Genetics of Healthy Aging in Europe

The EU-Integrated Project GEHA (GEnetics of Healthy Aging)

CLAUDIO FRANCESCHI,[a] VLADYSLAV BEZRUKOV,[b] HÉLÈNE BLANCHÉ,[c] LARS BOLUND,[d] KAARE CHRISTENSEN,[e] GIOVANNA DE BENEDICTIS,[f] LUCA DEIANA,[g] EFSTHATIOS GONOS,[h] ANTTI HERVONEN,[i] HUANNING YANG,[d] BERNARD JEUNE,[e] TOM B. L. KIRKWOOD,[j] PETER KRISTENSEN,[k] ALBERTA LEON,[l] PIER GIUSEPPE PELICCI,[m] LEENA PELTONEN,[n] MICHEL POULAIN,[o] IRENE MAEVE REA,[p] JOSÉ REMACLE,[q] JEAN MARIE ROBINE,[r] STEFAN SCHREIBER,[s] EWA SIKORA,[t] PIETERNELLA ELINE SLAGBOOM,[u] LIANA SPAZZAFUMO,[v] MARIA ANTONIETTA STAZI,[w] OLIVIER TOUSSAINT,[x] AND JAMES W. VAUPEL[y]

[a] *C.I.G.—Interdepartmental Centre "L.Galvani," University of Bologna, Via S. Giacomo 12, 40126 Bologna, Italy*

[b] *Institute of Gerontology, 04114 Kiev, Ukraine*

[c] *Centre Polymorphisme Humaine, Fondation Jean Dausset, 75010 Paris, France*

[d] *Beijing Genomics Institute, Chinese Academy of Sciences, 101300 Beijing, China*

[e] *Institute of Public Health, University of Southern Denmark, 5000 Odense C, Denmark*

[f] *University of Calabria, 87030 Rende, Italy*

[g] *University of Sassari, 07100 Sassari, Italy*

[h] *National Hellenic Research Foundation, GR-11635 Athens, Greece*

[i] *Tampere School of Public Health, University of Tampere, 33015 Tampere, Finland*

[j] *School of Clinical Medical Sciences, Gerontology "Henry Wellcome," University of Newcastle upon Tyne, NE4 6BE Newcastle upon Tyne, United Kingdom*

[k] *University of Aarhus, 8000 Aarhus C, Denmark*

[l] *Research & Innovation Soc.Coop. a r.l., 35136 Padova, Italy*

Address for correspondence: Claudio Franceschi, C.I.G.—Interdepartmental Centre "L.Galvani," University of Bologna, Via S. Giacomo 12, 40126 Bologna, Italy. Voice: +39-051-2094743; fax: +39-051-2094768.
claudio.franceschi@unibo.it

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[m] *IFOM—Fondazione Istituto FIRC di Oncologia Molecolare, 20139 Milano, Italy*

[n] *National Public Health Institute, 00251 Helsinki, Finland*

[o] *Research Centre of Demographic Management for Public Administrations, UCL—GéDAP, 1348-Louvain-la-Neuve, Belgium*

[p] *The Queen's University Belfast, BT 9 7BL Belfast, United Kingdom*

[q] *Eppendorf Array Technologies, SA—EAT Research and Development, 5000 Namur, Belgium*

[r] *University of Montpellier, Val d'Aurelle Cancer Research Center, 34298 Montpellier, France*

[s] *Kiel Center for Functional Genomics, University Hospital Schleswig Holstein, 24105 Kiel, Germany*

[t] *Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warsaw, Poland*

[u] *Leiden University Medical Centre, Leiden, the Netherlands*

[v] *INRCA—Italian National Research Centre on Aging, 60121 Ancona, Italy*

[w] *Istituto Superiore di Sanità, 00161 Rome, Italy*

[x] *Facultés Universitaire Notre Dame de la Paix, B-5000 Namur, Belgium*

[y] *Max Planck Institute for Demographic Research, 8057 Rostock, Germany*

ABSTRACT: **The aim of the 5-year European Union (EU)-Integrated Project GEnetics of Healthy Aging (GEHA), constituted by 25 partners (24 from Europe plus the Beijing Genomics Institute from China), is to identify genes involved in healthy aging and longevity, which allow individuals to survive to advanced old age in good cognitive and physical function and in the absence of major age-related diseases. To achieve this aim a coherent, tightly integrated program of research that unites demographers, geriatricians, geneticists, genetic epidemiologists, molecular biologists, bioinfomaticians, and statisticians has been set up. The working plan is to: (*a*) collect DNA and information on the health status from an unprecedented number of long-lived 90+ sibpairs ($n = 2650$) and of younger ethnically matched controls ($n = 2650$) from 11 European countries; (*b*) perform a genome-wide linkage scannning in all the sibpairs (a total of 5300 individuals); this investigation will be followed by linkage disequilibrium mapping (LD mapping) of the candidate chromosomal regions; (*c*) study in cases (i.e., the 2650 probands of the sibpairs) and controls (2650 younger people), genomic regions (chromosome 4, D4S1564, chromosome 11, 11.p15.5) which were identified in previous studies as possible candidates to harbor longevity genes; (*d*) genotype all recruited subjects for apoE polymorphisms; and (*e*) genotype all recruited subjects for inherited as well as epigenetic variability of**

the mitochondrial DNA (mtDNA). The genetic analysis will be performed by 9 high-throughput platforms, within the framework of centralized databases for phenotypic, genetic, and mtDNA data. Additional advanced approaches (bioinformatics, advanced statistics, mathematical modeling, functional genomics and proteomics, molecular biology, molecular genetics) are envisaged to identify the gene variant(s) of interest. The experimental design will also allow (*a*) to identify gender-specific genes involved in healthy aging and longevity in women and men stratified for ethnic and geographic origin and apoE genotype; (*b*) to perform a longitudinal survival study to assess the impact of the identified genetic loci on 90+ people mortality; and (*c*) to develop mathematical and statistical models capable of combining genetic data with demographic characteristics, health status, socioeconomic factors, lifestyle habits.

KEYWORDS: longevity; genetics; mitochondrial DNA; centenarians; aging

INTRODUCTION

The proportion of people aged more than 60 years in the European Union (EU) is currently close to a quarter and is likely to rise to a third within three decades. This demographic explosion makes critically important the identification of factors (biological and nonbiological) involved in aging devoid of major diseases and disabilities, thus contributing *to increase the number of old European citizens in good health.* Clues concerning such *healthy aging can be found by studying the selected group that survives over the age of 90 years*, the group that GEnetics of Healthy Aging (GEHA) project focuses on. Thus, it is timely to address the problem of the genetic determinants of healthy aging in humans with a critical mass of human and technological resources.

Human resources are an important element in the knowledge-based economy enshrined in *the Lisbon directives 2000.* The large size and vision of the GEHA project fits within the ambition and concept of *integrating and strengthening the European Research Area*. Indeed, GEHA coordinates a well-integrated network of demographers, physicians and gerontologists, geneticists, molecular biologists, statisticians, genetic epidemiologists, and bioinformaticians who are at the cutting edge of their various specialities. To our knowledge, GEHA represents *the strongest and most competitive consortium ever assembled in Europe* (and not only in Europe) *to investigate the genetic basis of the aging process and longevity in humans,* capable of reaching a critical mass from a technological and interdisciplinary point of view which is impossible to attain in single European countries.

Human longevity is *a complex trait* resulting from the interaction among environment, genetics and stochasticity,[1,2] which has specific and unusual characteristics.[3–5] Human longevity does not appear to be homogenously distributed from a geographical point of view,[6–8] and it seems to be clustered

in families enriched in long-lived parents and ancestors.[9] Human studies of longevity face numerous theoretical and logistical challenges, as the determinants of life span are extraordinarily complex.[10] Longevity can be achieved by different combinations of genetics, environment and chance, that vary, quantitatively and qualitatively, in different geographic areas according to the population-specific gene pool and to the socioeconomic level of the population.[3]

However, *large-scale* linkage studies of long-lived families, longitudinal candidate-gene association studies and the development of analytical methods provide the potential for future progress, assuming that both public and private genetic variants contribute to such a very complex trait as the healthy aging and longevity in humans.[10]

THE GEHA PROJECT: THE LARGEST INITIATIVE ON THE GENETICS OF HUMAN LONGEVITY

In July 2003 the 5-year GEHA-Integrated Project, supported through Priority 1 (Life Sciences, Genomics and Biotechnology for Health) of EU's FP6**,** Project Number LSHM–CT–2004–503270, was preliminarly approved by the European Commission. The project officially started on May 1, 2004 after a negotiation of several months, during which a Consortium Agreement among the participating Partners was agreed. It will end on April 30, 2009.

The aim of the GEHA Project is *to identify genes involved in healthy aging and longevity***,** which allow individuals to reach advanced old age in good cognitive and physical function and in the absence of major age-related diseases. The GEHA structure is conceived as a pipeline, where the first phase is the *recruitment of subjects (90+ sibpairs and younger unrelated controls)* over all Europe, that is, the collection of information on their phenotype (health status) as well as of biological samples (blood and/or cheek swab); the second phase is the *DNA extraction,* from the collected biological samples, *its quality control* and shipment to the GEHA partners in charge of the genetic analysis; the third phase is the *genetic analysis*, and, finally, the forth phase is the *analysis of data* by mean of new analytical methods and *ad hoc* developed mathematical models.

As far as we know, the GEHA consortium is the largest international collaborative study on the genetics of human longevity, and eventually will provide the largest database on this topic. It is worthy of note that in the last years, since the project was designed, proposed, and approved, additional data have been published, which strongly argue in favor of the existence of a genetic component of the extreme longevity in humans.

The GEHA Consortium and Its Bodies

The GEHA project is a large consortium of *25 partners (24 partners from Europe and 1 partner from China*). All these countries have traditions and laws quite different regarding privacy protection, ethical recommendations for genetic studies, access to demographic sources, Intellectual Property Rights (IPR) rules, among others. The GEHA project regarding the *genetics of human longevity*, requires the recruitment of very old sibpairs and the donation of their blood or other biological material on which to carry out the genetic analysis. Thus, GEHA deals with sensitive issues (ethics, privacy, etc.), which requires as much attention and care as possible. For all these reasons, in the first year of activity a particular attention was devoted to the *standardization of all the necessary tools,* and the *fulfillment or ethical requirements* both essential to start the recruitment of 90+ sibpairs and younger controls. A great effort was done to overcome the heterogeneity of the legislations established in the various countries involved in the project to guarantee the respect of privacy and confidentiality laws of the European citizens involved in the project.

In order to fulfill all the scientific, ethical, financial, and IPR requirements, and following the guidelines of the EU, the GEHA project was endowed with a complex organization structure composed by the following bodies:

Coordinator: Professor Claudio Franceschi; *Project Manager:* Dr. Alessandra Malavolta; *Scientific Manager:* Dr. Silvana Valensin;

General Assembly (GA) composed by 25 members (i.e., all the Principal Investigators, one person per Partner);

Steering Committee (SC) composed by 9 members (i.e., the leaders of the 12 Work Packages);

Ethics Steering Group (ESG) composed by 3 internal members plus 2 external members;

External Advisory and Gender Board (EAGB) composed by eminent scientists from the United States and Europe;

Legal and IPR Board (LIPR) composed by 3 members;

Financial Management Board (FMB) composed by 5 members.

The Institutions (Principal Investigator in parentheses) constituting the GEHA Consortium are:

(1) UNIBO-CIG, Interdepartmental Centre "L.Galvani," University of Bologna, Italy (Claudio Franceschi);
(2) CRLC, Department of Biostatistics, University of Montpellier, Val d'Aurelle Cancer Research Center, Montpellier, France (Jean Marie Robine);
(3) CAU, Kiel Center for Functional Genomics, University Hospital Schleswig Holstein, Kiel, Germany (Stefan Schreiber);

(4) CEPH, Centre Polymorphisme Humaine, Fondation Jean Dausset, Paris, France (Hélène Blanché);
(5) ISS, Istituto Superiore di Sanità, Rome, Italy (Maria Antonietta Stazi);
(6) LUMC, Molecular Epidemiology, Leiden University Medical Centre, Leiden, the Netherlands (Pieternella Eline Slagboom);
(7) MPIDR, Max Planck Institute for Demographic Research, Rostock, Germany (James W. Vaupel);
(8) NHRF, National Hellenic Research Foundation, Athens, Greece (Efsthatios Gonos);
(9) KTL, Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland (Leena Peltonen);
(10) NENCKI, Laboratory of Molecular Bases of Aging, Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland (Ewa Sikora);
(11) QUB, Department of Geriatric Medicine, The Queen's University Belfast, Belfast, United Kingdom (Irene Maeve Rea);
(12) UNICAL, Department of Cell Biology, University of Calabria, Rende, Italy (Giovanna De Benedictis);
(13) IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milano, Italy (Pier Giuseppe Pelicci);
(14) UNISS, Department of Anesthesiologic Surgery, University of Sassari, Sassari, Italy (Luca Deiana);
(15) UCL, Research Centre of Demographic Management for Public Administrations, UCL—GéDAP, Louvain-la-Neuve, Belgium (Michel Poulain);
(16) FUNDP, Department of Biology, Facultes Universitaire Notre Dame de la Paix, Namur, Belgium (Olivier Toussaint);
(17) UNEW, School of Clinical Medical Sciences, Gerontology "Henry Wellcome" & PEALS Research Institute, Bioscience Centre, International Centre for life, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom (Tom B.L. Kirkwood, Erica Haimes);
(18) SDU, Institute of Public Health, University of Southern Denmark, Odense C, Denmark (Kaare Christensen, Bernard Jeune);
(19) TAMPERE, Laboratory of Gerontology, Tampere School of Public Health, University of Tampere, Tampere, Finland (Antti Hervonen);
(20) R&I, Research & Innovation Soc.Coop.a r.l., Padova, Italy (Alberta Leon);
(21) INRCA-Italian National Research Centre on Aging, Molecular Genetic Laboratory, Ancona, Italy (Liana Spazzafumo);
(22) UAAR, Department of Molecular Biology, University of Aarhus, Aarhus C, Denmark (Peter Kristensen);
(23) BGI, Department of Genome Dynamics and Bioinformatics, Beijing Genomics Institute, Chinese Academy of Sciences, Beijing, China (Huanning Yang, Lars Bolund);

(24) EAT, Eppendorf Array Technologies, SA - EAT Research and Development, Namur, Belgium (José Remacle);
(25) IG, Institute of Gerontology, Kiev, Ukraine (Vladyslav V. Bezrukov).

The Major Goals of the GEHA Project

(1) To overcome the fragmentation of the research on the genetics of aging in Europe;
(2) To recruit an unprecedented number of long-lived sibpairs ($n = 2650$) from 11 European countries in 15 geographic areas whose DNA will be used to perform a genome-wide linkage scanning (Linkage analysis or Affected SibPair analysis) aimed to identify chromosome regions involved in longevity and healthy aging;
(3) To recruit a large number ($n = 2650$) of younger control subjects (mean age: 60–65 years) from the same geographic areas, whose DNA will be used for case–control association studies—linkage disequilibrium mapping (LD mapping), followed by positional cloning and mutation analysis to fine map the chromosome regions identified by Linkage analysis;
(4) To perform bioinformatic, functional genomics and proteomics, and molecular biology studies on the identified/putative longevity regions/genes and gene variants resulting from Linkage scanning and LD mapping;
(5) To test whether ethnically different populations (including those from Sardinia and Finland) share the same genes involved in aging and longevity;
(6) To verify if the genes involved in longevity and healthy aging in the European population are the same in an ethnically different population, such as the Han Chinese;
(7) To ascertain the role played in human longevity by candidate regions (D4S1564 in chromosome 4, as well as chromosome 11p15.5);
(8) To verify in a variety of European populations and at a large scale the role of mitochondrial DNA (mtDNA) inherited (haplogroups and subhaplogroups) and epigenetic variability, and to study their interaction with the newly emerging longevity nuclear genes;
(9) To identify gender-specific genes differently involved in the healthy aging and longevity of women and men;
(10) To stratify all the samples according to apoE genotype. All the 7950 subjects recruited by the GEHA consortium will be genotyped for polymorphisms of the apoE gene ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$) known to be correlated with reduced longevity in all the populations tested so far;
(11) To develop innovative analytical strategies (based on statistical method and mathematical models) capable of combining all the data collected (clinical, socioeconomical, related to lifestyle, demographic and genetic);

(12) To perform a longitudinal study to evaluate the importance of genetic factors on mortality of the recruited long-lived sibpairs.

RECENT ADVANCES IN THE GENETICS OF HUMAN LONGEVITY

We will briefly review recent data, mostly published after the GEHA project was presented and approved (2003), suggesting that:

(1) human longevity clusters in families;
(2) long-living siblings are likely enriched in longevity genes.

The list of papers we will refer to is far from being complete, and it is part of a much larger literature we cannot quote for space reasons.

Actually, an impressive and coherent series of epidemiological data from different populations (White Americans from New England, Mormons from Utah, Ashkenazi Jewish living in the United States, Icelanders, Japanese from Okinawa, Netherlanders from Leiden, Danish collected in the entire nation, Italians from Southern Italy) suggests the presence of a strong FAMILIAR component of human longevity. All these studies demonstrate that *first-degree relatives (parents, siblings, and offspring) of long-lived subjects* (but not the spouses of the long-lived subjects who shared with them most part of their adult life) *have a significant survival advantage***,** a higher probability to have been or to become long-living people and to have a lower risk regarding the most important age-related diseases, such as cardio- and cerebrovascular diseases (CVD), diabetes, and cancer, when compared to appropriate controls.[11–15] Thus, literature indicates that longevity is present in many generations of a single family in spite of the great variations in lifestyle and life expectancy as it occurred in the last century. In particular, it is remarkable that in the most recent studies on this topic, spouses of long-lived subjects were added as additional control group. The results indicate that this control group does not have any advantage/benefit in terms of survival and protection from the above-mentioned diseases, even if they shared with the long-lived partner most of their adult life.

In particular, as far as parents, siblings, and offspring of centenarians are concerned, the available data indicate that:

(1) *CENTENARIANS* have the following characteristics:

- A lower prevalence of cancer, CVD, insulin-resistance and diabetes, and a delay of about 1–2 decades of the onset of others pathologies, such as dementia and hip fractures.[16]
- Most of them do not show insulin-resistance and have anthropometric (BMI), metabolic (cholesterol, LDL-C, HDL-C, triglycerides, etc.), and cardiovascular (systolic and diastolic pressure) features that are optimal for their age.[17]

- Their successful aging seems to be largely influenced by their optimal balance between inflamm-aging and anti-inflammaging.[4] Centenarians appear to have the capability to set up responses capable of neutralizing or at least diminishing the deleterious effect of the low-grade, chronic inflammatory status, characteristics of the aging process (inflamm-aging), which in turn is largely a consequence of the level of subclinical antigenic stimulation sustained by bacteria, viruses, and other pathogens.
- The above-mentioned characteristics can explain the finding in centenarians of a different frequency of a variety of polymorphisms of genes involved in immune response, inflammation, coagulation, and lipid and glucose metabolism, in comparison with younger controls (association studies).[18–27] However, most of these studies need to be replicated in different populations and contrasting data have been obtained in different studies.
- A different frequency of germ line variants of mtDNA.[28]

To this regard it is important to remind that *it is still unclear* whether and how much the different populations of long-lived individuals (centenarians and nonagenarians) studied so far (Ashkenazi Jewish, Danish, French, Finnish, German, Irish, Islandic, Italians, Japanese, Mormons, among others) share the same genetic markers of longevity and *whether "public" and/or "private" (population specific) longevity genes and polymorphisms do exist in different populations and/or individuals.*

(2) *PARENTS OF CENTENARIANS* have a higher "risk" (about 7 times) to reach extreme longevity (90–99 years old)[13];

Parents' longevity is probably important and interesting from a biomedical point of view, as demonstrated by two recent studies:

- According to an investigation performed on 1402 members of 288 pedigrees within the framework of the Framingham Heart Study, genetic factors explained an additional 57% of biological age variability.[14]
- According to a study performed in 51,485 men and women aged 40–79 years, the risk of mortality from all death causes including stroke and CVD was 20–30% lower in men and women with parents who died at age equal or higher than 80 years (fathers) and equal or higher than 85 years (mothers), compared with subjects having parents whose age at death was lower than 60 years (fathers) and lower than 65 years (mothers). These findings indicate that parental longevity could be a predictor for reduced risk of mortality from stroke, CVD, and all causes of death.[15]

(3) *SIBLINGS OF CENTENARIANS* also have an advantage for survival and for attaining extreme longevity:

- In a study on 2092 centenarian siblings, it has been demonstrated that both males and females have a mortality 50% lower than that of 1900 subjects of the same birth cohort, and their relative survival probabilities

increase markedly at older ages, reflecting the cumulative effect of their mortality advantage throughout life. Male siblings of centenarians were at least 17 times as likely to attain the age of 100 years, while female siblings were at least 8 times as likely.[29]

- From the analysis of the pedigrees of 348 Okinawan centenarian families with 1142 siblings it resulted that both male and female centenarian siblings experienced approximately half mortality of their birth cohort-matched counterparts of the general Okinawan population.[8] Remarkably, this mortality advantage of centenarians siblings was sustained at all ages and decades, and did not diminish or disappear with age in contrast to many environmentally based mortality gradients (gender, ethnicity, nutritional factors, such as cholesterol, physical activity, economical status, education level), suggesting that the familiar component is mostly genetically related.
- In families with at least two long-living siblings (men aged 89 years or more and women aged 91 years or more), the rest of their siblings, their parents, and their offspring, but not their spouses (husbands and wives), showed a major survival and a mortality rate for all causes of death that was 35% less than in the general population[30] (see later).

(4) *OFFSPRING OF CENTENARIANS* presents a lower prevalence of CVD (56%), hypertension (66%), and diabetes (59%) and their median ages of onset for CVD, hypertension, diabetes, and stroke were significantly shifted forward by 5.0, 2.0, 8.5, and 8.5 years, respectively.[11]

- They had a 62% lower risk of all causes mortality, a 71% lower risk of cancer-specific mortality, and an 85% lower risk of coronary heart disease-specific mortality.[12]
- They had a favorable lipoprotein profile characterized by significantly larger HDL and LDL particle size and significantly increased homozygosity for the 405 valine allele (V allele) in the CETP gene (Cholesteryl Ester Transfer Protein),[31] and the −641 C allele in APOC3 gene,[13] similar to what has been observed in parents of centenarians.

At present, it is still unknown how much this familiar component of longevity and successful ageing is due to genetics. This is a crucial issue from a theoretical (biology) and practical (biomedicine and public health) point of view, and the GEHA project is aimed to contribute to its clarification.

On the whole, the above-mentioned data would suggest that the familiar component of longevity is fundamentally a GENETIC component. At the same time, they indicate that families enriched in long-living members and, in particular, in very old siblings, and offspring of long-lived parents represent study groups particularly suitable to investigate the determinants of the human longevity.

We will review in detail *three very recent papers that we consider of particular interest* in the relatively large literature on the genetics of longevity, and to which contributed some GEHA partners and members of the GEHA EAGB.

Schoenmaker *et al*.[30] *s*tudied families with at least two long-living siblings (men: 89 years and over; women: 91 years and over) and showed that the standardized mortality ratio for all siblings of the long-living participants was 0.66 and that a similar survival benefit was also observed in the parents (0.76) and in the offsring (0.65) of the long-living participants. The standardized mortality ratios of the spouses of the long-living subjects was 0.95. The authors conclude that: (*a*) it is unlikely that the familiar clustering of extended survival is caused by environmental factors, because the spouses of the long-living participants had a mortality risk comparable with the general Dutch population, whereas they share the same environment; and (*b*) families with two long-living siblings are genetically enriched for extreme survival.

Hjelmborg *et al*.[32] start from the consideration that although human familiy studies have indicated that a modest amount of the overall variation in adult life span (approximately 20–30%) is accounted for by genetic factors, it is not known if they become increasingly important for survival at the oldest ages. The genetic influence on human life span and how it varies with age was studied in cohorts of Danish and Finnish twins born between 1870 and 1910 (20,502 indiduals) followed until 2003–2004. Mean life span for male monozygotic (MZ) twins increases 0.39 years for every year his cotwin survives over age 60 years, and this rate is higher than the rate of 0.21 for dizygotic (DZ) males. Females and males have similar rates and these are negligible before age 60 for both MZ and DZ pairs. Having a cotwin surviving to old ages substantially and significantly increases the chance of reaching the same old age and this chance is higher for MZ than for DZ twins. The authors conclude that: (*a*) such a large population-based study shows genetic influence on human life span; (*b*) this influence is minimal prior the age of 60 years but increases thereafter; and (*c*) these findings provide a support for the search for genes affecting longevity in humans, especially at advanced ages; linkage studies in large samples of extremely long-lived siblings may be among the best approaches to identify such genes.

Christensen *et al*.[10] published a rich and comprehensive review which deliver several take home messages, including the followings:

(1) The determinants of life span are extraordinarily complex and human studies of longevity face theoretical and logistic challenges;
(2) Longevity clusters in some families but it is difficult to disentangle the effect of the shared environment and that of genetics;
(3) Owing to the complexity of the long-living phenotype, there is the possibility that different variants are involved in life-span variation in different populations;

(4) As the effect of the genetic component on longevity increases after the age of 60 years, nonagenarians and centenarians are particularly informative about longevity genes;
(5) Large sample size are needed to uncover alleles which occur only in a few percent of the population and that have a modest effect on survival;
(6) Large-scale and carefully designed study assessing long-lived siblings and controls, as well as studies on large cohorts of elderly people followed longitudinally, will be essential to progress in genetic studies of human longevity, especially if combined with high-throughput genotyping techniques;
(7) Genome-wide association studies are becoming feasible and are promising but logistically and financially demanding.

The great potentialities but also the difficulties of projects like GEHA are clearly depicted in such a review.

Putative Longevity Genes in Chromosome 4

One of the original aims of the GEHA project was to ascertain *the role played in human longevity by a candidate region in chromosome 4 (D4S1564)* according to previous observations by an American group. As a result of a *genome-wide scan* performed on *308 individuals belonging to 137 sibships* demonstrating exceptional longevity, a *borderline significant evidence* ($P = 0.044$) *for linkage was noted for chromosome 4 near microsatellite D4S1564* (4q25) that was underrepresented among long-living individuals when compared with younger controls.[33] This interval spans 12 million bp and contains approximately 50 putative genes. To identify the specific gene and gene variants impacting life span, the same group performed a haplotype-based fine-mapping study of the interval. The resulting genetic association study identified a haplotype marker within *microsomal transfer protein (MTP)* as a modifier of human life span. This same variant was tested in a second cohort of French centenarians from CEPH, and *the association was not replicated*.[34] MTP has been identified as the rate-limiting step in lipoprotein synthesis. The *low number of sibships used in this study*, together with the impossibility to replicate the results in the French samples, prompted several labs to replicate the study in different populations and in a larger sample of long-living individuals. The plan of the GEHA Consortium was to take advantage of the possibility to replicate the study in a sample about 20 times as large as the one used by the American group, in a variety of European populations.

In the meantime, since the GEHA project was presented, approved, and started several papers have been published by members of the GEHA consortium who, using their own previously collected samples of long-lived subjects, *failed to replicate the original observation of the American group* in different European populations.

Nebel *et al*.[35] performed a study on 1039 unrelated subjects of German ancestry between 95 and 109 years of age (mean age, 98.2 years), 373 (36%) being centenarians. In comparison with all other U.S. and European subjects analyzed in the literature, the MTP "risk" haplotype was found to be overrepresented only in U.S. controls, implying that the putative association reported by Geesaman *et al*.[34] was more likely to reflect recent changes in the genetic structure of the U.S. Caucasian population as a whole, rather than genetic effects upon survival to old age.

Bathum *et al*.[36] tested the hypothesis that MTP gene polymorphisms were associated with extreme longevity in a longitudinal study of nonagenarians and in an association study. Participants in the Danish 1905 cohort study (1651 participants aged 92–93 years) were genotyped for the two SNPs (rs2866164 and Q95H) in the MTP gene recently reported to be associated with longevity. The 1905 Cohort has been followed for 6.5 years, during which 83% of the cohort has died. Furthermore, a group of 575 middle-aged Danish twins (mean age 53.7 years) were tested as a younger control group. The risk haplotype had no significant survival disadvantage (*P* values: 0.56, 0.31, and 0.97 in the total population of nonagenarians, males, and females, respectively) after 6.5 years of follow-up. The distributions of the suggested risk alleles (rs2866164-G and Q95) and the resulting haplotypes were very similar and not statistically different between the two age cohorts. In conclusion, this longitudinal study of survival in the tenth decade of life and this association study in a genetically homogeneous population provided no support for an association between the MTP gene polymorphisms and extreme longevity.

Beekman *et al*.[37] investigated the linkage to 4q25 in 164 nonagenarian sibships of the Leiden Longevity Study (LLS). Moreover, the MTP −493G/T and Q95H allele and haplotype frequencies were compared in 379 nonagenarians, 525 of their offspring and 251 partners of their offspring of the LLS, and in 655 octogenarians and 244 young controls of the Leiden 85+ Study followed for at least 7 years and providing an opportunity to perform a prospective analysis. Both the linkage analysis and the association study were negative and the authors, after performing a meta-analysis arrived to the same conclusions of Nebel *et al*.,[35] i.e., that the problem of the original report was the admixture of the U.S. control population.

As far as the GEHA project is concerned, these data, on the whole, are important for several reasons and suggest that:

(1) Linkage analysis to detect longevity genes must be performed in a large number of sibpairs;
(2) Association studies are useful and more sensitive than linkage analysis, but must be performed and replicated in different ethnically homogeneus populations, and particular attention must be payed to population stratification in the control groups;

(3) the study of the chromosomal region 4q25 is no longer an urgent priority for the GEHA project.

Longevity Genes in Chromosome 11

One of the aim of the GEHA is to ascertain the role played in human longevity by *a candidate region in chromosome 11 (11.15.5).* The reason to concentrate the effort of the GEHA Consortium on this region derives from *several studies published by GEHA members* before and after the starting of the GEHA project that *point out that polymorphic variants of an unusually large number of genes present in such a region* of about 2Mbases, as, Sirtuin 3 *(SIRT3),* v-Ha-ras Harvey rat sarcoma viral oncogene homolog 1 (*HRAS1*), Insulin-like Growth Factor 2 (*IGF2*), Insulin (*INS*), and Tyrosine Hydroxylase (*TH*) *are associated with human longevity.*[38–43]

It is important to remember that these genes are the human homologs of genes that in a variety of animal models, appear to play an important role in life-span extension and in protection from from a variety of stressors.

The new data which have been published in humans by GEHA partners[44,45] since the GEHA project has been presented reinforce the interest for such a region of chromosome 11 and render the GEHA agenda extremely up-to-date.

The general hypothesis which can be tested by the GEHA Consortium is that the capability of some genes to be involved in life-span extension might have been conserved throughout evolution from yeast and worms to humans.

THE GENETICS OF HEALTHY AGING AND LONGEVITY AND THE mtDNA VARIANTS

One of the aim of the GEHA project is to investigate in a variety of European populations *the role of mtDNA germline variants (haplogroups, sub-haplogroups), and mutations (C150T)* in human longevity,[46] and to study their interaction with the newly emerging longevity nuclear genes.

Indeed, a remarkable result from studies of long-lived individuals is the association found between mtDNA-inherited variants (*haplogroup J*) and healthy aging and longevity in *Italian centenarians*.[47] Further data showed that *this association is likely population specific*, being present in long-lived subjects from *Ireland*[48,49] but *not in those from southern Italy*.[50] Moreover, *a C150T mutation* was found at a *much higher frequency in centenarians* than in young people.[51] The data also showed that *C150T* variant causes *a remodeling of the replication origin at position 151* and can be either inherited (polymorphism) or somatically acquired (mutation). A commentary to this article was

published by Wallace and co-workers[52] suggesting that *mtDNA-inherited variants (haplogroups) are likely not neutral* and subjected to climatic adaptation, and that C150T variant and/or J haplogroup *might have changed (reduced) oxidative phosphorylation (OXPHOS) efficiency* and thus reactive oxygen species (ROS) production, reducing oxidation stress, and increasing longevity. The higher frequency of 150T in aged subjects has been *confirmed* in a total of 321 very old individuals and 489 middle-aged controls from Finland and Japan.[53] In addition, *150T was shown to be associated with longevity in subhaplogroup J2*, in accordance with a specific study on mtDNA haplogroup J in centenarians.[54]

Thus the available data concordantly point out that mtDNA variants (C150T polymorphism and haplogroup J or subhaplogroup J2) are associated with longevity in a population-specific way. The reason(s) and geographic extension are still unclear. Another open question regards the degree of heteroplasmy of the C150T variant and its tissue specificity.

The GEHA project, with its unprecedent number of recruited samples of Caucasian origin and from different geographic areas represents *a unique opportunity to confirm and further extend those data*, which indicate a strong role of mtDNA variants in human longevity. Such a role of mtDNA (maternally inherited) is in line with data on the genealogy of supercentenarians (people older than 110 years of age), who show *a great survival advantage in the maternal lineage*.[9] Furthermore, the classification of mtDNA variants is undergoing continuous modifications and updating which eventually redefine the mtDNA phylogenetic tree. The most recent paper redefining haplogroups classification and names also suggests that the complete sequencing of mtDNA would be preferable instead of the mere haplogroup identification.[55] Unfortunately this kind of approach is not feasible at large scale due to the still high cost of mtDNA resequencing. The GEHA project has planned to perfom the resequencing of the whole mtDNA for a consistent fraction of the collected samples. Furthermore, these samples will be selected among homogeneous populations in order to confirm possible interactions between genetics and environment.[50]

Within this scenario and taking into account that *mtDNA haplogroups interact with polymorphisms of nuclear genes*[56,57] the GEHA consortium will eventually match all the data obtained on mtDNA genetics with those obtained as a result of the genotyping of the nuclear genome, in the 90+ sibpairs and in the controls. For this purpose, a new database has been created in order to allow storage, retrieval, and analysis of all the collected mtDNA as well as the cross-matching of these data with those coming from the nuclear DNA genotyping. This database will represent one of the largest collection of mtDNA sequence data, and by adding to it other already published sequences will constitute one of the largest mtDNA database worldwide. The consortium is also working to implement in the database some new functions which are currently

not available in any other mtDNA database such as the automatic haplogroups classification. This feature is the first step into the direction of making such a software a permanent service available, in due time, to any other user worldwide.

THE GEHA PROJECT: THE FIRST TWO YEARS OF ACTIVITY

Standardization of Tools and Procedure

The most important achievements of the GEHA project during its two and a half years of activity have been the following:

(1) Set up and standardization of *two Informed Consent Forms*, the first for 90+ sibpairs, and the second for the younger controls (in all the collecting country National languages and in English).
(2) Set up and standardization of *three Questionnaires*, one for 90+ sibpairs, one for younger controls, and the last for the family of the 90+ sibpair (in all the collecting countries National language and in English).
(3) Set up of the *GEHA phenotypic and genetic databases* plus a *database for mtDNA*. All databases strictly respect the privacy protection requirements established upon suggestions of the ESG and based on the European legislation.
(4) Set up and standardization of the procedures for the *collection and processing of the biological material* (blood samples and cheek swabs).
(5) Identification of the *centralised facility for DNA extraction*, quality controls, permanent banking, preparation of DNA plates, and their shipment to genetics platforms.
(6) Set up and standardization of the *protocol for DNA extraction* for nuclear DNA, and for mtDNA from peripheral blood lymphocytes and granulocytes.

Recruitment and DNA Preparation

The following activities were performed during the two and a half years of activity:

(1) Identification of the geographic areas suitable for the recruitment of 90+ sibpairs and ethnically matched younger controls, and assessment of the procedures to access the demographic data.
(2) Preparation of the documents (in both National and English language) for obtaining the approval of the local ethical committees.
(3) Participation to a specific Recruitment Course organized in Bologna in October 2004.

(4) Standardization of the procedure for processing and labeling the biological samples in a way suitable to guarantee the privacy respect, and assure a suitable shipment and storage of the samples.
(5) Recruitment of 1671 TRIOS, that is, 2 siblings, or more where available, plus 1 younger ethnically matched control subject, in their respective geographic areas (Achievement end of October 2006).
(6) Collection of 4874 blood samples and 328 cheek swabs from recruited subjects (Achievement end of October 2006).
(7) Shipment of the biological samples to the centralized facility for DNA extraction and quality control (in progress).
(8) DNA extraction, quality control, and preparation of DNA plates for genetic analysis (in progress).

THE GEHA DESIGN AND THE GEHA GENOME-WIDE LINKAGE SCANNING

In the last few years an enormous amount of data became available regarding the human genome, including data on millions of new single-nucleotide polymorphism (SNP) variants in different human populations (HAPMAP Project). Such an unprecedented extremely fast progress has been possible owing to the continuous refinement of the genetic methodologies as well as the methods of data analysis. Concomitantly, the conceptualization about genome-wide studies, their possibilities and limitations, has also progressed in a very fast way so that the entire scenario of genetic studies on complex traits has completely changed.

The GEHA project took an enormous advantage from such a rapid advancement in the field, but at the same time it was necessary to reshape the genetic strategy originally envisaged to perform the Linkage analysis (the use of 400 highly polymorphic microsatellites separated by an average distance of 10–20 cM). The GEHA geneticists carefully examined and compared the most recent available literature in the field and decided the genetic strategy and the platform to adopt according to reliability of the results, cost per SNPs and technician time, as well as the direct experience and the expertise of the GEHA partners.

Even if *the main goal of the GEHA project is to perform a Linkage analysis* with several thousand of highly informative SNPs using the 2650 90+ sibpairs, it is important to stress that the GEHA design allows to perform both linkage and association studies (using one member of the sibship and the younger unrelated control), according to the most advanced genetic approaches to complex traits, as illustrated in FIGURE 1. These possibilities (genome-wide genetic association studies) might be pursued in future developments/continuation of the project, using the unique collection of DNA samples recruited by the GEHA consortium.

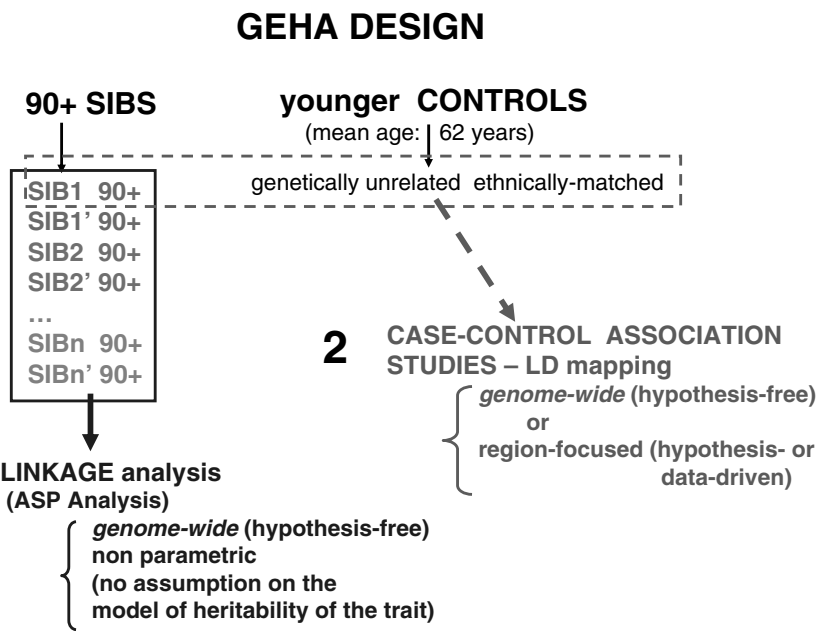

FIGURE 1. The design of the GEHA project allows to perform either genome-wide linkage studies, using the DNA collected from the 2650 90+ sibpairs, or association studies using the DNA collected from one (or both) member of each sibship and the DNA collected from the unrelated, ethnically matched younger control. The association studies can be either genome-wide or focused on specific chromosal region(s) or loci.

Indeed, linkage studies on large samples of extreme long-lived siblings may be among the best approaches to identify longevity genes. Linkage analysis looks for *coinheritance of chromosomal regions with the trait* in families, and it is more powerful than association analysis for identifying *rare high-risk disease alleles*. Association is an approach to gene mapping that looks for associations between a particular phenotype and allelic variation, that is, for differences in the frequency of genetic variants between *unrelated affected individuals and controls*, with the expectation that the risk-conferring allele (haplotype) will be more common in cases (the long-living people) than in controls (the younger subjects). Association analysis nowadays can be performed genome-wide and it is expected to be more powerful for the detection of *common* alleles that confer *modest disease risks.*

The advantage of linkage studies is that they are *less influenced by population admixture* than the association approach, while the advantage of association case–control studies is that they require much less genotyping to obtain equivalent power.

Within an evolutionary and Systems Biology perspective longevity likely results from *the interaction and cross-talk between two genomes:* (*a*) the Nuclear

genome; and (*b*) the Mitochondrial genome (mtDNA). Accordingly a major aim of GEHA is to ascertain the role of mtDNA inherited as well as epigenetic variability in human longevity taking advantage of the unprecedented number of very old sibpairs recruited by GEHA, belonging to different European populations. This part of the project will be discussed in details in the next paragraph.

The Analysis of mtDNA Variability

The GEHA consortium has the capacity to provide the largest dataset on mtDNA variation over age in different populations. To this purpose the main activities in the first 2 years of activity, were the following:

mtDNA Resequencing

Different approaches were developed by the GEHA consortium in order to obtain complete mtDNA sequencing. A *strategy of quality control of the sequences and the design of a database* for the storage and analysis of the sequences and their annotation were developed. mtDNA belonging to the specific populations will be resequenced for a total of about a thousand mtDNA sequences.

All other GEHA samples will be genotyped for mtDNA haplogroups and subhaplogrops, using a protocol based on polymerase chain reaction (PCR) amplification and *sequencing of the mtDNA D-Loop together with some principal restriction sites. An appropriate database* for storage and analysis of mtDNA genetic data is under development.

Analysis of C150T Mutation

A fast and relatively cheap DHPLC technique to screen heteroplasmy in the whole mtDNA molecule has been developed. This will allow to analyze possibly identified common "hot spots" of heteroplasmy (including the C150T mutation) in a large group of sibpairs and controls.

At present, first data on mtDNA resequencing, mtDNA haplogroups and subhaplogrous, and heteroplasmy of C150T are emerging.

ANALYSIS OF THE GENETIC DATA

The GEHA partners have a great experience in the analysis of data related to the genetics of human longevity and they pioneered the field by proposing

new methods, which takes into account the demographic data (mortality and survival probability).[58,59] These methods have been further extended[60–65] and will be used as a starting point for further development able to fully exploit all the data (phenotypic and genetic of both the nuclear and the mitochondrial genome), which will eventually be provided. This innovative approach will greatly increase the power of the genetic analysis, since the lack of statistical power is the most common problem in studies on genetic complex traits, such as human longevity.

Bioethical Issues and Implications

Ethics was a major and pervasive topic which dominated all the issues in these 2 years of the GEHA activity. The hard work and the superb expertise of the *ESG* were critical to solve a variety of important and complex problems related to recruitment and the planning of genetic studies. Many ethical issues emerged during the 2-year activity of the project and deserved deep discussions and a consequent search for a commonly agreed solution. Indeed, there is *a large heterogeneity of ethical rules* among the different countries taking part in the GEHA Consortium that must be taken into consideration whenever facing any decision involving ethical issues. In particular, the ESG produced specific suggestions and recommendations regarding:

(1) The key ethical questions about recruitment and informed consent;
(2) The establishment of criteria for privacy and confidentiality of data and their long-term storage;
(3) The establishment of criteria for let the general public appreciate the ethical implications of a genetics study such as GEHA;
(4) The issue of the use of biological samples after the end of the GEHA consortium.

Moreover ESG performed a thorough investigation of all the literature regarding the genetics of aging and longevity in humans, in order to have a comprehensive view of how the ethical issues and implications of this type of research have been addressed and solved all over the world. Specific papers have been published on this topic.[66]

Training

The long-term success of GEHA consortium depends on successful and integrated working and interchange of ideas between people with different expertise such as demographers, epidemiologists, geriatricians, geneticists, molecular biologists, mathematicians, statisticians, and bioinformaticians. In particular there is an urgent need *to train young scientists* in this field at the

cutting edge of the above-mentioned disciplines. Two different instruments have been used so far to train the young scientists:

(1) Exchanges of young scientists among GEHA partner labs and
(2) A Short Course on Demographic-Statistical Methods, held on September 12–30, 2005 at the Max Planck Institute for Demographic Research, Rostock, Germany, in which young members of the GEHA consortium participated.

Dissemination

The following dissemination initiatives were pursued:

(1) A *GEHA web site* www.geha.unibo.it has been set up since June 2004.
(2) Many articles devoted to the most advanced scientific projects in Europe mentioned the GEHA project in *daily newspapers and weekly magazines* as an example of cooperation at European level to achieve important goals for the health of citizens.
(3) *Several TV and radio programs* in United Kingdom, Germany, Italy, Finland, France, Poland, Ukraine, and Greece, among others, specifically devoted to the aging of the population, and to the biological basis of aging and longevity mentioned the GEHA project.

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