorrhage (SAH). In particular, the authors conclude that ADRB1 and ADRA2C polymorphisms are associated with an increased risk of cardiac abnormalities after SAH. These data support the hypothesis that cardiac dysfunction after SAH is a form of neurocardiogenic injury. Note that, the regional dysfunction reversible left ventricular view in SAH patients is phenotypically similar to that of TTC patients.

Sharkey and colleagues[42] evaluated, in a cohort of 41 patients with stress cardiomyopathy, the functional polymorphisms of adrenergic receptors β1 and α2c already implicated in the increased activation of the sympathetic nervous system, but have not found significant differences between TTC patients and controls.

Handy and colleagues[43], based on evidence from animal studies, have included in their study also the evaluation of the ADRB2, over that of ADRB1 and ADRA2C, assuming that during stress, when epinephrine is the main circulating catecholamine, regional differences in epinephrine sensitive β2-receptors could explain the myocardial response to cathecolamine surge seen in TTC. According to their study, the authors conclude that while a molecular defect in adrenergic signaling remains a plausible pathogenic mechanism, their data, as well as those of Sharkey and colleagues, indicate that the TTC is not probably based on genetic variations in adrenergic receptors.

Spinelli and colleagues[44] have analysed the genetic polymorphisms in ADRB1, ADRB2, GNAS and GRK5 genes in 22 TTC patients. The prevalence of most of the polymorphisms were similar between patients and controls, but for L41Q polymorphism of the GRK5, wherein the glutamine (wild-type) at position 41 is replaced by leucine, was significantly more frequent in the TTC group. Specifically, the authors have hypothesized that this polymorphism could attenuate the inotropic effect of catecholamines on cardiomyocytes in confirmation of what has been shown in isolated cells and in transgenic mice, in which the GRK5 L41 variant causes a negative inotropic effect under conditions of acute massive catecholamine release. Moreover, the same polymorphism is associated with an increase in βAR desensitization, which may predispose to TTC.

Recently, Figtree and colleagues[45], in a large Australian cohort of 92 TTC patients, did not find association of genetic variants in the oestrogen receptor α (ERα), β1AR, β2AR, and catechol-O-methyl transferase (COMT) genes, or with the previously implicated GRK5, with occurrence of TTC. Although their data showed no evidence for specific genetic variants in GRK5 or βAR playing a role in susceptibility of an individual to TTC, the authors believe that this interesting hypothesis cannot be disputed and further research are needed .

In our study, consistent with the data of Spinelli and colleagues[44], the percentage of TTC patients who presented the rs17098707 polymorphism of the GRK5 gene was significantly higher than controls (p=0.0372). Furthemore, the

analysis of data showed that "wild-type" genotype (AA) could be protective against TTC. In contrast, GRK5 L41 variant, in heterozygous (AT) and homozygous (TT) genotypes, did not seem to be predisposing to TTC although the T allele could represent one of the genetic risk factors for this cardiomyopathy (p=0.0174).

Study limitation

The main limitation of our study is the small sample size, however our results do not pretend to clarify the complicate issue concerning the genetic mechanisms that might modulate the pathogenesis of TTC, rather to provide the cardiological community with a further observation.

Conclusion

We found a significant difference in the frequency of GRK5 polymorphism between TTC patients and controls. These findings reinforce the hypothesis of the role of genetic predisposition in the pathogenesis of TTC. Thus, a defect in the adrenergic signal remains one of the most compelling pathogenetic hypothesis in this field. Further research larger multicentre studies are needs to better understand the role of genetics in the pathophysiology of this syndrome.

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Table

Table 1 The main clinical characteristics of the study population (n=20)

Age (mean ± SD)	65 ± 9
Female(%)	19 (95)
Emotional/physical stressors (%)	17 (85)
Smoker (%)	6 (30)
Hypertension (%)	14 (70)
Diabetes mellitus (%)	3 (15)
Troponin I peak (mean ± SD) (ng/ml)	5.98 ± 6.15
Ejection fraction in acute phase (mean ± SD) (%)	41.6 ± 7.6
Ejection fraction at discharge (mean ± SD) (%)	52.3 ± 7.2
In hospital events (%)	2 (10)
- pericarditis	2 (10)

Table 2 Frequency of L41Q polymorphism of the GRK5 in TTC and control subjects

Gene (SNP)	Group	WT (%)	Het (%)	Poly (%)	P-value
GRK5 (rs17098707)	Control	92.0	4.0	4.0	
	TTC	60.0	25.0	15.0	0.0372

SNP, single nucleotide polymorphism; GRK5, G-protein-coupled receptor kinase 5; TTC, Takotsubo cardiomyopathy; WT, homozygous for the wild type; Het, heterozygote; Poly, homozygous for polymorphism.

Table 3 Analysis of the significance of the frequency distribution of genotypes between patients and control group

Genotypes	OR	95% CI	P-value
WT	0.150	0.027-0.827	0.0296
Het	7.000	0.739-66.249	0.0866
Poly	3.500	0.334-36.688	0.345

WT, homozygous for the wild type; Het, heterozygote; Poly, homozygous for polymorphism; OR, odd ratio; 95% CI, 95% confidence interval.

Table 4 Frequency distribution of the alleles between patients and control group

N° analyzed chromosomes	Group	A Allele (%)	T Allele (%)	P-value
44	Control	93.2	6.8	
40	TTC	72.5	27.5	0.0174

A Allele, Adenin; T Allele, Thymine

CHAPTER 9

Genetic determined expression of pro and anti inflammatory mediators influence the clinical history and response to chemotherapy in patients with Sporadic Colorectal cancer (CRC)

Giusi Irma Forte, Loredana Vaccarino, Letizia Scola, Giorgia Santini, Manuela Bova, Patrizia Di Gangi, Laura Di Noto, Domenico Lio.

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Genetic determined expression of pro and anti inflammatory mediators influence the clinical history and response to chemotherapy in patients with Sporadic Colorectal cancer (CRC)

Giusi Irma Forte[1], Loredana Vaccarino[2], Letizia Scola[2], Giorgia Santini[2], Manuela Bova[2], Patrizia Di Gangi[2], Laura Di Noto[2], Domenico Lio[2].

[1] IBFM CNR-LATO, Cefalù (PA), Italy

[2] Departement of Biopathology and Medical Biotechnology, University of Palermo,Palermo, Italy

Running title: *Genetic background effects on clinical history and response to chemotherapy of sporadic CRC*

Correspondence: Prof. Domenico Lio
Department of Pathobiology and Medical Biotechnologies,
University of Palermo
Corso Tukory, 211
I 90134, Palermo Italy
Phone: +390916555914;
Fax:+390916555901;
e-mail: domenico.lio@unipa.it

All the authors contributed equally to this study

ABSTRACT

Colorectal cancer (CRC) is one of the highest cause of morbidity and mortality in Western world. As it is known, a tight relation exists between the inflammation control capacity and cancer, in terms of susceptibility, tumorigenesis, progression and metastatic dissemination. Indeed, in the early steps of the cancerogenesis a genetically determined major systemic inflammatory capacity, due to polymorphisms of genes coding for inflammation regulation key molecules, could be primarily involved in cancer susceptibility and development as well as the response to chemotherapy. Here the effect of genetic polymorphisms located on crucial pro-inflammatory and anti-inflammatory cytokine genes (IL-6 -174G/C, rs1800795; INF-γ +874A/T, rs2430561; IL-10 -592A/C rs1800872, -819C/T rs1800871, -1082G/A rs1800896; TGF-β R25P, rs1800471) on the expression changes of some pro-inflammatory (IL-6, INF-γ, Cyclooxygenase (COX)-2), and anti-inflammatory (IL-10, TGF-β) factors produced by peripheral blood mononuclear cells (PMBC) in a group of patients having sporadic colorectal carcinoma across the period of their clinical management was analysed. SNPs analysis shows a weak protective role against the development of sporadic CRC for the minor C allele of the G → C R25P; rs1800471 SNP, located within the signal peptide sequence of the TGF-beta gene (P: 0.021; Odds ratio: 0.3412; 95% C. I.: 0.1323 to 0.8800).

An increase of IL-10 expression and an inverse behavior of IL-6 within 3 months from the chemotherapy treatment was observed. In particular a significant higher IL-10 mRNA level characterize patients not free from disease (NFFD). Besides, we always observed significantly lower IL-10 mRNA quantity in FFD patients, respect to NFFD ones, both if they underwent to chemotherapy or not (P: 00010, 00045, 0.328). Furthermore, among patients undergone to chemotherapy, not FFD patients were characterized by a significantly higher frequency of the IL-10 GCC haplotype, responsible of a higher mRNA production. In conclusion, overall our results confirms the involvement of TGF-β1 and IL-10 signaling pathway with CRC disease. Suggesting that analysis of IL-10 expression in PMBC might be an useful marker in prediction of ongoing anti-tumor response.

INTRODUCTION

Colorectal cancer (CRC) is one of the highest cause of morbidity and mortality in Western world. Among the factors involved in the pathogenesis of sporadic cases, eating habits, genetics, environment and inflammation are certainly focused, being the CRC familial cases, carriers of an hereditable genetic cause, only the 20% of overall patients. (Jemal A. et al. 2008).

As it is known, a tight relation exists between the inflammation control capacity and cancer, in terms of susceptibility, tumorigenesis, progression and metastatic dissemination (Caruso C. et al. 2004; Terzić J. et al. 2010). A large debate exists on the role that immune mediators play across the tumor progression steps, so that the positive or negative influences of immune reactions should be evaluated case by case and contextualized respect to the tumor steps (Vasto S. et al. 2008), also distinguishing the host local and systemic response (Caruso C. et al. 2009).

Indeed, in the early steps of the cancerogenesis a genetically determined major systemic inflammatory capacity, due to polymorphisms of genes coding for inflammation regulation key molecules, could be primarily involved in cancer susceptibility and development. This relation is largely documented by several reported associations between functional pro-inflammatory polymorphisms located on genes coding for crucial cytokines, such as Interleukin(IL)-6, Tumor Necrosis Factor (TNF)-α, IL-8, IL-10, Tumor growth factor (TGF)-β, and an increased susceptibility to develop colorectal cancer (Landi S. et al. 2003; Belluco C. et al. 2003; Crivello A. et al. 2006) and by the observations that patients with ulcerative colitis and Crohn's disease are at increased hazard for developing colorectal cancer (Lakatos PL. et al. 2008).

Actually, in the colorectal mucosa, cells of the innate immune system, such as neutrophils, mast cells, natural killer (NK) cells, dendritic cells (DC), and tumor-associated macrophages (TAM) can be easily detected and sustain the anti-tumor inflammation, through the synthesis of growth, remodeling or angiogenic molecules. Such evidences reveal a constitutive activation of molecules (NF-kB, STAT-3) implied in sustaining inflammatory pathways in colorectal cancer tissues (Sakamoto K. et al. 2009; Yu H. et al. 2009). Other groups have demonstrated that IL-10 deficient mice are able to develop severe forms of atrophic gastritis, chronic enterocolitis and colon-rectum cancer (Hachimine D, et al. 2008). Moreover, the examination of normal colorectal mucosa

from patients with colon cancer reveals that it contains higher number of monocytes and neutrophils than controls (Roncucci L. et al. 2008). These multiple observations suggest a central role of inflammation in colorectal carcinogenesis.

Activated innate cells are the first to migrate in the colorectal mucosa, where they are known to have a particular role in the orchestration of the response against tumor. Recent studies on co-coltures with colon cancer and mononuclear cells demonstrated a tight interaction between each other, leading to an higher induced secretion of both pro-inflammatory (TNF-α, IL-6, Interferon (INF)-γ, IL-1β) and anti-inflammatory (IL-10, IL-1ra) cytokines, tightly depending by the dose of tumor cells present into the culture (Bessler H. et al. 2010).

Moreover, blood neutrophils, isolated from patients having inflammatory bowel diseases were found to produce an increased quantity of inflammatory mediators in in-vitro coltures (Nikolaus S. et al. 1998), suggesting that the systemic immune system is stressed in patients suffering of chronic inflammation.

Other studies observed that sera from patients with colorectal cancer showed increased IL-10 and sIL-2R levels in comparison with subjects with colorectal adenoma (Berghella AM. Et al. 1997), while IL-10 higher concentration was observed by Fortis and co-workers in sera from patients with different kind of solid tumors at the same stage (IV) (Fortis C. et al 1996). Furthermore, IL-6 blood concentration seems to be associated with high circulating Carcino-Embryonic Antigen (CEA) and advanced stage in colorectal cancer (Belluco C. et al 2000).

On the other hand, the anti-inflammatory response might have a paradoxical effect have a role in the progression of cancer, contributing to immune-surveillance escaping mechanisms. Particularly, two main actors in this scenario are the immune-suppressive cytokines IL-10 and TGF-β, able to inhibit the maturation and activation of antigen-presenting dendritic cells (DC), to promote the down-regulation of classes I HLA molecules and NK cells activity, with the end effect of allowing the tumor to escape immune recognition and attack. In this sense, even though the infiltrated T-cells into sporadic colon cancer tissues are generally associated to a good prognosis (Guidoboni M. et al. 2001; Atreya I. et al. 2008), local immune-suppressive conditions could shift the immune-balance inducing the differentiation of T regulatory subsets that might facilitate tumor progression and dissemination.

A field that it has not well explored is the relation between local and systemic response during the tumor evolution steps and therapy treatments is intriguing and not well elucidated.

A large rate of interaction between anti-cancer drugs and host immune adaptation exists. This fact is revealed by several studies which suggest that the progression of malignant tumors as well as the response to chemotherapy and targeted therapy is critically dependent on the immunological parameters, often derived from the host immune system behavior. Chemotherapy rapidly attacks dividing cells, causing thrombocytopenia, neutropenia or leukocytopenia, but different cytokine profiling are reported in response to drugs mainly used in the treatment of cancer diseases. For example, Panis and coworkers (2011) report an immediate plasmatic reduction of IL-1, IL-10 and TNF-α one hour later the treatment with doxorubicin, while they observe an increase of IL-10 blood level one hour later the administration of paclitaxel in breast cancer patients. On the other hand, Liu XL. and his group (2008) observed increased INF-γ and decreased IL-10 blood levels after chemotherapy with Folfox4 (Oxaliplatin, Leucovorin, and Fluorouracil) regimen in patients having gastric cancer.

This study was designed to analyse both the role of genetic polymorphisms located on crucial pro-inflammatory and anti-inflammatory cytokine genes able to direct the immune system response, in the in the sporadic colorectal cancer susceptibility. and assessment of the systemic immunological balance modification caused by the host-tumor interaction and by the chemotherapy treatment.

To aim these goal we firstly typed -174G/C_rs1800795 (IL-6); +874A/T_rs2430561 (INF-γ); -592A/C_rs1800872, -819C/T_rs1800871, -1082G/A_ rs1800896 (IL-10); R25P_rs1800471 (TGF-β) gene polymorphisms and then, evaluated the expression level changes of some pro-inflammatory (IL-6, INF-γ, Cyclooxygenase (COX)-2), and anti-inflammatory (IL-10, TGF-β) cytokines produced by peripheral blood mononuclear cells (PMBC) in a group of patients having sporadic colorectal carcinoma across the period of their clinical management.

MATERIALS AND METHODS

Subjects

A total of 120 patients affected by sporadic CRC and 280 healthy controls, age and sex matched, were enrolled in this study. The control group, constituted by informed volunteer persons, was recruited at the Biopathology and Medical and Forensic Biotechnologies Department of Palermo University, whereas the group of CRC patients was recruited at the Surgical and Oncology Disciplines Department of Palermo University.

Informed consent was requested to all the subjects enrolled. Table 1 reports, in a synoptic way, details describing our cohort of CRC patients.

In details, our series of patients was so constituted: among female patients (44), 16 (36.4%) had age at diagnosis ≥ 65 years old and 28 (63.6%) < 65 years old, whereas 15 (34%) had right sited tumors and 29 (66%) left sited tumors. On the other hand, among male patients (76), 41 (53,8%) had age at diagnosis ≥ 65 years old and 35 (46,2%) < 65 years old, moreover, 13 (16,9%) had right sited tumors and 63 (83,1%) left sited tumors. Furthermore, among patients with age at diagnosis <65 years old (63), 11 (17.5%) had right sited tumors and 52 (81.5%) left sited tumors, whereas among patients with age at diagnosis ≥ 65 years old (57), 17 (29,8%) had right sited tumors and 40 (70,2%) left sited tumors.

Study design

Specifically, for each patient enrolled, the blood samples collected were classified based on the following moments of harvesting: post- surgery (PS), < 3 months post-chemotherapy (<3MPC), >3 months post-chemotherapy (>3MPC). Then, the cytokine expression levels, evaluated by qPCR, were studied on the base of: 1) the timing of blood collection during the patient's management, 2) the undergoing to a chemotherapy treatment, 3) the Duke stage, and 4) the freedom from disease at the moment of blood collection.

In the end, it has been investigated the role of functional polymorphisms located on the IL-10 promoter (-592C/A_rs1800872; -819C/T_rs1800871; -1082G/A_rs1800896), structured in three characteristic haplotypes (GCC, ACC,ATA) (Turner DM. et al. 1997;

Eskdale J. et al. 1998), known to affect the promoter activity (Lio D. et al 2002), respect to the quantity of IL-10 mRNA produced in response to the chemotherapy treatment.
These mRNA levels, conditioned by genetics, might be indicative of the type of an host-tumor immune response and could give us prognostic indications about a better or worse response to chemotherapy.

Sample Analyses

Blood samples were collected and DNA was obtained from all the subjects recruited, whereas total mRNA was extracted from 24 healthy controls and 117 blood samples randomly obtained from a sub-group of patients according to the following moments of collection: post- surgery (PS), < 3 months post-chemotherapy (<3MPC), >3 months post-chemotherapy (>3MPC). Post-surgery' and post-chemo' blood collections have been performed, at least, 20 days later. Total RNA extracted from healthy controls has been used to obtain a calibrator for the relative quantification by the Comparative Cycles to Threshold (Ct) method.

Nucleic acids extraction

The blood specimens were collected in EDTA sterile tubes. Then, DNA molecules were extracted using a salting out protocol (Miller et al. 1988), resuspended in Tris–EDTA buffer and stored at -20°C. Instead, total mRNAs were exclusively obtained from fresh blood samples, following the PMBC recovery on Ficoll (Euroclone, UK) stratification. Then, total mRNA were purified by using silica-membrane columns (RNeasy Mini Kit, Qiagen, Germany), eluted in RNAse free water and stored at -80°C.

SNPs genotyping

Dedicated and pre-made competitive allele specific PCR (Polymerase Chain Reaction) assays (KASPar), developed by KBioscience (England) were used to perform the Allelic Discrimination tests for the typing of the following functional polymorphisms: -174G/C_rs1800795 (IL-6); +874A/T_rs2430561 (INF-γ); -592A/C_rs1800872, -819C/T_rs1800871, -1082G/A_ rs1800896 (IL-10); R25P_rs1800471 (TGF-β).
In details, KASPar SNP genotyping method is an end-point PCR, based on a fluorescent FRET (Fluorescent Resonance Energy Transfer) system. A mixture of unlabeled pri-

mers are specifically designed on the sequence of interest and it consists of: 1) two allele-specific forward primers, having a terminal common tag at the 5'end; 2) a common reverse primer; 3) two small oligonucleotides complementary to the 5' tag regions of the allele-specific forward primers, labeled with two different fluorophores (FAM, 6-carboxy-fluorescein; VIC, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein) 4) two small oligonucleotides labeled with quenchers at the 3' end, complementary to those described in section 3.

Unlabeled primers were specifically designed on demand by KBioscience (England).

With the advancing of PCR cycles the labeled oligonucleotides bind, in a competitive way, the tag regions of forward primers rather than the quenching tags and will remain incorporated in the new synthesis molecules, releasing an increasing signal.

Briefly, 10 ng of DNA for each sample were used in an end-point KASPar PCR reaction, containing optimized master mix and the specific primer assay mix for each SNP, according to manufactory protocol in a final volume of 8 µl. Then, the amplification reactions were performed in the Master Cycler Gradient (Eppendorf, Germany), using a standard program consisting of the following cycles: 1) 94 °C for 15'; 2) 94 °C for 20"; 3) Touchdown 65-57 °C per 60"; 4) Repeating steps 2 and 3 for 10 cycles (Drop -0.8°C/for each annealing cycle); 4) 94 °C for 20"; 5) 57 °C per 60"; 6) Repeating steps 4 and 5 for 26 cycles. Finally, plates were scanned at the temperature of 4°C in a 7300 Real-Time ABI Prism PCR System (Applied Biosystems, USA) to register the fluorescence emission for each well, then samples were graphically grouped by the SDS software vs.1.3 (Applied Biosystem, USA) in three genotypic clusters (homozygous and heterozygous subjects), easily recognizable in the Allelic Discrimination plot on the basis of the two probe's fluorescence intensity emission.

Furthermore, following the genotype determination for each IL-10 SNP, haplotypes were easily deduced respect to the three possible SNP combinations (GCC, ACC,ATA).

cDNA synthesis and Real Time quantification

cDNA molecules were synthesized by using SuperScript® II Reverse Transcriptase (Invitrogen, USA), according to manufactory protocol. Briefly, 500 ng of total RNA were placed in the presence of 1 µl of oligo-dT (500 µg/ml) and 1 µl of dNTP Mix (10 mM)

and the mixture, so constituted, has been warmed at the temperature of 65°C in order to eliminate mRNA secondary structures. Then, First- Strand Buffer 5X, 2 μl of 0,1M DDT (Dithiothreitol) and 200 U of Reverse Transcriptase were added in a final volume of 25 μl and the mixture incubated at 42°C for 50'.

Subsequently, 5 μl of the reverse transcription reaction has been used in Quantitative Real Time PCR (qPCR) assays, performed to quantify the relative mRNA levels in CRC patients by using the Comparative Ct method. In particular, we determined the fold change expression of target genes of interest (IL-6, INF-γ, COX-2, IL-10, TGF-β) normalizing respect to the housekeeping gene L13. Moreover, for the purpose of our study, a ΔCt mean value, calculated as the difference between the Ct mean value of the target gene and that one of the housekeeping gene from a total of 24 healthy subjects, has been used as calibrator ($\Delta Ct_{calibrator}$).

TaqMan probes and specific PCR primers have been designed for each sequence of interest, taking care to design primers on the sides of a long intronic sequence, in order to avoid amplification of such contaminant DNA molecules. Each Probe was 3' labeled with BHQ1 (Black Hole Quencher 1) and 5' labeled with FAM (target genes) or VIC (housekeeping gene) fluorophore.

Table 2 reports the details of primers pairs and probes sequences used in the Real Time assays.

Reactions have been prepared in a final volume of 25 μl in the presence of 300 mM probe, 900 mM primers, 0,5 μM ROX and 1X Platinum Quantitative PCR Super Mix-UDG (Invitrogen, USA) and run in a ABI Prism 7300 (Applied Biosystem, USA), utilizing a standard amplification program: 1) 2' at 50 °C; 2) 10' at 95°C; 3) 15" at 95°C; 4) 30" at 60°C; 5) Repeating steps 3 and 4 for 39 cycles.

Statistical analysis

The allele and genotype frequencies of the SNPs analyzed were evaluated by 3x2, 2x2 or nxn tables and the differences between the observed and expected distributions between the groups of subjects considered was assessed by applying the Pearson Chi-Square test or the Fisher's Exact Test. It was accepted a significance value of $P \leq 0.05$. The Hardy Weinberg equilibrium was tested on the observed frequencies registered for

the control' population, using the Pearson Chi-Square test. All the analysis were performed with the SPSS software (SPSS, Chicago, Illinois).

The Mann-Whitney nonparametric test was used in every cases where it was necessary to evaluate differences between the observed and expected medians from distributions of two series of measured values. In alternative, the Kruskal-Wallis test was utilized for the comparison of the medians obtained from more than two series of distributions, while the Dunn's Multiple Comparisons test has been used for the multiple comparison of pairs of distributions.

RESULTS

Association analysis

As a first step, we analyzed the genetic contribute of polymorphisms known to affect the cytokine mRNA production to the susceptibility of sporadic colorectal cancer disease.

Particularly, we have chosen selected crucial cytokines (IL-6, INF IL-10, TGF-β) able to profoundly influence the immune microenvironment surrounding neoplastic cells. we studied the genotypic and allelic frequencies distribution between patients and healthy subjects of the following polymorphisms of IL-6 -174G/C (rs1800795), INF-γ +874A/T (rs2430561), IL-10-592A/C (1800872) -819C/T (rs1800871) -1082G/A (rs1800896) and TGF-β) R25P (rs1800471) have been evaluated.

For each polymorphism, it has been possible to study a random selection of samples from the total number of subjects recruited in our series of 120 patients and 280 healthy controls. The Hardy Weinberg equilibrium, applied to the observed frequencies of the control' population, was always respected.

Table 3 reports the results of this association study. No significant genetic contribute has been observed for 5 out the 6 SNPs tested. Indeed, a significant different allelic distribution between patients and controls has been observed for the polymorphism G → C, responsible of an arginine vs. proline missense change (R25P) in correspondence of the codon 25 of the coding region of the TGF-β gene (*P*: 0.021; Odds ratio: 0.3412; 95% C. I.: 0.1323 to 0.8800). By this analysis, a weak protective role would emerge for the minor allele C in the susceptibility to the disease.

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The chemotherapy effect

In order to study long term effect of chemotherapy on the immunological systemic balance of colorectal cancer patients, we analyzed the mRNA levels of some pro-inflammatory (IL-6, INF-γ, Cyclooxygenase (COX)-2, and anti-inflammatory (IL-10, TGF-β) type crucial cytokines produced by peripheral blood PMBC after at least 20 days from a dose administration. This time lapse is considered sufficient for the returning to an immunological steady state, giving us the possibility to observe only late effects produced by the chemotherapy drugs. Then, we investigated the systemic immune

system behavior within three months and after, at least, three months from the last chemotherapy dose.

As it is shown in table 4, the effect of a chemotherapy treatment administration results in a significant increase of the IL-10 mRNA produced by PMBC, observable when the samples were collected within 3 months from the chemotherapy administration (<3MPC, 26 samples).

In fact, comparing the median value of IL-10 mRNA between this group of 26 samples (<3MPC), respectively with: a) the group of 44 samples collected after, at least, 3 months from the last administrated dose of chemotherapy (>3MPC); b) the group of 38 samples collected after, at least, 3 months from the last administrated dose of chemotherapy that were free from disease at the time of the blood collection (>3MPC FFD), c) the group of 72 samples, including those collected after, at least, 3 months from the last administrated dose of chemotherapy and those performed after the tumor removal by surgery (>3MPC + PS), the IL10 mRNA levels were, always, significantly higher in the first group of samples analyzed within 3 months after the last chemotherapy dose (<3MPC), *P*-values: a) 0.0007, b) 0.0001, c) 0.0062 respectively.

Note that, the three groups chosen for the evaluation of the inflammatory status produced within three months from the chemotherapy administration, were preferred because, in every cases, we could suppose that they, presumably, underwent to a re-establishment of the immune balancing, due to a sufficient time elapsed from the treatment (more than three months) or from the surgery (at least 20 days later), or because they were already free from disease.

On the other hand, if one considers the comparison between the full group of patients who underwent to a chemotherapy treatment, regardless of timing of treatment (CT, 70 samples), with those not chemo-treated who underwent to the blood collection following the surgical tumor removal (PS, 28 samples), the significance is lost, sign of a returning toward an IL10 mRNA serum normalization few months later, which justify a balancing of IL-10 mRNA quantity inside the comprehensive group of patients that underwent to the blood collection before and after three months from the last chemotherapy dose.

Also, it can be observed that the IL-6 mRNA levels move following an inverse behavior to those ones of IL-10 mRNA, although the differences are never significant. In particu-

lar, we observed lower levels of IL-6 mRNA within 3 months from the last dose of chemotherapy (<3MPC), probably caused by the IL-10 higher anti-inflammatory effect, whereas, the IL-6 levels tend to rise in the groups analyzed more than three months away from the chemotherapy administration (>3MPC). Instead, no relevant change is recorded for Cox-2, IFN-γ and TGF-β mRNAs.

The host-tumor interaction effect

In order to study the relation between the presence of an active tumor mass and a consequent systemic immune modulation, we firstly investigated the cytokine mRNA serum levels according to the Duke's stage. In a first approach we considered if a trend of cytokine expression could exist across the progression of the disease from the A to the D stage. No significant differences it was observed from the comparison of the medians of the distributions analyzed, neither it was observed significant difference from the comparison of medians from pairs of distributions (data not shown). Then, we evaluated the difference in the median values of the cytokine mRNA's quantity distributions between the earliest stages (A-B) of the disease versus the latest stages (C-D). No significant difference was assessed between earlier and later Duke's stages for none of the crucial cytokine analyzed. Table 4.

Subsequently, we investigated the immune modifications based on the presence/absence of an active disease. In particular, in our series 65 samples derived from patients free from disease (FFD) at the time of the blood collection, while 33 from patients not free from disease (not FFD). This approach revealed that the IL-10 mRNA blood levels were significantly higher in the group of patients not free of disease (*P*-values: 0.0010). However, it can be assumed that the higher levels of IL-10 could be affected by a modulator effect resulting from the chemotherapy administration, as the above results suggest. Nevertheless a biological effect due to the presence of an active tumor mass could be also hypothesized. Indeed, looking among the patients not-free from disease (not FFD) only one third (11/33) had undergone chemotherapy and the IL-10 levels were particularly high (3.5 ± 4) in this small subset of patients, as to underline the result of double effect mediated both by the chemotherapy treatment and by the biological host-tumor interaction. Moreover, if one would compare the IL-10 mRNA blood levels of this small group of chemo-treated patients not free from disease (CT: not FFD, 11 samples) with that of treated patients free of disease (CT: FFD, 23 samples), or either with

the group of patients not treated with chemotherapy free from disease (not CT FFD, 14 samples), the IL-10 mRNA levels were significantly higher in the first group in both the comparison (*P*: 0.0045; *P*: 0.00328). In particular, the last group of comparison can be considered to be constituted by patients sharing the opposite condition: to be not chemo-treated and disease free.

On the other hand, no significant change has been observed for the of IL-6, COX-2, IFN-γ and TGF-β mRNA levels in the group of patients described in this section.

Finally, we wanted to investigate if the individual genetic background, controlled by the IL-10 haplotypes, could also be implicated to generate the observed higher IL-10 levels in response to therapy.

As it is shown in table 5, among the chemo-treated patients the GCC haplotype (high IL10 producer) has been observed to be significantly higher represented in patients not free of disease, respect to the ACC / ATA haplotypes (lower producers of IL-10) (GCC: 21 vs. ACC / ATA: 15;); on the other hand, the ACC / ATA haplotypes are significantly prevalent among patients free from disease respect to the GCC one (39 vs.21) (P: 0.034). The genetic data suggest that a greater capacity to produce IL-10 is prognostically not favorable for the resolution of the disease after treatment (Odds ratio: 2,6; 95% C.I. from 1,11 to 6.077). In addition, the genetic data justify the observation of a particularly increased IL-10 expression in patients receiving chemotherapy not free of disease.

DISCUSSION

Although immune therapy is still retained not very effective and tumors are mainly fought by surgical removal and individualized therapeutic treatments, however systemic and local cytokines strongly modulate the anti-tumor response, conditioning the disease outcomes. To this regard, the individual genetic contribute to the immune system anti-cancer effort could be considered fundamental. This study aimed to clarify the relations between a genetic determined immune control capacity and the cytokine expression pattern, able to interfere with sporadic colon cancer disease in terms of susceptibility and prognosis.

To this aim, we retained important to, firstly, analyze the contribute to susceptibility of functional polymorphisms affecting the promoter activity and the transcription rate of inflammatory and anti-inflammatory cytokine genes such as (-174G/C_rs1800795 (IL-6); +874A/T_rs2430561 (INF-γ); -592A/C_rs1800872, -819C/T_rs1800871, -1082G/A_ rs1800896 (IL-10); R25P_rs1800471 (TGF-β), crucial in directing the type and the strength of response. As already described, no associations were observed between 5 out the 6 analyzed polymorphisms and the development of sporadic CRC in our cohort of Sicilian patients for which genotype and allelic distributions were not differently distributed between patients and controls. However, a weak protective role emerged for the minor C allele of the G → C R25P; rs1800471 SNP, located within the signal peptide sequence of the TGF-β gene (P: 0.021; Odds ratio: 0.3412; 95% C. I.: 0.1323 to 0.8800).

Recent genome-wide association studies have highlighted the important contribute of numerous SNPs belonging to the TGF-β superfamily, which may be responsible of a sizeable proportion of colorectal cancer cases (Tomlinson IP. 2008; Tomlinson IP. 2009). Previously, we had studied the polymorphism rs1800470, responsible for a Leu→Pro substitution in correspondence of codon 10, forming haplotype with the rs1800471 SNP studied in this paper (Crivello A. et al. 2006). In that study, we had observed a significant increased risk for the major allele (cod10-Pro) (P: 0.011; Odds ratio: 2.62 (1.40–4.91). Therefore, taking together both the results, we could suggest a weak protective role for the haplotype constituted by the two minor alleles (cod10-Leu/cod25-Pro). This haplotype represents the 12.8% in the Indian population according to Manchanda PK. (2008) and co-workers, whereas in the study conducted by Jin Q and co-workers (2004)

on Polish and Finnish populations, it is registered with a percentage close to zero, as these European populations were characterized by a low percentage of the C allele frequency for the cod25 SNP, similarly to our finding (0.03%). However, our cod25 SNP frequencies are confirmed by many other groups (Gendzekhadze NS. 2006, Alakulppi K. 2004; Park 2004).

These observations suggest a different weight of TGF-β SNPs on CRC susceptibility, strongly dependent by the SNP penetrance on a certain population.

Then, in order to evaluate the immune response in prognostic terms, we aimed to characterize the cytokine PMBC expression profile (IL-6, INF-γ, COX-2, IL-10, TGF-β) describing the host-tumor relationships and the response to therapeutic treatments.

To reach this aim, we have chosen to categorize patients according to the moment of the blood collection (PS, <3MPC, >3MPC), recording, at that time, the clinical condition in terms of Duke's stage and freedom from disease. This approach allowed us to easily compare sub-groups of patients with each other for the PMBC mRNA quantity, as CRC is a very heterogeneous disease, characterized by a high clinical and molecular variability, that do not allow to analyze the patients as a single group (Sheffer M. et al. 2009; Winder T. et al. 2010).

Other groups investigated the immediate effect of chemoterapy administration on the immune system, finding different cytokine patterns (Panis C. 2011, Liu XL. 2008), however this is, to our knowledge, the first time it is chosen to look at long term immune system modulation induced by chemotherapy.

Our results make evident an increase of IL-10 within 3 months from the chemotherapy treatment. In our study, all the blood collections were performed after, at least, 20 days from the administration of a chemotherapy dose or surgery, a time lapse sufficient to define this one as a long term effect mediated by the treatment. In addition, the IL-10 expression seems to return to normalization few weeks later, as it is suggested by the observation that no significant difference occurs in the comparison between patients analyzed post-surgery (PS, 28 samples) and chemo-treated subjects regardless of timing of treatment (CT, 70 samples).

On the other hand, we also detected an IL-6 inverse behavior respect to the IL-10 movements, even if the results are not significant. This observation could be justified by the IL-10 suppressive effect, as it was demonstrated by Li and co-workers (2010),

which have proved the IL-10 involvement into the monocyte/macrophage IL-6/Stat-3 signaling down-regulation, a pathway enhancing the IL-6 itself synthesis mainly implicated in the tumor cells migration.

Our results are not filtered according to the type of adjuvant regimen used, both for the fact that the regimens commonly administrated in the treatment of colorectal cancer were mainly based on the use of 5-fluorouracil (5-FU) plus leucovorin (LV, also called Folinic acid, FA) in combination with other drugs: oxaplatin (FOLFOX4/6), irinotecan (FOLFIRI, IRIFAF), adriamycin (MACHOVER), both because combinations of these regimens could be administrated in different times to the same patient, so that the number of samples obtainable post-filtering would be reduced and statistically not suitable.

Moreover, radiotherapy treatments and biologic targeted therapies were excluded from our analysis.

Then, in order to verify the hypothesis that cytokine gene expression patterns could be indicative of ongoing anti-tumor response, we investigated this topic looking at the relation with the presence of an active tumor mass and with the disease stage.

Several groups described altered IL-6, TNF-α, IL-2, IL-4, IL-10 and TGF-β in sera of CRC patients. However, many of them studied these marker quantities in comparison to controls. Indeed, the group of Csiszár A. (2004) detected more IFN-γ transcripts in PBMC samples from patients with colorectal cancer than in healthy controls, while Tsushima H. (2004) and co-workers observed significantly higher plasma TGF-β1 levels in patients with colorectal cancer than in normal controls. Some other groups tried to correlate the cytokine expression pattern with the disease evolution. The group of Berghella AM. (1997) showed increased IL-10 and sIL-2R levels in patients with colorectal cancer in comparison with subjects with colorectal adenoma, while the group of Belluco C. (2000) associated IL-6 blood concentration with high circulating CEA in advanced stage in colorectal cancer.

Here, we lacked to observe significant cytokine movements according to the Duke stage progression, but we revealed a significant higher IL-10 mRNA quantity among not free from disease (NFFD) patients respect to FFD ones. Considering that patients with a remaining tumor mass could still be under chemo-therapy treatment, we evaluated patients not FFD that also underwent to chemotherapy. The finding of particularly high PMBC IL-10 mRNA levels in this group of subjects suggest a double effect mediated

both by the treatment and by the presence of an active mass. Besides, we always observed significantly lower IL-10 mRNA quantity in FFD patients, respect to not FFD ones, both if they underwent to chemotherapy or not (P: 00010, 00045, 0.328). Our findings are supported by PBMC/ CRC cells co-cultures experiments made by the group of Blesser H. (2010), which revealed a more pronounced pro- and anti-inflammatory cytokine production depending upon an increased malignant cells quantity in the culture.

Furthermore, among patients undergone to chemotherapy, not FFD patients were characterized by a significantly higher frequency of the IL-10 GCC haplotype, responsible of a higher mRNA production, respect to FFD ones, which, instead, had a major percentage of ACC/ATA haplotypes (*P*: 0.034), an observation suggesting the IL-10 SNPs as prognostically informative genetic markers.

It can be supposed that these increased IL-10 circulating level, genetically affected, may contribute to enhance the tumor cells escaping from immune surveillance, however, IL-10 is known to play also a protective role into reduce the inflammatory molecules levels, inducing local inflammation, angiogenesis and cell migration (Li YY. et al. 2010; Nikolaus S. et al. 1998).

Specifically, IL-10 is known to play a double role in tumor diseases (Terzić J. et al. 2010), as if it is known to play a protective in the reduction of inflammation and susceptibility to tumor transformation (Hachimine D. et al. 2008), it seem to have a negative role in tumor progression later, as the results of this study confirm.

In conclusion, overall our results make evident the immune system role in the conditioning of susceptibility and disease's outcomes, revealing, one more time, the association of TGF-β1 signaling pathway with CRC disease (Bellam M. et al. 2010) and finding in IL-10 both a genetic and a circulating marker of ongoing anti-tumor response.

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Table 1. Patients' features

Patients' Features	Total N (%)
Age at diagnosis	
<65 years old	63 (52,5)
≥65 years old	57 (47,5)
Gender	
Female	44 (36,7)
Male	76 (63.3)
Tumor Location	
Right	28 (23,3)
Left	92 (76,7)
Duke' Stage	
A and B	58 (48.3)
C and D	62 (51.7)
Chemotherapy	
Yes	81 (67,5)
No	39 (32,5)
Freedom from disease (FFD)*	
Yes	77 (65.8)
No	40 (34.2)
Blood collection time*	
post- surgery (PS)	35 (30.0)
< 3 months post-chemotherapy (<MPC)	30 (25.6)
>3 months post-chemotherapy (>MPC)	52 (44.4)

** Blood collections were randomly obtained (when available) from a sub-group of patients during some steps of their own clinical management (PS; <3 MPC; >3 MPC). The FFD has been assessed at the time of blood collection.*

Table 2. Primers pair and probes used for the relative quantification of IL-10, INF-g, IL-6, TGF-b, Cox-2 PBMC levels

L13 primers pairs and probe
L13 Forward: 5'-GCTGGAAGTACCAGGCAGTGA- 3'
L13 Reverse: 5'-ACCGGTAGTGGATCTTGGCTTT- 3'
L13 Probe: 5'VIC -TCTTTCCTCTTCTCCTCCAGGGTGGCT- BHQ1_3'
IL-10 primers pairs and probe
IL10 Forward: 5'-CTGAAGACCCTCAGGCTGAG- 3'
IL10 Reverse: 5'-GCATTCTTCACCTGCTCCAC- 3'
IL10 Probe: 5'FAM -ACGGCGCTGTCATCGATTTCTTCCC- BHQ1_3'
INF-γ primers pairs and probe
INFγ Forward: 5'-TTCGGTAACTGACTTGAATGTCC- 3'
INFγ Reverse: 5'-GACAACCATTACTGGGATGCTC- 3'
INFγ Probe: 5'FAM –CGACCTCGAAACAGCATCTGACTCCT- BHQ1_3'
IL-6 primers pairs and probe
IL-6 Forward: 5'-ATTGACAAACAAATTCGGTACATCC- 3'
IL-6 Reverse: 5'-GGACTTGGAAGGTTTCTACCG- 3'
IL-6 Probe: 5' FAM –TGAAAGCAGCAAAGAGGCACTGGCA- BHQ1_3'
TGF-β primers pairs and probe
TGF-β Forward: 5'-GCCTGAGGCCGACTACTAC- 3'
TGF-β Reverse: 5'-TGTGTACTCTGCTTGAACTTGTC- 3'
TGF-β Probe: 5' FAM -CCAAGGAGGTCACCCGCGTGCTAAT- BHQ1_3'
Cox-2 primers pairs and probe
Cox-2 Forward: 5'-GCCTCCTTCAGCTCCACAG- 3'
Cox-2 Reverse: 5'-GGTGGGAACAGCAAGGATTTG- 3'
Cox-2 Probe: 5'FAM -ACGCCCTCAGACAGCAAAGCCTACC- BHQ1_3'

Table 3. Genotype frequencies in CRC patients and healthy controls

Table 3	**Con-**	**Cases**	***P***	***P allelic***
IL-10 (-592 A/C rs1800872)				
CC	80 (54.0%)	45	0.785	0.527
AC	58 (39.2%)	39		
AA	10 (6.8%)	7 (7.7%)		
IL-10 (-819C/T rs1800871)				
CC	105	43	0.901	0.694
CT	90 (43.1%)	40		
TT	14 (6.7%)	7 (7.8%)		
IL-10 (-1082G/A rs1800896)				
AA	108	36	0.462	0.306
AG	120	43		
GG	40 (14.9%)	20		
IL-6 (-174 G/C rs1800795)				
GG	125	52	0.370	0.250
GC	110	32		
CC	40 (14.5%)	13		
INF-γ (+874 A/T rs2430561)				
TT	70 (27.3%)	22	0.725	0.716
AT	128	36		
AA	58 (22.7%)	21		
TGF-β (R25P rs1800471)				
GG	200	77	0.080	0.021
GC	34 (14.3%)	5 (6.1%)		
CC	3 (1.3%)	0 (0%)		

Genotype frequencies observed in our Sicilian healthy cohort and in our sporadic CRC group of patients.
It was accepted a significance < 0.05.

Table 4. Analysis of mRNA expression in relationship to the chemotherapy effect and the Host-tumor interaction

	I° group (N)	II° group (N)	*P*-value	I° group (N)	II° group (N)	*P*-value	I° group (N)	II° group (N)	*P*-value	I° group (N)	II° group (N)	*P*-value	I° group (N)	II° group (N)	*P*-value
	IL-10			**IL-6**			**COX-2**			**INF-γ**			**TGF-β**		
The chemotherapy effect															
<3MPC vs >3MPC	1.59 ± 3.7 (26)	0.59 ± 0.2 (44)	0.0007	1.9 ± 1.39 (27)	2.16 ± 0.96 (50)	0.88	0.17 ± 2.15 (20)	0.2 ± 2.15 (41)	0.40	1.5 ± 2.23 (29)	1.72 ± 1.47 (50)	0.87	0.90 ± 1.08 (27)	1.17 ± 0.81 (50)	0.80
<3MPC vs >3MPC FFD	1.59 ± 3.7 (26)	0.52 ± 0.2 (38)	0.0001	1.91 ± 1.39 (27)	2.47 ± 0.99 (42)	0.64	0.17 ± 2.15 (20)	0.26 ± 2.58 (34)	0.36	1.5 ± 2.23 (29)	2.24 ± 4.8 (42)	0.49	0.90 ± 1.08 (27)	1.17 ± 0.84 (42)	0.85
<3MPC vs >3MPC + PS	1.59 ± 3.7 (26)	0.94 ± 0.6 (72)	0.0062	1.91 ± 1.39 (27)	2.03 ± 0.98 (80)	0.8	0.17 ± 2.15 (20)	0.27 ± 1.35 (70)	0.34	1.5 ± 2.23 (29)	1.2 ± 1.33 (84)	0.75	0.90 ± 1.08 (27)	1.31 ± 1.03 (84)	0.67
CT vs PS	1.03 ± 1.4 (70)	1.28 ± 1.59 (28)	0.19	2.12± 0.78 (77)	1.78 ± 3.3 (31)	0.99	0.19 ± 1.58 (62)	0.41 ± 1.21 (29)	0.60	1.6 ± 2.69 (80)	0.75 ± 2.5 (34)	0.13	1.08 ± 0.64 (77)	1.55 ± 2.22 (34)	0.13
The Host-tumor interaction															
DS: (A-B vs C-D)	1.15 ± 0.29 (21)	1.34 ± 1.95 (28)	0.80	2.2± 1.97 (25)	1.7 ± 5.4 (30)	0.56	0.16 ± 0.98 (22)	0.31 ± 1.37 (26)	0.98	0.55 ± 1.65 (26)	0.9 ± 0.86 (31)	0.52	0.77 ± 0.77 (28)	0.74 ± 2.17 (28)	0.65
FFD vs not FFD	0.79 ± 0.47 (65)	1.75 ± 1.48 (33)	0.0010	1.16 ± 1.53 (72)	1.61 ±1.25 (37)	0.54	0.27 ± 1.57 (61)	0.24 ± 1.28 (30)	0.28	1.2 ± 2.9 (75)	1.22 ± 1.87 (40)	0.61	1.25 ± 0.83 (77)	1.08 ± 1.87 (35)	0.66
CT: FFD vs not FFD	0.64 ± 1.28 (23)	3.5 ± 4 (11)	0.0045	2.5 ± 1.25 (26)	1.6 ± 2.06 (13)	0.87	0.34 ± 1.84 (21)	0.39 ± 0.54 (12)	0.92	0.95 ± 1.56 (28)	0.90 ± 1.8 (13)	0.48	0.74 ± 0.75 (27)	0.72 ± 4.9 (12)	0.65
Not CT FFD vs CT not FFD	1.15 ± 0.33 (14)	3.5 ± 4 (11)	0.0328	2.07 ± 2.7 (15)	1.6 ± 2.06 (13)	0.93	0.19 ± 0.77 (14)	0.39 ± 0.54 (12)	0.67	0.42 ± 0.24 (15)	0.90 ± 1.8 (13)	0.065	0.95 ± 1.17 (17)	0.72 ± 4.9 (12)	0.999

Data are expressed as median ± SEM. Inside brackets the number of samples involved. Significance has been calculated by Mann-Withney test.
Abbreviations: PS: < 3 months post-chemotherapy; <3MPC: < 3 months post-chemotherapy; >3MPC: >3 months post-chemotherapy; FFD: free from disease; DS: Duke Stage; CT: Chemo-Treated

Table 5 IL-10 Haplotype effects on chemotherapy response

Chemo-treated patients	Haplotype 1 GCC	Haplotype 2/3 ACC/ATA
Not Free from disease (n.18)	21 (58.3%)	15 (41.7%)
Free from disease (n.30)	21 (35.0%)	39 (65.0%)

Fisher's Exact Test on 2x 2 table. *P*: 0.034. Odd Ratio: 2,6 (95% C.I. from 1,11 to 6.077)

CHAPTER 10

Summary and general discussion

The extraordinary increase of the expected life span in developed countries underscore the importance of studies on ageing and longevity and the need for the prompt spread of knowledge about ageing in order to satisfactorily decrease the medical, economic and social problems associated to advancing years, because of the increased number of individuals not autonomous and affected by invalidating pathologies.

Centenarians are equipped to reach the extreme limits of human life span and, most importantly, to show relatively good health, being able to perform their routine daily life and to escape fatal age-related diseases. Thus, they are the best example of extreme longevity, representing selected people in which the appearance of major age-related diseases, such as cancer, and cardiovascular diseases among others, has been consistently delayed or escaped.

Aging is a post-maturational process that, because of a diminished homeostasis and increased organism vulnerability, causes a reduction of the response to environmental stimuli. The progressive decrease in physiological capacity and the reduced ability to respond to stresses lead to increased susceptibility and vulnerability to disease. Thus, mortality due to all causes increases exponentially with aging.

Aging involves all the cells, tissues, organs, and organisms and is modulated by external factors.

Ageing is accompanied by chronic low-grade inflammation state clearly showed by 2–4-fold increase in serum levels of inflammatory mediators such as cytokines and acute phase proteins in aged population, which act as predictors of mortality independent on pre-existing morbidity.

So far, we believe this is the most important cause of the elderly pro-inflammatory status that, interacting with the genetic background, potentially triggers the onset of age-related inflammatory diseases as atherosclerosis, cancer, (Franceschi et al., 2000a,b; De Martinis et al.).

The individual genetic background and the eventual influence of counteracting cytokines, associated with different degrees of control of the inflammatory response, could therefore play a determining role in the onset of age-related diseases and in the achievement of advanced age in the absence disease.

Genetic variations located within the promoter regions of pro-inflammatory and regulatory cytokines could influence inflamm-ageing and the susceptibility to age-related diseases.

The Metabolic Syndrome (MS) is a clinical condition characterized by the simultaneous presence of more alterations in one individual , mainly of metabolic origin, that directly increases the risk of cardiovascular disease, type 2 diabetes mellitus, and all cause mortality.

It further increases with age, with an incidence of 44% in the population aged between 60 and 69 years (Ford ES. Et al.2002).

MS is a state of chronic low grade inflammation as a consequence of complex interplay between genetic and environmental factors. Insulin resistance (IR), visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hyper coagulable state, and chronic stress are the several factors which constitute the syndrome.

It can be the basis for the emergence of many age-diseases.

This association could be due to the link between IR and inflammation, in fact, the same mechanisms that cause the IR determine both the synthesis and self-maintenance of pro-inflammatory cytokines and thus the chronic and persistent inflammatory response that characterizes the inflammaging.

It is not surprising that some studies have found an association between the metabolic syndrome and the development of cancer (Kreger BE et al. 1991), in particular of the Colon-Rectum Cancer (CRC); in addition to an increased risk of diabetes and cardiovascular disease (Ninomiya JK et al. 2004).

In this particular context, knowledge of genes polymorphisms, encoding cytokines, is a tool of extreme importance, not only for understanding the mechanisms underlying the pathogenesis of age related diseases, but also for future customization of personal treatment protocols, the true "goal" of research in the coming decades.

During my PhD, I faced this issue in many respects, first observing the evidence of genetic background on the individual capacity to reach successful aging and therefore, aiming to identify new common markers between these diseases.

To this end I conducted numerous association studies centered on the role of inflammatory balance in determining the onset of some of the age-diseases, such as cancer, cardi-

ovascular diseases, such as acute ischemic stroke, Thoracic Aortic Aneurysm, syndrome takotsubo, myocardial infarction, Sporadic Colon Cancer, and other successful and unsuccessful aging.

In the work entitled **"Genetics of longevity. Data from the studies on centenarians Sicilian"** (Balistreri CR et al. 2012) (chapter 2) we collected our studies conducted over 10 years on centenarians Sicilians. We evaluated the role of their individual genetic background on the immune response and longevity.

In particular, we studied some genetic variants of TLR4 gene, CCR5, Cox2, 5-Lo and analyzed the allelic and genotypic frequencies of polymorphisms that map the genes encoding IFN-γ and IL-10.

We have seen that over-expression of anti-inflammatory variants CCR5Δ32, +896 G allele (299Gly) TLR4, -765 C allele Cox-2 alleles -1708 G 5 and 21 C-Lo characterize males Sicilian centenarians. These SNPs regulating the immune- inflammatory response and appear to be associated with longevity.

In addition, our results show an increase of the subjects -1082G IL10 positive, associated with an increased production of IL-10, in males centenarians and suggest an increased production of IL10 as possible biomarkers of longevity.

In addition in **Chapter 3,** we evaluated, through the Luminex technology, serum levels of 17 cytokines in 44 nonagenarians Sicilian and 79 control subjects (aged 30 to 50 years) (Palmeri M. et al. 2012). We found a statistically significant increase in serum levels of IL6 (p=0.01), IL-12 (p=0.01) and IL13 (p=00,2) , and a decrease of IL4 (p=00,4) in nonagenarians in comparison with controls; whereas no modifications of other proinflammatory cytokines and chemokines were observed.

Our results suggest that the multiplex analysis of cytokine levels might be useful in defining a successful aging profile.

To better understand the genetic basis of complex diseases such as acute ischemic stroke and to outline a possible risk profile of stroke in patients with cerebrovascular risk factors, we evaluated, In **Chapter 4,** the role of SNPs of some pro-inflammatory/ anti-inflammatory and coagulation/fibrinolytic genes in susceptibility to acute ischemic stroke (Tuttolomondo A. et al 2012).

We observed a significantly higher frequency of IL-10 1082 AA genotype in stroke patients with a significant risk trend. We also reported a higher frequency in stroke sub-

jects with a significant risk trend of the TPA 7351-CT genotype and of IL-1RN-VNTR 86 bp 2/2 genotype.

Our results show, therefore, a close association between these SNPs and risk of ischemic stroke.

In a study published in "Cytokine" entitled "Myocardial infarction marker levels are influenced by prothrombin and tumor necrosis factor-a gene polymorphisms in young patients" (Vaccarino L. et al. 2012), **Chapter 5,** we analyzed the effects of the genotypes pro-inflammatory of the TNFα and pro-thrombin (FII) on the laboratory parameters of young patients affected by acute myocardial infarction in comparison with a group of control subjects; in order to be able to use the typing of these polymorphisms in combination with the specific chemistry parameters as prognostic markers.

The analysis of the genotype frequencies of the polymorphism -308A / G of TNF in patients and controls found an association between the genotype -308A positive ($p = 0.0048$), associated with an increased production of TNFα, and risk of AMI.

Furthermore, stratifying the data obtained with the measurement of blood parameters of patients affected by AMI on the basis of functional genotypes of TNF-α and FII, it indicated that TNF −308A positive genotype frequencies were increased in these patients and that a genetically determined higher production of TNF-α is associated in young subjects to a more severe cardiac damage as depicted by higher levels of troponin, Creatine kinase-MB Isoenzyme (mCK-MB) ($p < 0.0001$, $p < 0.0001$, respectively) and a significant increased plasma fibrinogen levels.

Similar and probably additive effects on might have a genetically determined increased production of pro-thrombin even if no significant differences in genotype frequencies of pro-thrombin (FII) 20210G/A polymorphisms were observed in this study.

All together these results, indicating the relationship among genetically determined TNFα and FII production and increased levels of tissue damage markers of AMI, suggest that a complex genetic background, might be involved in susceptibility to AMI in young men influencing the extension and severity of the disease and thus in unsuccessful aging.

In previous studies, our group seen that a genetic background protective for cardiovascular disease is an essential feature of successful aging (Candor et al., 2006a, d; Caruso et al., 2005).

Furthermore, in chapters 6 and 7 it was described another cardiovascular disease that characterizes aging: the Thoracic aortic aneurysm (TAA), a progressive disorder involving gradual dilation of ascending and/or descending thoracic aorta with dissection or rupture as complications.

Initially, in **Chapter 6,** we researched morphological phenotypes associated with the risk of aorta rupture and dissection in aged S-TAA individuals compared with those of control subjects, in order to identify a biomarker of rupture and dissection (Balistreri CR et al. 2014).

Our study showed that the phenotype III characterized by elevated medial degeneration, plurifocal apoptosis and increased metalloproteinase-9, it was significantly correlated with the severity of elastic fragmentation, hypertension, and smoking, and particularly with advancing age.

This might permit the assumption that phenotype III, with its typical histological abnormalities, is an optimal biomarker of rupture and/or dissection in aged individuals and is useful both for applying different surgical approaches and providing appropriate surgical indications.

In addition, in **Chapter 7,** we evaluated the role of five genetic variants of TGF-β pathways (TGF-β1 and 2 isoforms and receptors R1 and R2) and some common single nucleotide polymorphisms (SNPs) in the genes encoding IL-10 on susceptibility to TAA sporadic (Vaccarino L. et al. 2014).

Our study included cases affected by sporadic TAA and two control groups. The most relevant finding obtained allows us to propose that rs900 TGF-β2 SNP is associated with the risk of sporadic TAA.

This might open new perspectives for the analysis of sporadic TAA susceptibility factors and prevention.

Recently, we were interested in Takotsubo cardiomyopathy (TTC) in **Chapter 8**, a clinical syndrome that mimics acute myocardial infarction without obstructive coronary artery disease and it characterized by transient systolic dysfunction of the apical and/or midial segments of the left ventricle (Novo G . et al. 2014).

We evaluated the role of rs17098707 (L41Q) SNP of the G-protein-coupled receptor kinase 5 (GRK5) on genetic susceptibility to TTC, to favour a better understanding of

the pathogenesis of this peculiar syndrome, that could allow the development of more appropriated preventive strategies and tailored treatment.

Our study showed a significant difference in the frequency of the rs17098707 GRK5 SNP between TTC patients and controls, in particular, we have found that the L41 allele of GRK5 is significantly increased in TTC patients ($p = 0.0372$) than in control group.

So, polymorphisms of cytokines may play a role in susceptibility to this functional heart disease. The preliminary data presented by our research group at the recent 2nd Joint Meeting of Pathology and Laboratory Diagnostics (Palermo 17th-20th, September 2014) indicate that the presence of a complex GRK5 genotype T / * - IL10 CC may be a predisposing factor for syndrome of Tako-Tsubo, while the presence of GRK5 AA-IL10 A / * could have protective effects.

Finally, in **Chapter 9** in the manuscript "Genetic determined expression of pro and anti inflammatory mediators influence the clinical history and response to chemotherapy in patients with Sporadic Colorectal cancer (CRC)" (Forte GI et al. Submitted) as a first step, we analyzed the genetic contribute of polymorphisms known to affect the cytokine mRNA production to the susceptibility of sporadic colorectal cancer disease.

No significant genetic contribute has been observed for 5 out the 6 SNPs tested. Indeed, a significant different allelic distribution between patients and controls has been observed for the polymorphism G → C, responsible of an arginine vs. proline missense change (R25P) in correspondence of the codon 25 of the coding region of the TGF-βgene (*P*: 0.021; Odds ratio: 0.3412; 95% C. I.: 0.1323 to 0.8800). By this analysis, a weak protective role would emerge for the minor allele C in the susceptibility to the disease.

Secondly, we studied long term effect of chemotherapy on the immunological systemic balance of colorectal cancer patients, in particular, we analyzed the mRNA levels of some pro-inflammatory (IL-6, INF-γ, Cyclooxygenase (COX)-2, and anti-inflammatory (IL-10, TGF-β) type crucial cytokines produced by peripheral blood PMBC after at least 20 days from a dose administration.

It is shown a significant increase of the IL-10 mRNA produced by PMBC, observable when the samples were collected within 3 months from the chemotherapy administration as the effect of a chemotherapy treatment administration.

Also, it can be observed that the IL-6 mRNA levels move following an inverse behavior to those ones of IL-10 mRNA, although the differences are never significant. In particular, we observed lower levels of IL-6 mRNA within 3 months from the last dose of chemotherapy (<3MPC), probably caused by the IL-10 higher anti-inflammatory effect, whereas, the IL-6 levels tend to rise in the groups analyzed more than three months away from the chemotherapy administration (>3MPC). Instead, no relevant change is recorded for Cox-2, IFN-γ and TGF-β mRNAs.

Finally, we wanted to investigate if the individual genetic background, controlled by the IL-10 haplotypes, could also be implicated to generate the observed higher IL-10 levels in response to therapy.

It is shown, among the chemo-treated patients the GCC haplotype (high IL10 producer) has been observed to be significantly higher represented in patients not free of disease, respect to the ACC / ATA haplotypes (lower producers of IL-10) (GCC: 21 vs. ACC / ATA: 15;); on the other hand, the ACC / ATA haplotypes are significantly prevalent among patients free from disease respect to the GCC one (39 vs.21) (P: 0.034). The genetic data suggest that a greater capacity to produce IL-10 is prognostically not favorable for the resolution of the disease after treatment (Odds ratio: 2,6; 95% C.I. from 1,11 to 6.077). In addition, the genetic data justify the observation of a particularly increased IL-10 expression in patients receiving chemotherapy not free of disease.

So, this data indicate that an increase genetically determined the production of IL-10 may influence the effectiveness of the therapy.

Moreover, as reported in the manuscript in preparation "Cytokine serum profile in a group of patients with colorectal carcinomas treated with the standard chemotherapy" (Vaccarino L. et al.) A serum profile opposite to that identified in aging successfully could affect the response to chemotherapy in patients with carcinoma of the colon.

It is clear from that observed in the numerous reported case-control association studies that the contribution of key genes that encode cytokines such as TNF, IL-10 and TGF-beta, involved in

regulating the inflammatory response have a great influence of the onset of many age-related diseases and therefore, certainly in successful aging.

Conclusions

The studies reported in this thesis are put into the general framework above outlined on the analyses of physiopathological mechanisms implicated in age-related diseases, the biological events involved in aging, and genetic predispositions in longevity achieving.
In particular, numerous case-control studies that I performed have been focused on the role of the inflammatory balance in determining the onset of some age-related diseases, as well as on aging with and without success. On this matter, the old subjects and the centenarians group, most of all have been informative about the role of genes that may influence successful aging.
Furthermore, the results published and summarized below confirm the importance of cytokines, such as central actors in the regulation of the immune response, as key mechanism in pathogenesis of age-related diseases and making in evidence the crucial role of some functionally relevant polymorphisms in cytokine genes in "inflamm-aging".
In future, we have a strong hope that such scientific evidence may soon be translated into methods for the identification of disease risk or treatment of age-related pathologies in order to improve the health as well as psychological status and living conditions of old persons.

CHAPTER 11

Sommario e discussione generale

Lo straordinario aumento dell'aspettativa di vita nei paesi più avanzati rende conto dell'importanza degli studi sull'invecchiamento e la longevità essendo necessario ottenere una rapida diffusione delle conoscenze dei meccanismi patologici e fisiologici di invecchiamento per affrontare in modo soddisfacente i problemi medici, economici e sociali associati, a causa dell'aumento del numero di persone in età avanzata non autonome e affette da patologie invalidanti.

I Centenari che sembrano essere "attrezzati" per raggiungere i limiti estremi della vita umana relativamente in buona salute, rappresentano il miglior esempio di persone in grado di sfuggire o rallentare la comparsa delle principali malattie legate all'età, come il cancro, le malattie cardiovascolari.

L'invecchiamento è un processo di post-maturazione che, a causa di una diminuzione dell'efficienza dell'omeostasi ed un aumento della vulnerabilità dell'organismo, provoca una riduzione della risposta agli stimoli ambientali. La progressiva diminuzione delle capacità fisiologiche e la ridotta capacità di rispondere alle sollecitazioni portano ad una maggiore suscettibilità e vulnerabilità alle malattie. Pertanto, la mortalità da qualsiasi causa aumenta esponenzialmente con l'invecchiamento.

I processi di invecchiamento coinvolgono tutte le cellule, tessuti, organi e organismi ed è modulata da fattori esterni.

L'invecchiamento è accompagnato da uno stato di infiammazione cronica di basso grado come chiaramente dimostrato dall'aumento di 2-4 volte dei livelli sierici di mediatori infiammatori, come citochine e proteine della fase acuta della popolazione in età, che agiscono come predittori indipendenti di mortalità sulla morbidità preesistente.

Lo stato pro-infiammatorio negli anziani, interagendo con il background genetico, potenzialmente innesca l'insorgenza di malattie infiammatorie legate all'età come l'aterosclerosi ed il cancro, (Franceschi et al., 2000a, b ; De Martinis et al)..

Il Background genetico individuale e l'eventuale influenza di citochine anti-infiammatorie, sembrano associati a diversi gradi di controllo della risposta infiammatoria e potrebbero, pertanto svolgere un ruolo determinante nell'insorgenza di malattie legate all'età ed al raggiungimento di età avanzata in assenza di malattie.

Variazioni genetiche situate nelle regioni promoter di citochine pro-infiammatorie e regolatorie potrebbero influenzare l'inflamm-aging e la predisposizione alle malattie legate all'età.

La Sindrome Metabolica (SM) è una condizione clinica caratterizzata dalla presenza contemporanea di più alterazioni in un solo individuo, principalmente di origine metabolica, che aumenta direttamente il rischio di malattie cardiovascolari, diabete di tipo 2, e mortalità per tutte le cause.

La frequenza della sindrome è età-dipendente, con un'incidenza del 44% nella popolazione di età compresa tra 60 e 69 anni (Ford ES. Et al.2002).

La SM è uno stato di infiammazione cronica di basso grado come conseguenza della complessa interazione tra fattori genetici e ambientali. La resistenza all'insulina (IR), l'adiposità viscerale, la dislipidemia aterogenica, disfunzione endoteliale, suscettibilità genetica, pressione sanguigna elevata, stato iper coagulabilità, e lo stress cronico sono i diversi fattori che costituiscono la sindrome.

Può essere la base per la comparsa di molte malattie di età.

Questa associazione potrebbe essere dovuto al legame tra IR e infiammazione, infatti, gli stessi meccanismi che causano l'IR determinano sia la sintesi sia l'auto-mantenimento della produzione di citochine pro-infiammatorie e quindi la risposta infiammatoria cronica e persistente che caratterizza l'inflammaging.

Non è sorprendente che alcuni studi hanno dimostrato un'associazione tra la sindrome metabolica e lo sviluppo di neoplasie (Kreger BE et al 1991.), In particolare del tumore del colon-retto (CRC); oltre ad un aumentato rischio di diabete e malattie cardiovascolari (Ninomiya JK et al. 2004).

In questo particolare contesto, la conoscenza dei polimorfismi di geni che codificano per citochine, è uno strumento di estrema importanza, non solo per la comprensione dei meccanismi alla base della patogenesi delle malattie legate all'età, ma anche per una futura personalizzazione dei protocolli di trattamento, il vero "obiettivo" di la ricerca nei prossimi decenni.

Durante il corso di dottorato, ho affrontato la questione sotto molti aspetti, in primo luogo analizzando il ruolo del background genetico sulla capacità individuale di raggiungere l'invecchiamento di successo e, quindi, al fine di individuare nuovi marcatori comuni tra le patologie che possono essere ricondotte al “common soil” della SM.

A tal fine ho condotto numerosi studi di associazione incentrati sul ruolo di polimorfismi genici in grado di modificare l'equilibrio infiammatorio nel determinare

l'insorgenza di alcune delle patologie età-associate, come il cancro, le malattie cardiovascolari, l'ictus ischemico acuto, aneurisma dell'aorta toracica, la sindrome TakoTsubo, infarto del miocardio, s cancro poradico del colon, che caratterizzano l'invecchiamento con o senza successo.

Nell'articolo **"Genetics of longevity. Data from the studies on centenarians Sicilian"** (Balistreri CR et al. 2012) **(capitolo 2**) abbiamo raccolto i nostri studi condotti in 10 anni sui centenari siciliani. Abbiamo valutato il ruolo del loro background genetico individuale sulla risposta immunitaria e la longevità.

In particolare, abbiamo studiato alcune varianti genetiche dei geni TLR4, CCR5, Cox2, 5-Lo ed analizzato le frequenze alleliche e genotipiche dei polimorfismi che mappano i geni che codificano IFN-γ e IL-10.

Ciò che I nostri studi dimostrano indicano la sovraespressione di varianti anti-infiammatori CCR5Δ32, 896 G allele (299Gly) TLR4, -765 C alleli Cox-2 alleli -1708 G 5 e 21 C-Lo che caratterizzano I centenari maschi siciliani. Questi SNP che regolano generalmente in senso anti-infiammatorio la risposta agli stressors sembrano essere associati con la longevità.

Inoltre, i nostri risultati mostrano un incremento dei soggetti -1082G IL10 positivi, associata ad un aumento della produzione di IL-10, in maschi centenari e proporre un aumento della produzione di IL10 come possibili biomarcatori di longevità.

Inoltre nel **capitolo 3**, abbiamo valutato, attraverso la tecnologia Luminex, i livelli sierici di 17 citochine in 44 novantenni siciliane e 79 soggetti di controllo (di età compresa tra 30 a 50 anni) (Palmeri M. et al. 2012). Abbiamo trovato un aumento statisticamente significativo dei livelli sierici di IL6 ($p = 0,01$), IL-12 ($p = 0,01$) e IL13 ($p = 00,2$), e una diminuzione di IL4 ($p = 00,4$) in nonagenari rispetto ai controlli; mentre non sono state osservate modificazioni di altre citochine proinfiammatorie e chemochine.

I nostri risultati suggeriscono che l'analisi multiplex dei livelli di citochine potrebbe essere utile nella definizione di un profilo di invecchiamento con successo.

Per meglio comprendere le basi genetiche di malattie complesse come l'ictus ischemico acuto e di delineare un profilo di rischio possibile di ictus nei pazienti con fattori di rischio cerebrovascolari, abbiamo valutato, nel **capitolo 4**, il ruolo di SNPs di alcuni

pro-infiammatori / anti-infiammatori e di fattori della coagulazione /fibrinolisi nella suscettibilità all'ictus ischemico acuto (Tuttolomondo A. et al 2012).

Abbiamo osservato una frequenza significativamente più alta del genotipo IL-10 1082 AA in pazienti colpiti da ictus, con un significativo trend di rischio. Abbiamo anche segnalato una maggiore frequenza nei soggetti con ictus con una significativa tendenza rischio della TPA 7351-CT genotipo e del genotipo IL-1RN VNTR-86 bp 2/2.

I nostri risultati mostrano, quindi, una stretta associazione tra questi SNP e il rischio di ictus ischemico.

In uno studio pubblicato su "Cytokine" dal titolo "Myocardial infarction marker levels are influenced by prothrombin and tumor necrosis factor-a gene polymorphisms in young patients" (Vaccarino L. et al. 2012), (**capitolo 5**), abbiamo analizzato gli effetti dei genotipi pro-infiammatoria del TNFά e pro-trombina (FII) sui parametri di laboratorio di pazienti giovani affetti da infarto miocardico acuto in confronto con un gruppo di soggetti di controllo; per essere in grado di utilizzare la tipizzazione di questi polimorfismi in combinazione con i parametri di routine come marcatori prognostici.

L'analisi delle frequenze genotipiche dei polimorfismi -308A / G del TNF in pazienti e controlli ha dimostrato un'associazione positiva tra il genotipo -308A ($p = 0,0048$), associato ad un aumento della produzione di TNFά., ed il rischio di AMI. Inoltre, stratificando i dati ottenuti con la misurazione dei parametri ematici di pazienti affetti da AMI sulla base di genotipi funzionali di TNF-α e FII, ha indicato che I pazienti TNF˜308A positivi hanno una più elevata produzione dei marker di citolisi ed infiammazione associati ad un più grave danno cardiaco come descritto da più elevati livelli di troponina, creatina chinasi-MB Isoenzima (MCK-MB) ($p <0,0001$, $p <0.0001$, rispettivamente) e un significativo aumento dei livelli plasmatici di fibrinogeno.

Effetti simili e probabilmente additivi potrebbe avere un aumento geneticamente determinato della produzione di pro-trombina.

Tutti insieme questi risultati, che indicano il rapporto tra l'incremento geneticamente determinato della produzione di TNF e FII e l'aumento dei livelli di markers di danno tissutale di AMI, suggeriscono che un background genetico complesso, potrebbe essere coinvolto nella suscettibilità alla AMI in giovani uomini influenzando anche l'estensione e la gravità della malattie e condizionandone l'aspettativa di vita.

In studi precedenti, il nostro gruppo aveva dimostrato che un background genetico protettivo per la malattia cardiovascolare è una caratteristica essenziale dell' invecchiamento con successo (Candore et al, 2006a,d;. Caruso et al., 2005).

Inoltre, nei **capitoli 6 e 7** è stata presa in considerazione un'altra malattia cardiovascolare che caratterizza l'invecchiamento: l'aneurisma aortico toracico (TAA), una malattia che coinvolge la progressiva dilatazione dell'aorta ascendente e / o aorta toracica discendente con dissezione o rottura come complicazioni.

Inizialmente, nel **Capitolo 6**, abbiamo ricercato fenotipi morfologici associati con il rischio di rottura dell'aorta e dissezione rispetto a quelli di soggetti di controllo, al fine di identificare un biomarker predittivo di queste complicanze (Balistreri CR et al. 2014) .

Il nostro studio ha dimostrato che il fenotipo III caratterizzato da elevata degenerazione della media aortica, apoptosi plurifocale e aumento della presenza di metalloproteinasi-9, era significativamente correlata con la gravità della frammentazione delle fibre elastiche, l'ipertensione e il fumo, e soprattutto con l'avanzare dell'età.

Questo potrebbe far ipotizzare che il fenotipo III, con le sue tipiche anomalie istologiche, è un biomarker ottimale di rottura e/o dissezione in individui di età avanzata utile anche per un'appropriata scelta di approccio chirurgico.

Inoltre, nel **capitolo 7**, abbiamo valutato il ruolo di cinque varianti genetiche di percorsi TGF-β (TGF-β1 e 2 isoforme e recettori R1 e R2) e alcuni comuni polimorfismi a singolo nucleotide (SNP) nei geni codificanti IL-10 on suscettibilità al TAA sporadica (Vaccarino L. et al. 2014).

Il nostro studio ha incluso casi colpiti da TAA sporadico e due gruppi di controllo. Il dato più rilevante ottenuto ci consente di proporre che il polimorfismo RS900 TGF-β2 SNP è associato al rischio di sporadici TAA.

Ciò potrebbe aprire nuove prospettive per l'analisi delle sporadiche fattori di suscettibilità TAA e la prevenzione.

Recentemente, I nostri studi si sono concentrati sulla Sindrome tako-tsubo, TTC, (**capitolo 8**), una sindrome clinica che imita l'infarto miocardico acuto senza malattia coronarica ostruttiva e caratterizzata da una transitoria disfunzione sistolica dei segmenti apicali e / o Mediali del ventricolo sinistro (Novo G . et al. 2014).

Abbiamo valutato il ruolo di rs17098707 (L41Q) SNP del recettore G-protein-chinasi coupled 5 (GRK5) sulla suscettibilità genetica alla TTC, per favorire una migliore comprensione della patogenesi di questa sindrome particolare, che potrebbe consentire lo sviluppo di più appropriato strategie di prevenzione e trattamento personalizzato.

Il nostro studio ha mostrato una differenza significativa nella frequenza della rs17098707 GRK5 SNP tra i pazienti TTC e controlli, in particolare, abbiamo dimostrato che l'allele L41 di GRK5 è significativamente aumentata nei pazienti TTC (p = 0,0372) rispetto al gruppo di controllo.

Più recentemente abbiamo dimostrato che anche polimorfismi di citochine possono svolgere un ruolo nella suscettibilità a questa patologia. I dati preliminari presentati dal nostro gruppo di ricerca al recente 2 ° Congresso Congiunto di Patologia e Diagnostica di Laboratorio (Palermo 17-20, Settembre 2014) indicano che la presenza di un complesso GRK5 genotipo T / * - IL10 CC può essere un fattore predisponente per la sindrome di Tako-Tsubo, mentre la presenza di GRK5 AA-IL10 A / * potrebbe avere effetti protettivi.

Infine, nel **capitolo 9** nel manoscritto "Genetic determined expression of pro and anti inflammatory mediators influence the clinical history and response to chemotherapy in patients with Sporadic Colorectal cancer (CRC)" (Forte GI et al. Inviato per la pubblicazione) abbiamo analizzato il contributo di polimorfismi noti per influenzare la produzione di mRNA di mediatori della risposta infiammatoria alla suscettibilità della malattia cancro colorettale sporadica.

Non è stato osservato un significativo contributo genetico per 5 dei 6 SNPs testati. Infatti, una distribuzione allelica significativamente differente tra pazienti e controlli è stata osservata solo per il polimorfismo G → C, responsabile di una sostituzione missense arginina vs. prolina (R25P) in corrispondenza del codone 25 della regione codificante del gene TGF-beta (P: 0.021; Odds ratio: 0,3412; 95% CI: 0,1323-0,8800).

In secondo luogo, abbiamo studiato l'effetto a più lungo termine della chemioterapia sull'omeostasi immunologica sistemica dei pazienti affetti da Ca del colon-retto, in particolare, abbiamo analizzato i livelli di mRNA di alcuni mediatori pro-infiammatori (IL-6, INF-gamma, ciclossigenasi (COX) -2, ed anti-infiammatori (IL-10, TGF-beta) prodotti da PMBC di sangue periferico dopo almeno 20 giorni dalla una somministrazione della dose di chemioterapico.

I dati ottenuti mostrano un significativo aumento di mRNA IL-10 prodotto da PMBC, osservabile quando i campioni sono stati raccolti entro 3 mesi dalla somministrazione della chemioterapia.

Inoltre, si può osservare che i livelli di mRNA IL-6 hanno un comportamento inverso , anche se le differenze non sono mai significative. In particolare, abbiamo osservato bassi livelli di mRNA IL-6 entro 3 mesi dall'ultima dose di chemioterapia (<3MPC), probabilmente causato dal superiore effetto anti-infiammatorio di IL-10, mentre i livelli di IL-6 tendono a risalire in gruppi analizzati più di tre mesi dopo la somministrazione della chemioterapia (> 3MPC). Invece, nessun cambiamento rilevante è stao registrato per I messaggeri di Cox-2,IFN-gamma e TGF-beta.

Infine, abbiamo voluto verificare se il background genetico individuale, controllato dagli aplotipi IL-10 potrebbe anche essere implicato nella modulazione della risposta alla terapia adiuvante.

È stato dimostrato, tra i pazienti trattati con chemioterapia l'aplotipo GCC (alto produttore IL10) è significativamente più rappresentato in pazienti non liberi da malattia, rispetto agli aplotipi ACC / ATA (bassi produttori di IL-10) (GCC : 21 vs ACC / ATA: 15;); d'altra parte, gli aplotipi ACC / ATA sono significativamente prevalenti tra i pazienti liberi da malattia rispetto a quello GCC (39 vs.21) (P: 0.034). I dati genetici suggeriscono che una maggiore capacità di produrre IL-10 non è prognosticamente favorevole per la risoluzione della malattia dopo il trattamento (OR: 2,6; 95% CI da 1,11 a 6,077). Inoltre, i dati genetici giustificano l'osservazione di un particolare aumentata espressione di IL-10 in pazienti sottoposti a chemioterapia non liberi da malattia.

Quindi, questi dati indicano che un aumento geneticamente determinata la produzione di IL-10 può modulare l'efficacia della terapia.

Infine, come riportato nel manoscritto in preparazione "Cytokine serum profile in a group of patients with colorectal carcinomas treated with the standard chemotherapy" (Vaccarino L. et al.) Un profilo sierico opposto a quello individuato nell'invecchiamento con successo potrebbe influenzare la risposta chemioterapia in pazienti con carcinoma del colon.

Appare quindi chiaro che nel loro insieme gli studi di associazione caso-controllo indicano che geni chiave che codificano per citochine come TNF, IL-10 e TGF-beta,

coinvolti nella regolazione della risposta infiammatoria possono avere una grande influenza sull'insorgenza di molte malattie legate all'età e quindi, sull'invecchiamento con successo.

Conclusioni

Gli studi riportati in questa tesi rientrano nel quadro generale sopra delineato delle analisi dei meccanismi fisiopatologici implicati in malattie legate all'età, gli eventi biologici coinvolti nell'invecchiamento, e la predisposizione genetica alla longevità.

In particolare, numerosi studi caso-controllo si sono concentrati sul ruolo della regolazione della risposta infiammatoria nel determinare l'insorgenza di alcune malattie legate all'età, nonché sull'invecchiamento con e senza successo. A questo proposito, i soggetti anziani e il gruppo centenari, soprattutto sono stati informativi sul ruolo dei geni che possono influenzare l'invecchiamento con successo.

Inoltre, i risultati pubblicati confermano l'importanza delle citochine, come attori centrali nella regolazione della risposta immunitaria, come meccanismo chiave nella patogenesi delle malattie legate all'invecchiamento ed evidenziano il ruolo cruciale di alcuni polimorfismi di geni funzionalmente rilevanti per l'"inflamm-aging".

In futuro, abbiamo una forte speranza che tali evidenze scientifiche possano tradursi in metodi per l'identificazione del rischio o il trattamento di patologie legate all'invecchiamento, al fine di migliorare le condizioni di vita e lo stato di salute delle persone anziane.

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Curriculum vitae

Loredana Vaccarino was born on 06nd May 1981 in Partinico (PA), Italy.
In 2000, she got a diploma at high science school.
She started Biological studies at the University of Palermo in the same year and completed in October 2006, obtaining degree cum laude.
In January of the next year, she started specialist training in the Institute of Pathology at the University of Palermo (Head: Prof. D. Lio).
In 2006, she qualified as a professional Biologist. From 2006 to 2011, she completed a specialization school in Clinical Pathology at the University of Palermo (Head: Prof. D. Lio).
In January 2012, she started her doctorate course under the supervision of Prof. D. Lio.