

**Clinical, Epidemiological and Genetic  
Investigations on Thalassemia and Malaria in Italy**

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*Abstract.* The first epidemiological studies on thalassemia distribution in Italy carried out by Ezio Silvestroni and Ida Bianco after World War II confirm the geographic correlation previously reported by different clinicians between the high frequency of this hereditary condition and malarial infection. The phenomenon was studied by the Italian geneticist Giuseppe Montalenti who, stimulated by the hypothesis advanced by J.B.S. Haldane that the frequency could not be due to an abnormal rate of mutation but probably to selective advantage of the thalassemic heterozygotes, began collaborating with Silvestroni and Bianco in order to determine the origin of thalassemia distribution in Italy. In the present article a reconstruction is made of the early studies carried out in Italy on the genetics of thalassemia and a discussion is presented of the first hypotheses concerning the relationship between thalassemia and malaria. An examination is then made of the results and methodological difficulties surrounding the research coordinated by Montalenti at Ferrara, also thanks to funding from the Rockefeller Foundation, as well as those carried out by Ruggero Ceppellini and Marcello Siniscalco in Sardinia.

According to Weatherall and Clegg (1999), and Weatherall (2004b), the question of the relationship between thalassemia and malaria remained uncertain until, thanks to the methods of molecular genetics and the macroepidemiological studies carried out in the Southwest Pacific on alpha-thalassemia, "provided unequivocal evidence for protection". Perhaps this is true. The fact remains that, historically speaking, the idea that a specific human disease, malaria, might have a selective role and therefore modify the genetic composition of a population by favouring certain genotypes rather than others, was suggested for the first time with reference to the relationship between thalassemia and malaria. The demonstration of the selective action of malaria came, as we know, from Anthony Allison's research on sickle cell anemia in East Africa (Allison, 1954). Nevertheless, in Italy, the first studies were carried out to determine the possible origin of the high frequency of the thalassemic gene in certain areas of the country, and if the cause could possibly be, as hypothesized J.B.S. Haldane in 1949 (Haldane 1949a; 1949b), a selective advantage of the heterozygotes due to the concomitant presence of malarial infection. The story of how the "malaria hypothesis", also known as the "Haldane hypothesis", has been pieced together by several authors (cf. Weatherall, 2004a for details). Less is known of several premises and of consequences of the hypothesis of a causal relationship between malaria and thalassemia, from the standpoint of the epidemiological observations and research carried out in Italy.

**The evolution of the knowledge of thalassemia genetics: the Italian contribution**

In 1932, when George Whipple suggested the term 'thalassemia' (Whipple e Bradford, 1932, 1936), Thomas Cooley noticed that the disease he had clinically defined showed a clear familial incidence (Cooley and Lee, 1932). Later, Heinrich Lehndorf supposed that Cooley's anemia was an inherited disease due to a genetic mutation (Lehndorf, 1936).

The Mendelian inheritance gradually became apparent. Ferdinando Micheli and collaborators (1935), Angelini (1937), and Jean Caminopetros (1937) observed distinctive, albeit minimal, haematological traits in healthy parents. Then Maxwell Wintrobe (1940) and William Dameshek (1900-1966) (1940) recognized the relationship between these haematological traits and minor forms of thalassemia. Jean Caminopetros (1937) had already assumed that clinically healthy people could transmit the disease as a Mendelian recessive factor.

The Mendelian inheritance of thalassemia was suggested for the first time by Alan Moncrieff and Lionel Ernest Whitby in 1934. Assuming the hereditary condition for thalassemia, Ignazio Gatto (1941, 1942) associated its lethality with homozygosis ("homozygote-disease"), while the heterozygote represented only minor and non pathological traits ("heterozygote-stigmata") (Gatto, 1942).

Halfway through the Forties, Italian and US physicians independently demonstrated the mechanism of inheritance. In 1943, Ezio Silvestroni and Ida Bianco, described an inborn and hereditary hematological anomaly in healthy people that they subsequently called microcythemia (1945a, 1946a). At the same time, Silvestroni and Bianco showed the genetic relationship between thalassemia and microcythemia, studying several people with Cooley's anemia (Silvestroni and Bianco, 1946b; 1946-47). At the end of these investigations, Silvestroni and Bianco documented the Mendelian inheritance of microcythemia as the heterozygotic condition, and the homozygotic condition in Cooley's disease (Silvestroni, Bianco, 1947). These results confirmed the evidence obtained in similar studies conducted in USA by Dameshek (1943), and especially by William Valentine and James Neel at the University of Rochester (Neel & Valentine, 1944; Valentine & Neel, 1945).

Silvestroni and Bianco carried out a series of epidemiological studies all over Italy (Silvestroni and Bianco, 1946-47; 1947; 1948a; 1948b; Silvestroni, Bianco, Montalenti, Siniscalco, 1950), using a specific method to detect the microcythemic trait through the reduction of globular fragility (Silvestroni and Bianco, 1945). The results of the research, which by 1950 had involved some 50,000 persons, enabled the two to map, for the first time ever, the disease's distribution for a whole country. This distribution revealed a disturbing epidemiological profile, with numerous microcythemic foci scattered all over the country, particularly in the areas of the Po Delta and the islands, Sardinia and Sicily, where the incidence of carriers exceeded 20% of the population. The map revealed a strong geographic correspondence between the frequency of the thalassemic features and endemic malaria. This singular correlation, which had already been observed by clinicians, was now documented by thorough and wide-ranging epidemiological research, thus raising even more clearly the question of maintaining the frequency of a gene that, at the time, doomed homozygotes to death within the first two years of life.

### **Early observations on the association between malaria and thalassemia**

The problem of the links between Cooley's anemia and malaria had been debated in Italian medical literature since the 1920s.

Different studies have considered the possibility that malaria was an etiological factor for thalassemia, as it was recognized that a particularly high frequency of cases of Mediterranean anemia existed in a number of malarial zones in families affected by Cooley's anemia (Auricchio, 1928, Careddu, 1929). These coincidences were confirmed by subsequent observations pointing to the presence or the frequency of the disease in malarial zones (Dolce, 1939, Frontali, 1940), or the existence of thalassemic foci in Italy (Sicily, Sardinia, Ferrara area, Puglia) corresponding to endemic malaria zones (Francaviglia, 1939, Auricchio, 1940, Pachioli, 1940). A similar situation was observed in Greece (Choremis and

Spiliopoulos, 1936). An even more significant fact was the exact localization of Cooley's anemia in intensely malarial zones such as Sardinia, in which the frequency of Cooley's disease was very high in the malarial coastal areas and practically absent in the non malarial internal mountainous zones (Careddu, 1940; 1941). Working at the pediatric clinic of Cagliari University, Cadeddu collected a large number of case histories from all over Sardinia and systematically analysed all the relevant environmental variables: "in the entire mountain area of the island" – he wrote – "in which malaria occurs sporadically there is an absence of observations except for one observation from Macomer but due to the family community moving there from a malarial zone. [...] The towns from which our cases have been drawn almost all have an altitude of less than 100 m. above sea level." (Cadeddu, 1940, p. 509)

The hypotheses regarding the relationship between malaria and thalassemia put forward by clinical practitioners who had observed the geographic links between them remained restricted to the medical domain, that is, at the level of pathogenetic explanation physicians had too little knowledge of genetics and evolutionary biology to allow those working in a clinical environment to elaborate interpretations capable of placing the focus on the links between a population's gene pool and selective pressures.

On the basis of the frequent reports of the malarial plasmodium and the alleged therapeutic effects of quinine in children diagnosed as having thalassemia (in this case it must be recalled that thalassemia was often confused above all in paediatrics with other pathologies having anemia as a symptom), Choremis and Spiliopoulos (1936) postulated that the chronic malaria of the parents triggered a hereditary predisposition of their children's homopoeietic system. This was then rendered manifest by the superimposition of specific environmental conditions, such as malnutrition. Caminopetros however immediately challenged the idea of the direct etiology on the basis of the evidence of the absolute inefficacy of quinine therapy on thalassaemic subjects, and followed the completely opposite approach of inoculation of the malaria plasmodium (Caminopetros, 1937)<sup>1</sup>.

The hypothesis of a direct etiology had to come to terms with a series of other anomalies pointed out by Gino Frontali and F. Rasi working at the pediatrics clinic of Padua University (Frontali and Rasi, 1939). In the first place the absence of thalassemia in populations with a high incidence of malaria or conversely the existence of cases of Mediterranean anemia unrelated to any malarial influence and, lastly, the inefficacy of antimalarial therapy observed by many clinicians (Vallisneri, 1940). Marino Ortolani, director of the Ferrara Provincial Institute for Childhood pointed out that the hypothesis of direct malarial etiology was based on a body of case histories collected through a systematic diagnostic error that led to a series of obvious cases of malaria being listed as Cooley's anemia and for this reason apparently sensitive to quinine therapy (Ortolani, 1941).

In 1929 Giovanni Careddu suggested an interesting indirect action by malaria on the germ cells of the parents that was capable of leading to an alteration of the hemopoietic and osteogenetic mesenchyma. Federico Vecchio, at the pediatric clinic of Naples University, in 1947 made explicit reference to germ plasma mutation, referring to Herman Muller's experiments with radiations and the recent (at the time) demonstration that thermal shock seemed capable of producing genetic mutations. It was postulated that gene variation indeed emerged as a consequence of "a direct action of malarial parasitism carried out by the repetition, especially in the pre-quinine era, of comparatively violent thermal shocks." (Vecchio, 1947, pp. 52-53) In the light of the growing evidence that thalassemia was not exclusively a Mediterranean disease, but one of the most widespread hereditary diseases in the world, Vecchio claimed that the cause should be sought in the exposure "of several ethnic groups to given environmental factors" (Vecchio, 1947, p.53), rather than in a given genotypic constitution of the populations affected.

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<sup>1</sup> Caminopetros' malariotherapy of Cooley's anemia was based on an accidentally observed fact. One of his patients who had accidentally been infected by malaria displayed a prolonged remission of the erythroblastic reaction. And given that medullary hyperactivity seemed to represent the original pathogenetic stage of Cooley's anemia, Caminopetros decided to induce malaria to slow down the hyperactivity of the bone marrow, and thus the erythroblastic reaction and erythroblastosis in the circulation.

Again in 1939 Michele Bufano actually considered the link between malaria and thalassemia a case of inheritance of acquired characters, postulating that malaria produced a series of pathological transformations of the bone marrow capable of being transmitted to the offspring and giving rise to the clinical symptoms of Cooley's anemia. On the other hand, in a long discussion in *Clinica Pediatrica*, Renato Pachioli, a lecturer at Bologna University, suggested viewing Cooley's anemia also as a hereditarily transmissible malarial hemodystrophy originating from a mutation somehow induced by malaria (Pachioli, 1940).

Pachioli claimed that this hypothesis would allow a single interpretative model to be used to explain the apparently hereditary nature of Mediterranean anemia and the singular similarity between several fundamental parts of the pathogenetic processes in malaria and in Cooley's anemia, in particular the alterations of erythropoiesis. The explanatory hypothesis was thus constructed by linking together several early speculations on the genetic determinisms of Cooley's anemia with a series of pathological data. On the one hand, there was the idea put forward by Heinrich Lehndorff in 1936 that Cooley's anemia is the effect of a genetic mutation following which the erythroblastic system becomes incapable of producing mature red corpuscles. On the other, the (incorrect) observations made above all by Virgilio Chini, seemed to show that malarial infection electively damaged the hemopoietic system and that this was somehow transmitted to the offspring, thus determining bone lesions that could be likened to the pathognomonic lesions of Cooley's anemia. This idea however allowed Pachioli to claim that the eradication of malaria would lead to a gradual reduction in the frequency of the thalassemic gene: "the causal relations, although indirect, between malaria and Cooley's anemia [...] allow it to be envisaged that the rehabilitation of malaria-infested zones can, in the course of generations, lead to the gradual exhaustion of this morbid hereditary defect" (Pachioli, 1940, p. 422).

Although in a completely nebulous and speculative explanatory framework, Cesare Cocchi put forward the hypothesis that Cooley's anemia, just like favism, represented a defensive process typical of subjects in populations that had been exposed at length to malaria. The idea was that "Cooley's anemia, with its clinical and pathological features, can manifest itself only in subjects prepared by a broad malarial inheritance in the sense that it represents a particular reaction of enhanced defence (instead of increased vulnerability). What I mean is that it is possible to imagine that the same morbid cause (toxic, infectious or due to deficiency) determines this disease, in that particular way, only in subjects that have malarial forebears, and are thus better protected (we know they are better protected against malaria itself than a subject who is not a descendant of malarial patients), better prepared to defend themselves against all hemolizing action" (Cocchi, 1941, pp. 286-287).

On the basis of Cocchi's hypothesis, in 1943 Marino Ortolani carried out research "on the immune state regarding malarial infection in subjects, some of whom present the classic Cooley type anemia symptoms and others affected by erythroleukemic myelosis with or without hyperhemolysis" (Ortolani, 1946, p. 38). Ortolani repeatedly tried to graft the "plasmodium vivax" into some patients by inoculating them with blood taken from soldiers from the Greek-Albanian front suffering from malaria. Without stating the number of cases precisely, he reported having failed to infect the subjects involved in the research. Malarial patients lacked the clinical signs of malaria, while the search for parasites in the blood was always negative, even after spleen contraction as well as the search via medullary and spleen puncture. The only exception was a 10 year old girl who, after being subjected to a cycle of 4 inoculations over four months of blood that was particularly rich in parasites drawn from four malaria patients, developed malaria two years later, displaying clinical symptoms and showing the presence of the benign tertiary plasmodium. The author also reported the case of a baby with Cooley's anemia who died about two months after being inoculated with infected blood and who tested negative for the plasmodium even on autopsy via bone marrow examination (ibid, p. 38).

In his comment on these results Ortolani explicitly cited the passage from Cocchi's work mentioned above, then claiming that his study seemed to support the hypothesis that Cooley's anemia ought to be considered as a hereditary form of defence against malaria that developed through long exposure of certain populations to the infection (ibid., p. 38). Although unsystematic and ethically dubious, Ortolani's experiment takes on an exceptional

historical value as it preceded by about ten years Allison's work demonstrating the greater resistance of sickle cell anemia patients to malarial infection. For reasons that we shall try to understand in the following, Ortolani's experiment was not replicated even in Italy. The only trace of it we found in the literature was in a 1957 article by Ezio Silvestroni and Ida Bianco on the dissemination of the thalassemic trait (Silvestroni and Bianco, 1957).

The absence of any consistent classification of the pathogenetic mechanisms of thalassemia as a function of the etiology and pathogenesis of malarial infection materially stood in the way of any emerging hypothesis that malaria could represent a selective fact capable of favouring a mutation of the erythropoietic system. Moreover there was no exact superimposition of the malarial zones on the zones with a high frequency of Cooley's anemia or the microcythemic trait. Reported exceptions were the Rome rural area and the Tuscan Maremma, which for centuries had been intensely malarial but characterized by a low incidence of Mediterranean anemia and microcythemia. In his introduction to the course of medical pathology and clinical methodology at Bari University, Virgilio Chini (1939a), one of the greatest Italian experts in Mediterranean anemia of the time, postulated that the absence of any link between the distribution of malaria and that of Cooley's anemia could be accounted for by biological difference among the types of malaria distributed through the various different geographic regions. The same year, Chini reported certain similarities in the radiological examinations of 40 of his malaria patients with those observed in Cooley's anemia (1939b)

In 1941, Franco Toscano reported a geographic correspondence between malaria and Rietti-Greppi-Micheli disease, or thalassemia intermedia, postulating a link with a single etiopathogenesis deriving from an ancestral malaria (Toscano, 1941).

Again with reference to the malarial hypothesis, two obvious theoretical anomalies were pointed out: the relatively small number of Cooley's patients also in the more intensely malarial zones and the fact that not all the offspring of chronic malarial patients were affected by Mediterranean anemia. Even though also here there was no lack of researchers, such as Paradiso (1942), who believed that the fact that Cooley's anemia was found in non malarial zones did not rule out the possibility that malaria might be part of the more or less remote ancestry of these patients.

It was also a rather common occurrence to attempt to use ethnic and anthropological factors to account for the distribution of the thalassemic trait and its link to malaria. Ezio Silvestroni, Ida Bianco and Nereo Alfieri, archaeologist and director of the Spina Museum in Ferrara, where the two researchers had carried out an intense screening activity, noticed that the regions with a high incidence of thalassemia corresponded to those colonized by the Greeks and that, like certain cities in the Ferrara area or the Po Delta, such as Adria, had had intense trading relations with Greece in Roman times (Silvestroni, Bianco and Alfieri, 1952). Although interesting, this hypothesis provided no solution to the problem of the maintenance of high gene frequency. The idea was that the selective pressure of the malaria was subsequently grafted onto the effect of the founder, however at that stage again raising the problem of the demonstration of the links between malaria and thalassemia. However, it still remained to explain the high frequency of thalassemia in Sardinia, which was inhabited by a population of different ethnic origin, and the high frequency of the trait in the Far East, which was just beginning to be recognized in the research of the time.

### **Collaboration of Silvestroni and Bianco with Montalenti: at work on Haldane's hypothesis**

In 1947 Silvestroni and Bianco made the first arrangements regarding collaboration with the Institute of Genetics of Naples University, directed by Giuseppe Montalenti<sup>2</sup>. It had been the lack of appreciation shown and the criticism made by the medical community of the genetic aspects of their work that led the two clinical pathologists to seek the support of a specialist in the field. Montalenti was the most authoritative Italian geneticist. Furthermore, he was already familiar with and had fully understood the importance of the genetic discoveries made by

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<sup>2</sup> Ida Bianco, personal communication to one (S.C.) of the authors.

Silvestroni and Bianco, namely the implications and extraordinary potential for the purpose of new research of the data collected by the two in the screening campaigns begun in the summer of 1943.

Montalenti advised them above all to investigate the causes of the persistence of the microcythemic foci identified by Silvestroni and Bianco, in spite of the selection of the thalassaemic genes at each generation due to the impossibility of the homozygotes reproducing. He also suggested studying several well-known Mendelian traits in the populations investigated by the two clinicians, such as sensitivity to phenylthiocarbamide. And also to check any possible link with the microcythemic trait (Silvestroni and Bianco, 1950).

Between the end of July and the beginning of August 1948, just a few months after the beginning of collaboration with the two Rome physicians who had described the microcythemic condition, Montalenti met John Burdon Sanderson Haldane at Pallanza, on the occasion of a Symposium on the ecological and genetic factors of animal speciation, organized by Adriano Buzzati Traverso in the biophysics section he directed at the Physiopathology Study Centre of the National Research Council. In this forum Haldane presented a report on the links between disease and evolution, which above all hinged on the role of diseases and infectious agents as factors of selection and thus of evolutionary change (Haldane, 1949). In the discussion that followed, Montalenti asked Haldane if he deemed it hypothetically possible for the disease to have played a role in maintaining the microcythemic gene. Haldane answered affirmatively, adding that the advantage of the microcythemic heterozygote could also derive from an enhanced capacity for iron absorption in populations with diets deficient in this element.

Haldane's article "Disease and Evolution" is the one most frequently cited as that in which the malaria hypothesis, also known as the Haldane hypothesis, was first formulated even though in that context it was not Haldane but Montalenti who first suggested the link between thalassemia and malaria. In actual fact, the English geneticist several months earlier had already put forward the hypothesis at the International Genetics Congress held on 7 - 14 July 1948 at Stockholm. When discussing the problem of the mutation rate in man with reference to Neel and Valentine's studies on thalassemia, namely the hypothesis advanced by the two US geneticists that, in order to maintain such a high frequency, the mutation rate would have to be 1 : 2,500 and that this rate might have an ethnic basis (Neel and Valentine, 1947), Haldane considered the rate to be too high, proposing that the erythrocytes of thalasseemics were resistant to malaria parasites and that the disease's high frequency was the result of a selective heterozygote advantage.

The Haldane hypothesis emerged as an obvious alternative once it could be ruled out that polymorphism was due to a 'special' mutation rate. It is not clear when the idea of this hypothesis came to Haldane. On the basis of the reading of the article *Disease and Evolution*, published by the Italian review *La Ricerca Scientifica*, Allison gave credit to Montalenti for suggesting the malaria hypothesis to Haldane (Allison, 2004). According to Weatherall, the hypothesis was originally conceived of at Stockholm, during the World Genetics Congress held several weeks before that of Pallanza. In fact, Weatherall's thesis is not documented, in the sense that it must in any case be demonstrated that the address published in the proceedings of the Stockholm Congress corresponds to that delivered publicly at the Congress. Some doubt as to whether Haldane got the idea before Pallanza seems to emerge from the fact that in the article *Disease and Evolution* he does not propose the example. Why? If he had already presented it at Stockholm, where also Montalenti was present, why did he not talk about it again in Italy? And was it Montalenti who had to remind him of it? The fact is that, for the molecular genetics historian and philosopher Sahotra Sarkar there is "archival evidence" that Haldane developed the hypothesis before going to Stockholm<sup>3</sup>.

Could it not be that, after his discussion with Montalenti, Haldane modified the written text of his address to the Stockholm Congress, including the hypothesis of heterozygote advantage vis-à-vis malarial infection? The question can be resolved only if documentary evidence is available concerning what Haldane actually said in his report to the Stockholm Congress.

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<sup>3</sup> Sahotra Sarkar, personal communication to one (G.C.) of the authors.

The first results produced by the collaboration between Silvestroni and Bianco and Montalenti and his Naples Genetics Institute were published on 29 April 1950 in *Nature*. In the light of the epidemiological data the question arose of the high incidence of the thalassemic gene (indicated as M in the article, as opposed to  $m$ , the symbol of the normal hemoglobin gene) despite its constant elimination with the death of the homozygotes prior to the reproductive age. The authors advanced four hypotheses: "1) the gene might have, in the heterozygous condition, a positive selective value; 2) the mutation frequency from  $m$  to M might be such as to balance the loss; 3) mating may not occur at random; 4) the fertility of some genotypes may be higher."

These actually rejected the possibility of the heterozygotes enjoying any selective advantage, as they seemed to contradict the evidence of Silvestroni and Bianco with reference to the health conditions and average age at death of microcythemics, the former being far worse and the latter much lower than the average for subjects that are not carriers of the thalassemic trait. The second possibility seemed equally unlikely in view of the excessively high frequency of mutation required to maintain the incidence observed in the thalassemic gene. And in any case, even if the hypothesis of such a high mutation frequency was ultimately accepted, it would become extremely difficult to explain why it was so high in certain geographic areas and not in others. Silvestroni and Bianco's data also clashed with the third hypothesis, that of non random mating, that is, the possible tendency of microcythemics to mate among themselves. The same evidence seemed instead to support the fourth hypothesis, that of the heterozygote genotype leading to higher fertility since the heterozygous couples had an average number of children that was appreciably higher than that of couples with no thalassemic traits and those of homozygous couples.

The results, the hypotheses and the conclusions regarding the causes of the balancing of selection of the thalassemic gene were again proposed two years later in a broader-based report published in *Eugenics* (Bianco, Montalenti, Silvestroni and Siniscalco, 1952). Numerous theoretical difficulties related to the level of knowledge available at the time stood in the way of any consideration of Haldane's hypothesis. One first problem was the large phenotypic variability of carriers of the thalassemic traits. Furthermore, nothing was known about the complex heterogeneous nature of genetic and molecular phenomena underlying thalassemic phenotypes. Thinking and debate centered around a microcythemic gene, around a single defect in a specific gene, based on the idea of an extremely simple gene. For example nothing at all was known about the existence of alpha-thalassaemia, which was instead typical of the Sardinian populations being studied. The idea of a disease that is today classified as part of a set of different hematological disorders and associated with almost 300 different mutations, could not even be remotely contemplated.

And then to what extent was gene expression influenced by other genes of the environment? In 1941 Haldane demonstrated that it was possible to determine the origin of the variable expression of a gene by measuring the intensity of character correlation between parents and offspring or among siblings. In Haldane's view multiple allelism occurred when a correlation coefficient close to one was observed. If instead the intermediate value approached 0.5, the likelihood of gene modifiers became quite possible. With lower correlation coefficients the phenotypic variability must have depended on non genetic factors. The available information in this connection was, at the time, largely inadequate precisely because of the difficulty of defining and measuring the phenotypic traits to be linked together. In the case of globular resistance, the only trait that could be measured with sufficient precision, a correlation of about 0.30 emerged, at the limit of a significant scale of relations. The investigations thus seemed to point to the action of modifier genes in the variability of phenotypic expression, as anticipated by Silvestroni in 1949 (1949b).

In 1953, at Bellagio, on the occasion of the IX International Genetics Congress, when presenting a long report on microcythemia genetics based on Silvestroni and Bianco's research, Montalenti continued to express doubts regarding the idea of a possible heterozygote advantage. The first reason was the partial superimposability in Italy of the epidemiological map of microcythemia on that of malaria. This is a topic that, as we have already seen, had been deemed to be central by clinicians in order to refute the hypothesis of

possible links between malaria and Cooley's anemia. Another argument against the hypothesis was the total absence of any causal explanation of the way in which the microcythemic gene could increase resistance to malarial infection. Understanding of the possible heterozygote advantage in this sense could be provided at the level of macroscopic functions. From this point of view it was practically impossible to identify any characteristic traits of the heterozygote such as would lead to a selective advantage. Furthermore, this opinion was shared at the time by Neel who wrote that: "in our own experience (Valentine and Neel, 1948) individuals with thalassemia minor have averaged two grams of hemoglobin less than normal persons. While there is undoubtedly a large margin of safety in normal hematological physiology, it is difficult to see how such a departure from the norm can per se be of adaptive value to the organism. The possibility remains that the hematological trait is linked to some yet unrecognised characteristic of distinct value" (Neel, 1951)

Above and beyond the theoretical aspects of the problem, the group coordinated by Montalenti addressed the issue of microcythemic gene maintenance by seeking a possible alternative survival of microcythemics versus non carriers. A preliminary analysis was carried out at the end of 1952 on the data collected during the 1951 and 1952 campaigns in the Ferrara area, with a total of about 9,000 subjects being tested. As Montalenti himself (1953) acknowledged, there were gaps in the investigation. The older age group seemed under-represented, and there were few data on subjects under the age of 30. Furthermore, data were collected on married couples with both spouses living, thus making it possible that any selective advantage of one of the two genotypes would be overshadowed. Although controversial, the data nevertheless seem to indicate a greater fitness of carriers of the microcythemic gene compared with normal subjects and thus suggested the need to develop research in that direction.

In 1953 a more thorough examination of couples with genotypes  $mmxmm$ ,  $mmxMm$ ,  $MmxMm$ , carried out on about 2800 families, did not show up any significant difference in fertility. This seemed to refute the initial hypothesis that the maintenance of the microcythemic gene was indeed due to a greater fertility of couples carrying the thalassemic trait. With the data observed in the same families studied between 1951 and 1952, a complex analysis of the fitness of the different genotypes was also attempted. The idea was to be able to calculate the theoretical fitness required to maintain in equilibrium a given gene frequency in a population of which the gene elimination rate was known. Also in this case, the computations, in any case incorrect in a preliminary work published in *Nature* in 1954 (Silvestroni et al., 1954), produced controversial results which made it impossible to adequately demonstrate the advantage of heterozygosis, but nevertheless suggested pursuing research in that direction.

A statistical survey carried out to study microcythemia distribution as a function of age seemed indirectly to lean in favour of an advantage of the heterozygote versus malarial infection, as the thalassemic trait was more frequent on average among individuals over the age of 40. It must be pointed out that, in 1946-47, a five-year plan had been launched in Italy to eradicate malaria by means of DDT spraying, which had practically eliminated *P. falciparum* starting from 1951. As a result the selective of malaria to maintain the polymorphism of the microcythemic trait disappeared and the populations were tending towards a different equilibrium (Research project, AM B125).

### **Research of Carcassi, Cappellini and Pitzus**

During the XX Symposium held at Cold Spring Harbor on the topic "Population Genetics: The Nature and Causes of Genetic Variability in Population", Anthony C. Allison summarized the data on polymorphism in man on the basis of his discovery, reported the previous year, that polymorphism balanced by a sickle-cell trait is due to a selective advantage of the heterozygote versus the serious malaria form (*P. falciparum*). The Italian geneticist Ruggero Ceppellini took part in the discussion, summarizing the genetic studies on thalassemia carried out in Italy and reporting for the first time the results of research carried out in 1954 in Sardinia (Ceppellini, 1955). The experiment carried out by Carcassi, Ceppellini and Pitzus was extremely elegant and was based on the studies that had allowed Allison to demonstrate



the role of *P. falciparum* as a factor responsible for the high frequency of the sickle cell trait in the zones he had studied in East Africa (Allison, 2004). In practice, the Italian researchers made comparisons among the inhabitants of four villages in the province of Nuoro who had been relatively isolated from the reproductive point of view. Two of these villages, Orosei and Saltelli, lay at the bottom of a valley that had been intensely malarial until 1947, with one of the highest rates of *P. falciparum* infection in Europe. In these villages, the percentage of microcythemics was around 20% (18.8 and 21.3, respectively). The other two villages, Desulo and Tonara, were situated quite close as the crow flies (about 50 km), although at an altitude of 1000 metres and had had only rare cases of malaria. The frequency of microcythemics among the inhabitants of these two villages was about 5% (3.75% and 4.67%, respectively.)

It is significant that these researchers were the first to grasp that the observation made as early as 1940 concerning the coincidence of the absence of malaria and thalassemia at higher altitudes, if suitable genetically isolated communities were selected, represented a useful context in which it was possible to rule out environmental factors other than malaria. Having eliminated on the basis of a thorough study of the frequency of blood groups the hypothesis of ethnic heterogeneity as a possible explanation of the different frequency of the microcythemic trait, Cercassi, Ceppellini and Pitzus claimed that the only hypothesis displaying favourable empirical elements is that malarial infection exerted a selective pressure to the advantage of microcythemics (heterozygotes). "The fundamental similarity – said Ceppellini in 1955 – of dietary, social and economic conditions points toward malaria as the most important environmental difference between the two groups. Naturally the first data are only of limited value and more careful and extensive investigations must be carried out before malaria can be accepted as the environmental agent responsible for the high value of the mutant gene established in the two valley villages" (Ceppellini, 1955, p, 253).

The results of their research were presented in full in a 1957 publication. The authors claimed that the gene frequencies of the thalassemic allele in the plainland villages could be maintained at the high values observed "only if the heterozygote, in the given environment represented by the two countries in question, is advantaged with respect to the normal subject by a selective coefficient of around 12.5%" (Cercassi et al., 1957, p. 211). They emphasized that Plasmodium infection was "the element of an environmental nature that more conspicuously diversified the two zones" (plain land and mountain), and was "instead uniform for couples in villages inside the zones" (ib. p. 212). In a more expert fashion they dwelled upon excluding the hypothesis of an ethnic heterogeneity through an analysis of blood group frequency, which indeed they succeeded in demonstrating.

The research carried out by Cercassi, Ceppellini and Pitzus defined the experimental framework within which the Montalenti group was to work during the late 'fifties, also thanks to funding from the Rockefeller Foundation.

### **Studies on microcythemia genetics in Italy funded by the Rockefeller Foundation**

In 1954 Giuseppe Montalenti requested funding for a multiyear research programme<sup>4</sup>, stressing the privileged condition in which Italy now found itself in the field of thalassemia research owing to the peculiar geographic distribution of this disorder and the quantity of data already collected, as well as the acquisitions of Silvestroni and Bianco (Montalenti, Preliminary draft for a research plan on the genetics of mycrocythemia, AM, B125).

For Montalenti, the first problem to solve was "the cause of the high frequency of the gene in some regions; fluctuations of frequency in time should if possible be ascertained. Cause of the maintenance of high frequency in spite of continuous elimination of the gene (lethal in homozygous condition) should be investigated. A selective advantage to peculiar environmental conditions (malaria? Food quality and/or quantity?) or in regard to other hereditary traits (especially blood groups and anthropological traits) should be investigated as fully as possible. This was the necessary premise for any action towards the control of an hereditary disease due to a single gene such as Cooley's disease (Thalassemia major).

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<sup>4</sup> Already in 1951 Montalenti had obtained a grant from the Rockefeller Foundation to study microcythemia genetics.

(Montalenti, Preliminary draft for a research plan on the genetics of microcythemia, AM, B125)

The importance of this research and of the results did not escape the notice of the Rockefeller Foundation board. Via R.R. Struthers, director of the European bureau in Paris, they actually suggested to Montalenti that he should increase the research funds available to Silvestroni and Bianco and to fund their studies directly, and no longer through Naples University.<sup>i</sup> Furthermore, Struthers suggested boosting the development of “the eugenic aspect of the microcythemic problem, the establishment of official registers of persons carrying this gene, marriage counseling in some form”.<sup>ii</sup> This was precisely what also Silvestroni and Bianco were most interested in, and had already started doing.

In May 1954, Montalenti succeeded in obtaining five-year funding for a total of 15 million lire (the equivalent of about €195,500 in July 2004) to be applied largely to microcythemia studies.<sup>iii</sup> Contrary to Struthers' suggestions, Montalenti decided to use the grant almost exclusively to fund basic research, ignoring the clinical and eugenic aspects.<sup>iv</sup> Moreover, he managed the five-year fund directly, thus ruling out any possibility of setting up a direct funding channel between the Rockefeller Foundation and the Silvestroni-Bianco group.

The research actually got under way in 1956, the “essential and conditional” point was that “the research funded by the Rockefeller Foundation was supposed to provide the scientific basis for a possible check of the frequency of the ‘M’ gene in a limited population. Therefore the entire investigation must be aimed at extending knowledge of microcythemia genetics, of variations in gene frequency in the populations and on the possible influence of the environment on gene manifestation.” (Montalenti, Programma di ricerche sulla Microcythemia 1956-1959, AM B125)

The preliminary plan was subdivided into a series of preparatory investigations and into the study of the possible selective advantage of heterozygotes. The former would determine the demographic characteristics of the population in one or more “control” centres, measure the essential anthropological data, study population movement, estimate the coefficient of consanguinity, determine the degree of completeness of the data collected on the distribution of the ‘M’ gene, determine the number of Cooley’s anemia patients in the population and examine its agreement with the theoretical premises and lastly perfect diagnosis by means of biochemical examination.

On the basis of Montalenti’s general instructions, Silvestroni and Bianco drew up a preliminary research programme involving a series of preparatory investigations of human hemoglobins for the purpose of a more accurate biochemical, as well as hematological, identification of true microcythemics as opposed to possible carriers of other abnormal hemoglobins, and to be able to distinguish heterozygous from homozygous microcythemics. (Research programme on microcythemia and collaboration plan for the first year of Rockefeller funding, AM, B125).

The programme also entailed a summer research campaign aimed at making as complete an examination as possible of the entire population of a new village in the Ferrara area having a high percentage of microcythemics. The data thus obtained would shed new light on the problem of random mating and on computing the coefficient of consanguinity. The campaign thus involved an anamnestic investigation of the families and consultation of the municipal records in order obtain fresh data for the study of fertility in marriages, death and birth rates at the various ages, the differential morbidity between normal subjects and microcythemics, the causes of death and to determine the real number of deaths due to Cooley’s anemia in the population examined (Research programme on microcythemia and plan of collaboration for the first year of Rockefeller funding, AM, B125).

Lastly, Silvestroni and Bianco developed the blood smear technique for studying the intensity of the morphological manifestations of the microcythemic gene, to be carried out in accordance with the following three directives:

- a) study of the intensity of the hereditary line characters (between parents and offspring; in 3 or more successive generations; among siblings);

- b) study of the intensity of the characters as a function of age and gender;
- c) study of the intensity of the characters as a function of economic and environmental conditions.

In the first six months of 1956 Silvestroni and Bianco carried out the preparatory investigations of human hemoglobins. During the summer of the same year they then carried out a research campaign at Berra, a municipality in the province of Ferrara with a population of 4,150 (Silvestroni and Bianco, 1957). Silvestroni and Bianco examined the whole population, at the same time carrying out a study of the municipal records, a series of blood tests on samples taken from microcythemic subjects, the anthropological examination of the test subjects, investigation of the links between the hematological and anthropological characters between parents and offspring and among siblings. Owing to their completeness and the extension of the research to a circumscribed population, the data obtained represent study material of exception genetic-statistic and hematological interest, above when it is considered that the two researchers had already 'typed' the population of other municipalities in the province including Pomposa, Codigoro, Caprile and other towns for a total of about 50,000 subjects examined.

The issue of determining the existence of a possible heterozygote advantage becomes central in the programme of investigations funded by the Rockefeller Foundation. Among the documents conserved in the Montalenti records in the envelope of the so-called "Microcythemia" fund, the only research project drafted separately and not included in the more general research programmes conserved in the Microcythemia fund refers precisely to questions concerning Haldane's malaria hypothesis (Research project, AM B125).

The research project specifically concerning Haldane's hypothesis was divided into three separate investigations:

- 1) ascertainment of heterozygote fitness versus the fitness of normal homozygotes;
- 2) the relationship between microcythemic genotype and death due to malaria;
- 3) verification whether death due to malaria was sufficient to guarantee the observed frequencies of microcythemia.

These were complex investigations involving different dimensions of the phenomenon and that require sifting through variables that were hard to quantify. In order to determine the fitness of heterozygotes versus normal homozygotes, the research project for example required the construction of mortality rate tables for homozygotes and heterozygotes and thus a comparison of the differential survival of the two genotypes. This made it necessary to determine the fate of individuals 'typed' during the various screening campaigns carried out by Silvestroni and Bianco and ultimately discover the cause of death or, if they were married with children and thus if the children were carriers or not. This was an extremely complex task considering the strong migratory flows from the towns that had been subjected to "hematological census" and the difficulty encountered by the municipalities in recording, reconstructing and reporting the histories and movements of their citizens. Since the total set of these data referring to at least 10,000 individuals over a period of five years was available, it was theoretically possible to ascertain any advantage or disadvantage of the heterozygote also for causes other than malaria.

An even more complex task was the determination between microcythemic genotype and death due to malaria. In this case the research project involved the complete registration – from personal data to the genotype – of all typed individuals above the age of 50. Furthermore, for each of these individuals, the registry records were searched for the presence of siblings who had died of malaria, if possible going back as far as the prequinine area. This task proved particularly difficult and often impossible owing to wartime damage to the municipal registers (Research project, AM B125).

Similar difficulties accompanied the verification of whether death due to malaria was sufficient to guarantee the observed frequencies of microcythemia. In this case, the research project entailed the collection of the number of deaths due to malaria and of those due to all other causes, going back in time even as far as the nineteenth century and thus to the prequinine era. The data collected in this way were then classified in a double entry table, by year of

death and by age. The classification by age had to be done painstakingly and, up to five years of age, with a class for each year. The aim of this investigation was to relate the frequency of microcythemia in the older age groups to the progress of and death rate due to malaria before the introduction and spread of quinine. Indeed, theoretically, microcythemic frequency should be a function of the death rate due to malaria before the advent of quinine and at the same time the death rate trend due to malaria should be related to the effect of age on the frequency of microcythemia in the present population (Research Project, AM B125).

In spite of several inevitable gaps due to the complexity of the research, the processing of the data obtained through the complete observation of several Ferrara centers produced results that supported the hypothesis of malaria as the environmental cause of the high frequency of the gene that was lethal in the homozygous state.

### **The results of Siniscalco's genetic studies on the distribution of thalassemia, of G-6-PD deficit and of malaria**

The experience gained in the Ferrara area was of fundamental importance in the development of subsequent investigations. However, the experience of Cercassi, Ceppellini and Pitzus had shown that the natural laboratory for studying the problem was Sardinia. A population having a peculiar genetic inheritance, the result of geographic isolation, with peculiar cultural characteristics and settlement habits, such as the existence of small villages isolated for geographic and cultural reasons with populations having a high degree of kinship.

By the end of 1956 Marcello Siniscalco was planning and implementing research aimed at studying the interactions at genetic and population levels between thalassemia, favism (G-6-PD deficit) and malaria. Carrying out a data collection campaign in 19 Sardinian villages (Siniscalco et al., 1961) and later in 52 (Siniscalco et al., 1966), he showed that in the various geographic districts the high incidence of thalassemia and favism correlated positively with previous malarial morbidity, and negatively with the altitude at which the villages were situated. This morbidity was recognized on the basis of epidemiological investigations carried out in the 'thirties when malaria was raging on the island of Sardinia. For example, versus frequencies of 3-4% of these hereditary conditions observed in the mountain villages of Gennargentu such as Fonni, Desulo, Tonara and Lanusei, frequencies as high as 15-20% were found in Baronia and over 30% in Campidano (Siniscalco et al. 1961; Siniscalco et al., 1966).

The analysis and interpretation of the data collected by Siniscalco were criticized by the anthropologist Peter Brown. In particular, Brown (1981) challenged the assumption underlying Siniscalco's study, namely the negative correlation between thalassemia, G-6-PD deficit and village altitude, assuming that altitude was sufficient as a substitute measure of the morbidity due to malaria. Working on data obtained directly from the investigations of the hygienist Claudio Fermi between 1933 and 1937, Brown showed that morbidity rates cannot be considered a direct function of altitude and that on the basis of the correlations with actual morbidity no statistically significant relations emerged between the distribution of thalassemia, G-6-PD deficit and malaria in Sardinia.

Brown correctly demonstrated that it is not the altitude directly, but the ecology of the vector, which can account for the correlations with altitude in so far as the temperature and the presence of stagnant water affect the spread and behaviour of *Anopheles labranchiae*. On the grounds of the archaeology and ancient history of Sardinia, Brown postulated that the mutations responsible for thalassemia and favism were introduced during the Carthaginian conquest in the fifth century B.C. and the deforestation of large areas of the island to grow wheat created a favourable habitat for the vector of the malaria parasite. This in turn was responsible for maintaining a high gene frequency. In essence, Brown's thesis is that malaria is not the only explanation for the distribution of thalassemia and favism, but that there was an external gene flow on which the natural selection due to malaria was grafted.

## **The malaria hypothesis and the consequences of eradicating the plasmodia and of thalassemia prevention**

As, according to Haldane's hypothesis, malaria was the selective factor underlying the increased frequency in a population of the thalassemic or microcythemic trait, it was obviously wondered what the consequences might be on the spread of the genetic variant of the eradication of malaria from Italian territory. "If the malaria hypothesis as a selective ecological factor in favour of thalassemia is correct, now that malaria has been completely eradicated as a cause of death in Sardinia, it would be expected that the gene frequency would decrease rapidly. Starting from a frequency of 7% in the present population the frequency should be halved in the course of just seven or eight generations". This is what Latte wrote about the situation in Sardinia in 1968. He added that the elimination of the gene would be speeded up considerably if thalassemic couples, conscious of the risk of giving birth to offspring affected by Cooley's anemia, voluntarily abstained from having children. However, it was not sufficient, as Latte pointed out, not to have mating between thalasseemics to avoid the birth of children suffering from Cooley's anemia. "This is of course a eugenic measure for the individual and for the individual family, but not for the species, as in this case the frequency of the gene would remain unaltered in the course of the generations, or else might even increase if the biological fitness of heterozygous carriers were still high". The "biological fitness of the heterozygote genotype" should have been estimated under the existing conditions. Ultimately, a "perhaps less repressive eugenic measure could be to favour the emigration of individual affected family nuclei from microcythemic areas to non microcythemic areas, and vice versa the immigration of normal family nuclei to thalassemic areas". These movements were possible inside Sardinia itself where zones "with a high frequency of thalassemia and villages with very low frequencies" had been clearly identified. (Latte, 1968, pp. 410-11)

The problem of increased microcythemia frequency due to preventive action was instead discussed by Ida Bianco's group. It had been postulated, on the basis of certain computations to estimate the impact of a malarial focus on microcythemic gene frequencies such as to produce particularly high frequencies even in a comparatively short time (25-30 generations), that the flooding of the Pomposa area after the Po river broke its banks in 1150 and caused the rapid formation of many stagnant water pools might had led to selective pressure to increase the frequency of the microcythemic gene, already present through importation or the result of autochthonous mutation. The very person who had put forward this hypothesis, Robin Bannerman (1961), observed that microcythemia in the world was subject to recurrent fluctuations of environmental factors that, over a period of several centuries, could lead to the appearance or regression of microcythemic foci. In this sense, while "the recent land reclamation and the consequent disappearance of endemic malaria must already have broken the preceding gene equilibrium in the populations, and also produced a trend towards the reduction in the gene frequencies of microcythemia; consequently, the introduction of pre-marriage prophylaxis in a system like this that is no longer in equilibrium but displays a descending trend, will fail to produce new increases but at best will halt the decrease in gene frequencies at the present levels." (Bianco, Graziani and Marini, 1976, p. 529).

## **Conclusions**

After 1949, the year in which J.B.S. Haldane put forward the hypothesis that the distribution of thalassemia in Italy was the result of a thalassemic heterozygote advantage, the Italian geneticists Giuseppe Montalenti, Ruggero Cappellini and Marcello Siniscalco carried out a series of studies to verify this hypothesis. The first studies were carried out in collaboration with the clinicians Ezio Silvestroni and Ida Bianco who had been the first to define the genetic bases and the distribution of thalassemia in Italia, while subsequent, more targeted, research was carried out in Sardinia.

The results could not be considered as definitive proof that the thalassemic trait provides protection from malaria above all because of the epidemiologically and genetically complex signs displayed by thalassemia. Nevertheless, by means of this research a huge quantity of data was collected and analysed, and the cognitive and politico-cultural foundations laid for an anti- thalassemia campaign to be launched (Canali and Corbellini, 2003).

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<sup>i</sup> Communication of 22 January 1954 from the director of the European Office, R.R. Struthers, to Montalenti. B125, Fondo Montalenti, Bib. Storia della Medicina, Università degli Studi di Roma "La Sapienza".

<sup>ii</sup> Communication of 22 January 1954 from the director of the European Office, R.R. Struthers, to Montalenti. B125, Fondo Montalenti, Bib. Storia della Medicina, Università degli Studi di Roma "La Sapienza".

<sup>iii</sup> Communication of 24 May 1954 from the secretary of the Rockefeller Foundation, Flora M. Rhind to Chancellor and director of the Genetics Institute of Naples University. B125, Archivio Montalenti, Bib. Storia della Medicina, Università degli Studi di Roma "La Sapienza".

<sup>iv</sup> In this connection see research programmes and annual reports on the work performed in B125, Fondo Montalenti, Bib. Storia della Medicina, Università degli Studi di Roma "La Sapienza".