Radiazioni - Ricerca e Applicazioni

Vol XIX n. 3 – Dicembre 2016

Pubblicazione periodica quadrimestrale

Editrice: *Società Italiana per le Ricerche sulle Radiazioni*
Registrazione del Tribunale di Roma n. 406 del 6 Agosto 1998

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SOMMARIO

EPIGENETICS, EVOLUTION AND IONISING RADIATION

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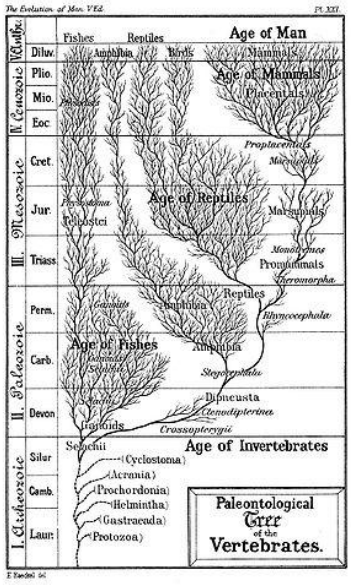
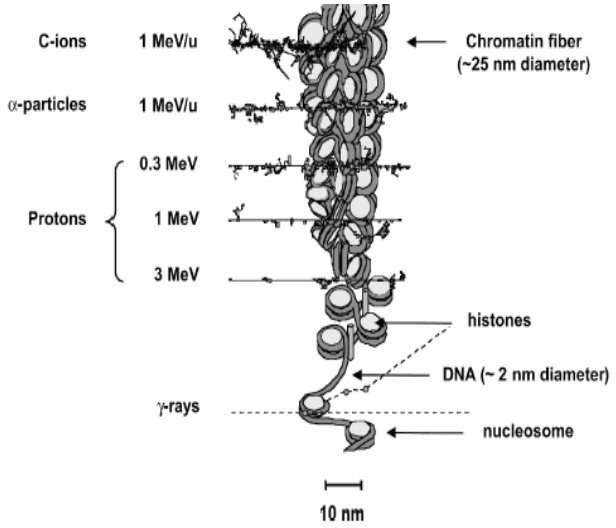


Paleontological Tree of Vertebrates (c. 1879) by Ernst Haeckel (Transferred from en.wikipedia to Commons by User:Legoktm using CommonsHelper. Public Domain,https://commons.wikimedia.org/w/index.php?curid=4246342)



Schematic representation of tracks of sparsely and densely ionizing radiations compared with relevant biological targets (from M. Belli, O. Sapora and M.A. Tabocchini, *Molecular Targets in Cellular Response to Ionizing Radiation and Implications in Space Radiation Protection*, J. Radiat. Res., **43**: Suppl., S13-S19, 2002)

Foreword

Scientific research is becoming more and more specialized, because of the need of getting deeper and deeper our knowledge of the world. Development of powerful research tools, supported by terrific technological advances, also leads to development of very specialised subjects. But, as every coins has two sides, an excessive specialization could make us overlook the general goal of our research.

Our scientific Association, SIRR, has the merit of pursuing, as its main mission, a multidisciplinary approach to the radiation research area, thus aiming at a more general description of the relevant phenomena with the contribution of various disciplines that otherwise, taken alone, are necessarily limited to their specific points of views.

It is worth to note that when a biological phenomenon is considered it should be viewed in an evolutionary perspective, since evolution is the central organizing aspect of biology. Therefore, for a multidisciplinary approach of biological effects of radiation, it is desirable that also the scientists working in disciplines other than biology realize the importance of this aspect. This is why this paper, while focussed on epigenetic effects of ionising radiation, gives special consideration to the relationship of epigenetics and radiation with biological evolution. Interestingly, also scientific research itself can be viewed as an evolutionary process, similarly to other human cultural activities. For practical reasons this paper is split in two parts: Part A (Epigenetics and Evolution) and Part B (Epigenetics and Ionising Radiation). Part A is intended to give the necessary background in order to put in the proper perspective the epigenetic effects of ionising radiation considered in part B, rather than to provide a comprehensive review of the influence of epigenetics in evolution understanding.

Part A: EPIGENETICS AND EVOLUTION

Introduction

By the end of the last century, it was known that DNA by itself does not determine all characteristics of an organism, including the human one. The role emerged of those characteristics that crucially determine which genes are expressed by which cell type, and when. In a word, epigenetics was considered besides genetics. The term "epigenetics" was coined in 1942[1] but its contemporary usage is quite recent, and for some years it has been used with variable meanings [1]. The Greek prefix "epi*"* can signify upon, above, near to, in addition. Whatever the prefix meaning is, (upon the genome, above the genome, or in addition to the genome) epigenetics in its current usage "collectively describes changes in the regulation of gene expression that can be passed on to a cell's progeny but are not due to changes to the nucleotide sequence of the gene" [2].

Even if the use of this term to describe processes that are heritable has been controversial, a consensus definition was formulated at a Cold Spring Harbor meeting in 2008 where the epigenetic trait was intended as "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence".

Epigenetic changes are meiotically heritable and mitotically stable alterations in gene expression. Epigenetic research can help explain how cells carrying identical DNA differentiate into different cell types, and how they maintain differentiated cellular states. Growing evidence now shows that maintenance of such cellular memory depends on epigenetic marks [3,4].

Epigenetics is thus considered a bridge between genotype and phenotype. Indeed, epigenetic factors influence life span and major problems of health in several species [5].

In recent years many potential examples of epigenetic inheritance have been documented and the potential implications for human development and health were quickly realized. There is growing evidence that acquired epigenetic alterations participate with genetic ones to cause altered patterns of gene expression that are key features of cancer [6].. Human epigenetic disease, and modes of treating them is receiving increasing attention (see the recent review in [7]).

Epigenetics is one of the fastest-growing areas of biological sciences. It has a great potential impact on our understanding of life evolution on Earth and has emerged as one of the most important aspects in biological studies of development and disease, linking environmental exposures during the course of life to long-term health. Quite recently, a special interest has been focussed on a specific type of exposures: exposures to ionising radiation.

Epigenetic mechanisms

In recent years there have been important advances in the understanding of epigenetic mechanisms able to regulate gene activity and expression during development and differentiation, or in response to environmental stimuli. These mechanisms include:

- DNA methylation, i.e., the addition of methyl groups to the DNA, mostly at CpG sites. DNA methylation was the first identified epigenetic alteration and is also the most widely studied. It is now a consolidated notion that genomic DNA methylation patterns are not randomly distributed and that its level is linked to transcriptional silencing (hypermethylation) or chromosomal instability (hypomethylation). DNA methylation also plays a crucial role in the development of nearly all types of cancer. [8]. Although DNA methylation in the mammalian genome was discovered in 1948

[1] The term epigenetics was originally coined by Conrad Hal Waddington in 1942 to describe the bridge between genotype and phenotype during development.

(9), it was much later that several studies demonstrated that DNA methylation is involved in gene regulation and cell differentiation (see e.g., [10]). DNA methylation is considered a heritable epigenetic mark since methylation modifications that regulate gene expression are usually heritable through mitotic cell division. For example, in vertebrates DNA methylation can be passed down from a cell to its daughter cells (11). DNA methylation can also persist from the germ line of one of the parents into the zygote (genetic imprinting).

- histone modifications, including, inter alia, acetylation, methylation, and phosphorylation. They can affect DNA-related processes, such as replication, transcription, repair by affecting chromatin organization. For example histone acetylation, the first epigenetic modification shown to be connected with biological activity (12), removes positive charges and reduces histone interaction with the negatively charged DNA, thereby opening the chromatin structure and allowing its transcription

- modulation of non-coding RNAs, such as microRNAs (miRNA). These are small RNA molecules (usually 21-23 nulceotides) that play a role in regulating gene expression (13) by transiently inhibiting translation of a messenger RNA molecule or by inducing its degradation. Also long non-coding (lnc) RNA molecules may have a epigenetic role (14), since they bind to the transcripts in the nucleus as they emerge from the DNA.

The first two mechanisms are involved in the so-called "chromatin remodelling", that is an important way how genes are regulated. It should be noted that the first mechanism acts at a transcriptional level, while the second one involves post-translational modifications. The third mechanism is instead a post-transcriptional regulation of gene expression. It is worth to note that these different mechanisms involve different cell compartments.

Emerging evidence indicates that these different mechanisms can be interrelated. For example, DNA methylation pattern can be determined by histone modifications such as acetylation and methylation (15).

The Epigenome

Epigenetic marks can be acquired throughout life and are potentially reversible or can be transmitted to the offspring (see later). The "epigenome" is defined as the set of the total epigenetics characteristics added to an organism's genome. It reflects the overall epigenetic state, and refers to global analyses of epigenetics markers across the entire genome.

Epigenomic information can be seen as the epigenetic interpretation of the genome, representing a sort of "second code" that handles the DNA-based information in different biological contexts. It can provide new insights into many diseases, and the discovery of new means to control them. It is also essential to understand the extent to which the epigenome has shaped human populations over generations and in response to the environment.

Research carried out in the last decades has pointed out some epigenetic changes characteristic of tumours. For example in tumour cells disruption of normal DNA methylation patterns is found (see [15] for a review). The term "epigenome of cancer" is currently used to describe such epigenetic features (16)

Defining epigenome maps in different normal and disease tissues and cell types is the objective of some large projects such as the International Human Epigenomic Consortium, IHEC (17) and the NIH Roadmap Epigenomics Project (18).

Epigenetics and phenotype[2]

It has been demonstrated that in plants (*A. thaliana*) transgenerational epigenetic variation in DNA methylation may provide a mechanism for phenotypic diversity in the absence of genetic mutations (19)

Direct evidence in humans that the same genotype can show different phenotypes having specific epigenomes comes from studies where a number of monozygotic twin pairs were compared. Although these twins shared a common genotype, several types of phenotypic discordance were observed, such as differences in susceptibilities to disease and a wide range of anthropomorphic features. These differences tended to increase with the twins' age (20). Also, the notion was supported that microenvironment at early stages of embryonic development can be quite important for the establishment of different epigenetic marks found in dizygotic twins (21).

All these findings indicate the importance of epigenetics in our understanding of how different phenotypes can be originated from the same genotype. Studies carried out in plants (A. thaliana) showed that transgenerational epigenetic variation in DNA methylation may provide a mechanism for phenotypic diversity in the absence of genetic mutations (19)

The interrelationships between nutrition and epigenetic phenomena, such as DNA methylation, histone modifications and chromatin remodelling are well documented [22, 23]. Nutrients are not simply food ingredients: they can directly affect physiologic and pathologic processes by various mechanisms, including those modulating gene expression through epigenetics effects.

An even more intriguing aspect is that of epigenetic effects driven by good mind-body practices and by good environmental hygiene. These practises, exploiting the so-called "relaxation response", have been used for millennia to prevent and treat diseases. Evidence has been provided that the relaxation response elicits specific gene expression changes that may relate to long term physiological effects (24)

Epigenetic inheritance and possible mechanisms

It is now widely accepted that epigenetic modifications may take place during embryonic development, with changes depending on its developmental stage (25) and that this is potentially important for changes later in life. (23,26)

Animal studies have shed light on possible mechanisms for these observations in humans. For example, studies on mice revealed that epigenetic marks on sperm DNA seem the likeliest carrier of paternal information, even if some effects of paternal stress on offspring can be mimicked by injecting embryos with RNAs found in sperm (27, 28).

Another study carried out in mice showed that an environmental stress that resulted in aggressive behavior in males caused the same behavior in their offspring which revealed some changes in the DNA methylation patterns of particular genes (29).

The mounting evidence that epigenetic alterations (also called "epimutations") can arise at high frequency, in response to environmental challenges or 'genomic shocks' or even by experience may suggest that what we eat, the air we breathe, or even the emotions we feel, may influence not only our gene expression but also that of our descendents.

Epigenetic inheritance is realized through some mechanisms still under study. Perhaps the most frequently considered, though some others have been described (30), is the "self-sustaining feedback loops", where the mRNA or protein product of a gene can stimulate its own transcription without any need of DNA changes. However, this mechanism alone has been considered

[2] "Phenotype" is an organism's actual observed properties, such as morphology, development, or behaviour, while "genotype" is the part (DNA sequence) of the genetic makeup of a cell, and therefore of an organism or individual, which determines, besides epigenetic and environmental factors, a specific characteristic (phenotype) of that cell/organism/individual (adapted from Wikipedia).

insufficient to allow transmission of epigenetic states throughout the length of development and in the germline of complex organisms (31)

These epigenetic mechanisms may be inherited from cell to cell, regulate gene expression patterns to govern the cell development. While all the 250 or more different cell types in the human body contain essentially the same genome, and arise from the progeny of a single fertilized egg, their epigenetic profiles (called "epigenomes"- see later) appear very different.

Stability of epigenetic changes

A central question is whether the epigenetic changes are stable in the successive generations or they are eventually lost when the stimulus is removed. There is clear evidence in plants that many epigenetic variants can be stably inherited over numerous generations even in the absence of selection (32, 33).

Indeed, there are many examples of transmission of epigenetic changes not only in plants, but also in animals and mammalians, including humans, having however some important differences (see for a review [7]).

Many studies on humans concerning parental (maternal or paternal) epigenetic effects, where changes are transmitted from mothers or fathers to their offspring, revealed inter-generational effects (from F0 to F1 generation), but they do not necessarily imply an impact to the germline (from F0 to F1, F2 and successive generations), i.e. evidence of transgenerational inheritance[3]. At present a lively debate exists about the identification of heritable epigenetic changes as true transgenerational inheritance especially in humans [7]. Even if incontrovertible evidence for true transgenerational epigenetic inheritance of acquired marks remains scant in vertebrates. (34), epigenetic effects that can be transmitted from one generation to another (even if not transmitted through the germline) are anyway considered of potential great interest in medical sciences (7).

From studies at molecular level it is known that epigenetic changes are not typically as stable as changes in DNA sequence, but this is not a general rule. For example, some stretches of DNA do remain unmethylated for many generations, but other sequences revert to their "wild type" methylation state. DNA methylation is typically removed during zygote formation and re-established through successive cell divisions during development.

A fundamental role in reverting the epigenetic state is played by the germ cell reprogramming. It refers to erasure and remodelling of epigenetic marks during mammalian development. The biological purposes of this phenomenon likely include the erasure and re-establishment of parental genomic imprints in germ cells (35).

Reprogramming is required to remove epigenetic signatures acquired during development, or imposed by the environment, so the embryo development properly reflects the genetic characteristic of each species. If germline reprogramming fails, epigenetic marks can be retained, and could be transmitted from one generation to the next. Difference in reprogramming between different species can give difference in epigenetic heritability.

The circumstance that epigenetic inheritance is relatively common in plants likely reflects their limited reprogramming of epigenetic changes in the germline, so that they can be propagated for many generations.

[3] The prevailing definition of transgenerational epigenetic inheritance is: epigenetic information mediated through germline that contributes to variation in phenotype. [7].

For example, it was found that the characteristics of a mutant of Linaria vulgaris[4] is related to a particular gene that is extensively methylated and transcriptionally silent. This epigenetic modification is heritable and co-segregates with the mutant phenotype [36].

Another example is that of *Arabidopsis thaliana* where multiple DNA methylation changes induced across the genome can be stably inherited over at least eight generations (33).

In animals there are many examples of epigenetic traits that appear to respond to environmental, and especially nutritional signals experienced by former generations, but there are relatively few examples of heritable epigenetic variation at individual genes (37). In contrast to plants, efficient reprogramming occurs in mammals in the early embryo and in the germ line, decreasing the possibility of inheritance of epigenetic marks (37).

Transmission of acquired states has been observed in some animals, such as nematodes, For example the worm *C. elegans* can undergo a process known as olfactory imprinting, and this behaviour can then be transmitted over more than 40 generations (38).

Occurrence of genomic mutations, implying DNA sequence changes, is generally a rare process, mostly either neutral or deleterious. Also most epigenetic changes ("epimutations") are either neutral or deleterious, but transgenerational epigenetic inheritance can also be adaptive, as response to fluctuations in environment, nutrition and to other challenges, therefore enabling an organism or population to survive in a dynamic condition. They have been proposed to provide the so-called "soft inheritance" (39,40) a more flexible system different from the usual "hard inheritance" [5]. It was found that epigenetic mutations in plants (*A. thaliana*) are about 100,000 times more likely than DNA sequence mutations, but still low enough to be subject to natural selection (41).

Interestingly, a recent study on corals showed that "inshore" corals are better able to adapt their gene expression to suit new environments than "offshore" corals (42). Inshore corals , dwelling close to the shoreline, are subjected to more-variable temperatures and increased pollution, so that it is reasonable to link this condition to the ability to dynamically alter their gene expression of environmental stress response genes.

It is also interesting to note that the concept of epigenetic transmission and soft inheritance has been generalized to include learning through social interactions, since this can lead to similarity between generations and to animal traditions. They are reviewed and discussed in detail by Avital and Jablonka (43) and Jablonka and Lamb (44).

Can epigenetic changes affect the DNA sequence ?

Another issue is whether or not epigenetic changes in an organism can alter the basic structure of its DNA. There are now well documented cases where epigenetic changes can lead to permanent modifications in the genetic sequence. For example, a methylated cytosine can spontaneously change to a thymine through deamination (26), a reaction that is faster than in a non-methylated cytosine (45).

[4] This is a famous case of epigenetic inheritance that was described over 250 years ago by Carl Linnaeus. This variant has a floral structure with five spurs instead of one spur, and Linnaeus named it 'Peloria', the Greek word for 'monster'.

[5] The term 'soft inheritance' was first proposed by Ernst Mayr (1904–2005) to describe a system especially suited to adapt to fluctuations in nutrition, predation, or disease, which occurs relatively unpredictably and may endure for more than one generation. Soft inheritance includes both non-DNA variations and developmentally induced variations in DNA sequence. This notion is still viewed as controversial by some biologists, because it was considered as associated with Lamarck's theory. By contrast, "hard inheritance" is essentially Mendelian, and keeps constant the genetic material except for rare random mutations.

Other possible mechanisms are:

- involvement of transposable elements[6] of DNA. Various types of endogenous and exogenous (including environmental) stress may affect the movement of these elements, which is recognized as a major cause of genomic change. It is claimed that epigenetic variations can determine when and where these genetic changes occur (46).

- epigenetic silencing with loss of function of genes (47). If these are genes controlling the cell cycle and DNA repair then predisposition of genetic mutation occurs.

- development of genetic mutations by promoting genome instability. For example it was found that the environmental induction of epimutations in rat sperm promotes genome instability, such that genetic mutations are acquired in later generations. (48).

All these observations suggest that environment and epigenetics have a more important role in mutations, disease and evolution than previously appreciated.

Theories of Evolution and the Modern Synthesis

The possibility that epigenetic changes can be passed on to future generations may transform our understanding of inheritance and evolution. Therefore, it is not surprising that the contribution of epigenetic effects to the biological evolution is presently a subject of lively debate.

There is evidence that life on Earth started approximately 4000 million years ago. Indeed, recent findings point to 4100 million years ago (49).

Subsequent evolutionary processes gave rise to diversity at every level of biological organization. The current scientific consensus is that all present day organisms stemmed from a common ancestor, possibly derived from a system of self-replicating molecules, such as RNA[7] , that is able both to serve as a template and to catalyze its own replication (see e.g., [50,51]).

Lamarck's theory, developed in 1809, is considered as the first real evolutionary scheme. It was based on two ideas: i) use versus disuse to develop characteristics that are useful, and ii) heritability of the acquired traits.

In 1859 Charles Darwin published his book "On the Origin of Species" in which he formulated the scientific theory of evolution through natural selection acting on variable populations. In the following years Darwin's theory overcome the Lamarck's one. However the original theory did not yet include the concepts of genetic heredity in the form ~~as~~ subsequently formulated in 1865 by Gregor Mendel. By studying how pea plants breed together, he reported that traits, i.e., the inherited characteristics of an organism, were inherited in a predictable manner through the independent assortment and segregation of elements . These were later known as genes[8], and the complete set of genes within an organism's genome is its genotype.

Mendel also showed that traits do not "mix" like paint when they are handed down from parents to offspring, but rather are "shuffled" like cards.

The modern working definition of a gene is a portion (or sequence) of DNA that codes for a known cellular function or process (in practice a specific protein or RNA). Indeed, after the discovery of

[6] A transposable element (or transposon) is a DNA sequence that move from one location on the genome to another. These elements, also known as "jumping genes," were first identified by Barbara McClintock, a discovery that earned her a Nobel Prize in 1983.

[7] This view is generally referred to as the"RNA World" hypothesis, proposed by Rich in 1962 [52] and other scientists (including Francis Crick) and substantiated in the early 1980s when Altman and Cech demonstrated the RNA catalytic properties, a finding that earned them the 1989 Nobel Prize in Chemistry.

[8] According to the definition of Gerstein et al [55] "A gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products".

the double-helical structure of DNA, a clear understanding was gained of how genetic information is copied and passed on to offspring.

The modern view of the Darwin's theory is that evolution in organisms occurs through changes in heritable traits that are controlled by genes. Darwinian evolution together with Mendelian genetics form the so-called Neo-Darwinism or "Modern Synthesis"[9], whose pillars are: i) variation exists within populations of organisms with respect to morphology, physiology, and behaviour (phenotypic variation); ii) different traits (controlled by genes) confer different rates of survival and reproduction (differential fitness[10]); iii) these traits can be passed from generation to generation (heritability of fitness).

According to the Modern Synthesis, the Mendelian heredity preserves the diversity of forms available in a population, while during evolution by means of natural selection the traits that enhance survival and reproduction become more common in successive generations of a population. Also, genotypes are assumed to translate more or less directly into phenotypes, and evolutionary change stemmed from the slow, gradual accumulation of random genetic mutations.

Challenges to the Modern Synthesis

In spite of the internal coherence of the Modern Synthesis, there are a number of observations made in animals and humans, that have challenged this paradigm. Several interesting reviews on the subject have been published in various issues of the online version of The Scientist Magazine in the last few years (53). Some examples are mentioned here.

- *Rapid Evolution*

Few years ago a research team reported (54) that on islands off the coast of Florida, they uncover a swift adaptive changes among "Carolina anole" (a type of lizard) populations, whose habitats were disturbed by the introduction of another lizard species. Because of its competitor, the Carolina anole started perching higher up in trees, and its toe pads changed to enable better grip. All this occurred in a 15 year period, or about 20 lizard generations. In 2014 the researchers found that the larger, stickier toepads were acquired and passed on to the next generation, suggesting a real evolution, not a simple plasticity phenomenon. This finding is not consistent with evolution driven by slow, random genetic mutation.

Another similar observation was made on the little songbirds called house finches. They spread throughout the United States from Mexico in the 1940s and after 19 generations (15 years) they developed a new beak shape as adaptations to the new environment at a surprisingly rapid rate [56], likely too rapid to have resulted from just random mutations.

- *Previous experience of mothers and fathers*.

A famous example comes from the Dutch survivors of the "Dutch Hunger Winter" of World War II. The Nazis occupying the Netherlands imposed an embargo on food shipments, causing a severe famine for many months. Children of pregnant women exposed to this famine were more susceptible to obesity, diabetes, cardiovascular disease, and even schizophrenia than were people who were born just before or conceived after the famine. These modifications persisted into old age [57-59]. However, the fact that environmental conditions affecting a mother can influence the phenotype of her offspring has not been considered a great surprise, as the mother's womb is said to be the baby's first environment. But there are interesting cases where the lifestyle or history of the father has been implicated in disease risk of his children. Studies of stress, diet, and additional

[9] Julian Huxley cemented the term in 1942 with the publication of his book "Evolution: The Modern Synthesis"..

[10] "Fitness" is the organism's ability to survive and reproduce, which determines the size of its genetic contribution to the next generation. "Adaptation" is the process that enhances organism's fitness. Adaptation results from the evolutionary process.

environmental stimuli such as toxin exposure are suggestive of the idea that a father's environment can influence the phenotype of his offspring (60). In 2006 a UK research team concluded that the experience of young boys could affect not just their health in later life, but also the health of their sons and grandsons. A study performed on an isolated community in northern Sweden demonstrated that the longevity of the subjects under study was influenced by their paternal grandfathers' access to food (61).

The impact of Epigenetics on evolution understanding

Transgenerational epigenetic modifications (epimutations) may play a more significant role in evolution than has so far been suspected.

Epigenomic regulation is involved in several processes central to evolutionary biology including phenotypic plasticity, evolvability and the mediation of intragenomic conflicts in human populations (62).

Epigenetics can explain why human beings look and act quite different from their closest relatives, the other great apes, while all these species share most of their genome. In effects, humans and chimpanzees share around 99% of their DNA. There are indications that the differences between great apes do not come solely from differences in what genes they have, but also in how they are regulated (63). Recent studies revealed human-specific epigenetic patterns in histones (64) and DNA methylation (65). These and other studies that have compared the epigenetic modifications of the human genome with that of other great apes, pointed out the importance of epigenetics in our recent evolutionary history, suggesting that regulatory changes may have played a key role in the acquisition of human-specific trait.

On the other hand, it is becoming clear that genomic sequence variants exist that encode and presumably regulate distinctive epigenetic patterns. For instance, numerous single-nucleotide polymorphisms that affect DNA-methylation patterns have been discovered in human populations. These studies begin to reveal a dynamic interplay between genomic and epigenomic factors across long and short evolutionary timescales.(66) .

Although biologists have generally discredited Lamarck's ideas, the increasing evidence of epigenetic phenomena, being outside the Modern Synthesis paradigm, has prompted reconsideration of the matter, even suggesting rejecting the Darwinism in favour of the Lamarckism. This clearly appears an excess, as it is unlikely that all the genomic changes during evolution are due to epigenetic mechanisms.

It has been noted that examples of soft inheritance, "although Lamarckian in their environmental determination, involve short-term adaptations that supplement the evolutionary processes of Darwin and Mendel. Thus, they are distinct from Lamarck's proposed overall mechanism of evolution" (67)

A number of researchers have recognized the need to come to an "extended" evolutionary synthesis (see, e.g., [68-70] arguing that "evolutionary biology today has to incorporate soft inheritance, saltational changes due to systemic mutations, and various types of genetic exchange and cooperation" and that "rather than trying to continue to work within the framework of a Synthesis that was made in the middle of the last century, we now need a new type of evolutionary theory, one that acknowledges Darwinian, Lamarckian and saltational processes." (71). It has been noted that in this Extended Evolutionary Synthesis (EES) "the word 'extended' implies quite clearly that there is no rejection of the previous synthesis" (72).

Witness of the lively debate existing on the subject is a discussion published by Nature in 2014 [73] on the need to rethink the evolutionary theory, where several scientists expressed different and contrasting points of view, some of them speaking in favour of the EES and others in favour of the standard theory.

In spite of some recent criticisms to the importance of soft inheritance (74) probably the most realistic vision is that a combination of both epigenetic and traditional genetic inheritance may contribute to the variations seen in species today. (75). In substantial agreement are those "inclusive" approaches that, starting from the observation that "genetic variation and natural selection are not the whole core, and adaptations are not the exclusive explanation of evolutionary biology" propose the inclusion of more patterns to the standard theory (76).

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APPROCCIO FENOMENOLOGICO ALLA DEFINIZIONE DI MODELLI DI PREDIZIONE DELLA TOSSICITÀ INDOTTA DA RADIOTERAPIA: UNA VISIONE CRITICA

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Nel campo della modellizzazione della tossicità radioindotta, spesso indicata come modellizzazione NTCP (dall'inglese *Normal Tissue Complication Probability*), è pratica comune il costruire modelli utilizzando una combinazione della conoscenza biologica e dei dati clinici ottenuti da osservazione di popolazioni di pazienti. Attualmente, nel tentativo di aumentare l'accuratezza di questi modelli, si assume spesso che le complicanze radioindotte siano causate dall'interazione di molteplici fattori, attraverso meccanismi biologici dei quali non si ha adeguata conoscenza, ma che possono essere dedotti dai dati raccolti in modo sistematico durante la pratica clinica e che includono generalmente dati di tipo epidemiologico, dati dosimetrici e in alcuni casi anche marcatori di tipo biologico/genetico.

Ne risulta che i modelli NTCP sono sempre più fondati su osservazioni e associazioni di tipo statistico e sempre meno sulla conoscenza di tipo biologico che sta alla base della interazione tra la radiazione ionizzante e il tessuto vivente. I modelli sono in questo caso di tipo fenomenologico, nel senso che in generale riescono a descrivere bene i dati osservati, ma non sono pienamente spiegati dalla attuale conoscenza radiobiologica. Il fatto che questi modelli siano in grado di descrivere bene i dati osservati nella clinica è sicuramente un punto di vantaggio, va tenuta però in giusta considerazione la loro natura statistica e quindi è importante aver presente in ogni caso il contesto clinico nel quale sono stati derivati.

Il punto di debolezza principale dei modelli fenomenologici, specialmente nel caso di modelli multivariabile, è che una molteplicità di modelli è derivabile in maniera consistente dallo stesso dataset di osservazioni cliniche. Si utilizzano in questi casi tecniche di valutazione della performance e della affidabilità per scegliere il modello candidato più adatto. Ne segue comunque, in ogni caso, la possibilità che modelli, che sono in grado di descrivere molto bene i dati sui quali sono stati ottimizzati, risultino poi inconsistenti quando testati su altre popolazioni cliniche.

Un primo aspetto da considerare è legato alla possibilità di *overfitting* nella procedura di determinazione del modello. Questo aspetto è dominante quando la popolazione sulla quale è stato

derivato il modello e quella sulla quale il modello viene testato sono in realtà popolazioni di pazienti i cui dati sono stati raccolti nelle stesse identiche condizioni. Per esempio, si tratta di due campionamenti casuali derivati dallo stesso trial clinico. In questo caso, il fenomeno per cui il modello NTCP descrive bene il dataset di training (=addestramento del modello), ma invece diviene poco descrittivo del dataset di test, può essere attribuito solo al fatto che il modello è stato adattato alla popolazione di sviluppo con dettaglio eccessivo (=*overfitting*), rendendolo incapace di seguire relazioni più generali. In questi casi le tecniche statistiche aiutano a limitare l'*overfitting* e a produrre modelli che abbiano alta probabilità di descrivere in modo soddisfacente sia la popolazione di training che la popolazione di test. Sono in generale tecniche che hanno lo scopo di limitare il numero di variabili da inserire nei modelli e che guidano nella selezione delle variabili più importanti e meno legate alla specifica selezione del dataset. I modelli generati secondo queste procedure, e che includono una validazione su una sotto-popolazione derivata da campionamento casuale della popolazione di studio, sono detti internamente validati. La validazione interna non dice nulla della capacità di un modello di descrivere popolazioni diverse, garantisce solo che il modello è stato derivato seguendo le componenti principale delle associazioni tra caratteristiche dei pazienti e insorgenza di tossicità, minimizzando il rischio di fitting del "rumore" presente nel dataset sperimentale.

Un secondo aspetto da prendere in esame è la possibile instabilità dei modelli, legata alle incertezze di selezione dei modelli stessi. E' infatti da notare che non è rara la situazione in cui esistono diversi modelli NTCP che rispettano la validazione interna, ma che danno diverse predizioni di tossicità, tutte ugualmente buone, soddisfacenti e accettabili per quanto concerne la descrizione della popolazione di training e test. In questi casi non esiste possibilità di scelta oggettiva tra i modelli alternativi da un punto di vista statistico, utilizzando cioè misure di performance classiche quali la varianza spiegata, la verosimiglianza, la calibrazione o la discriminazione.

In questi casi il riferimento è la capacità di generalizzazione del modello, cioè la sua performance su popolazioni esterne indipendenti. Questa operazione è sempre molto delicata e a rischio di fallimento quando ad essere generalizzati sono modelli di tipo fenomenologico. La struttura statistica del dataset su cui il modello è stato sviluppato e quella della popolazione indipendente su cui viene valutato sono spesso dominanti rispetto alle associazioni valutate dal modello stesso. Queste differenze sorgono perché le popolazioni di diversi centri ospedalieri sono diverse, i trattamenti spesso differiscono, ci sono criteri di selezione discordi per i trattamenti (nonostante l'esistenza di linee guida nazionali ed internazionali), esistono diversi modi di registrare le complicanze ai tessuti sani e differenti tempi/diversa intensità di follow-up dei pazienti. Inoltre, mentre alcune discrepanze tra le strutture statistiche delle popolazioni possono essere misurate e tenute in debita considerazione, in particolare tutte quelle legate alle variabili indipendenti incluse nel modello, esistono ulteriori variazioni che possono sorgere in maniera non prevedibile ed essere legate a fattori nascosti.

Un ultimo punto debole dei modelli NTCP di tipo fenomenologico è dato dal fatto che una associazione tra tossicità radioindotta e variabili di tipo epidemiologico/dosimetrico/biologico/genetico non implica mai di principio una causalità. Questo comporta una particolare attenzione nel momento in cui un modello fenomenologico viene utilizzato per ridurre la tossicità data da un trattamento. La fase di modifica del trattamento, e la registrazione degli eventi di tossicità dopo tale modifica, deve essere eseguita con particolare attenzione e cautela, al fine di verificare che ne derivi una effettiva riduzione dell'incidenza di effetti indesiderati. In letteratura esistono comunque esempi positivi di utilizzo dei modelli NTCP di tipo statistico. Un caso noto è l'associazione tra sanguinamento rettale tardivo dopo radioterapia per tumore alla prostata e la porzione di retto che riceve dosi superiori a 70-75 Gy. Diversi trial clinici hanno segnalato in modo indipendente questa associazione negli anni 2000-2005 [1-3], cui è seguita una attenzione nella fase di pianificazione ai volumi di retto inclusi nelle alte isodosi e una loro riduzione. Questo ha portato ad un sostanziale abbattimento dell'incidenza di sanguinamento rettale tardivo, nonostante l'aumento delle dosi di prescrizione al target prostatico.

I modelli NTCP fenomenologici sono dunque una fonte ricca e di valore per le informazioni che danno riguardo alle associazioni tra gli effetti indesiderati del trattamento radioterapico e le variabili che descrivono la situazione clinica del paziente, il suo trattamento, il suo profilo genetico/molecolare, ma risulta difficile garantire la loro validità su larga scala, e per fare questo è necessario seguire un approccio corretto di validazione continua di questi modelli.
Va in primo luogo accettato che questi modelli sono delle congetture, cioè delle ipotesi che sembrano essere corrette, ma che di fatto devono essere verificate e provate ad un livello più ampio. Le congetture sono una parte essenziale del normale processo scientifico. Nella elaborazione di teorie e modelli, si accettano congetture semplici, si testano e, se queste falliscono, si procede ad una loro sostituzione con congetture più complesse. Si riprende dunque il circolo di test delle nuove congetture, di conferma o meno della loro validità e di eventuale aggiornamento del modello in costruzione.
Si tratta dunque di tradurre queste procedure del processo scientifico nel campo dei modelli NTCP di tipo statistico (figura 1). Un modello viene sviluppato su una popolazione e lo si verifica su di una popolazione seguente, anche ottimizzata secondo indicazioni del modello. Nel contempo si una raccolta accurata dei nuovi dati. Ne deve seguire una verifica dell'accordo tra le probabilità di tossicità previste dal modello e le incidenze osservate di effetti collaterali indesiderati. Dopo la raccolta di una quantità di dati sufficiente, si dovrebbe essere in grado di decidere se il modello può essere accettato, e considerato validato perché soddisfacente, oppure se è necessaria una modifica del modello, da valutarsi sulla base dei dati provenienti dalle due popolazioni congiunte. Questo ciclo di apprendimento può essere ripetuto più volte, in modo da aggiungere accuratezza al modello ad ogni iterazione.

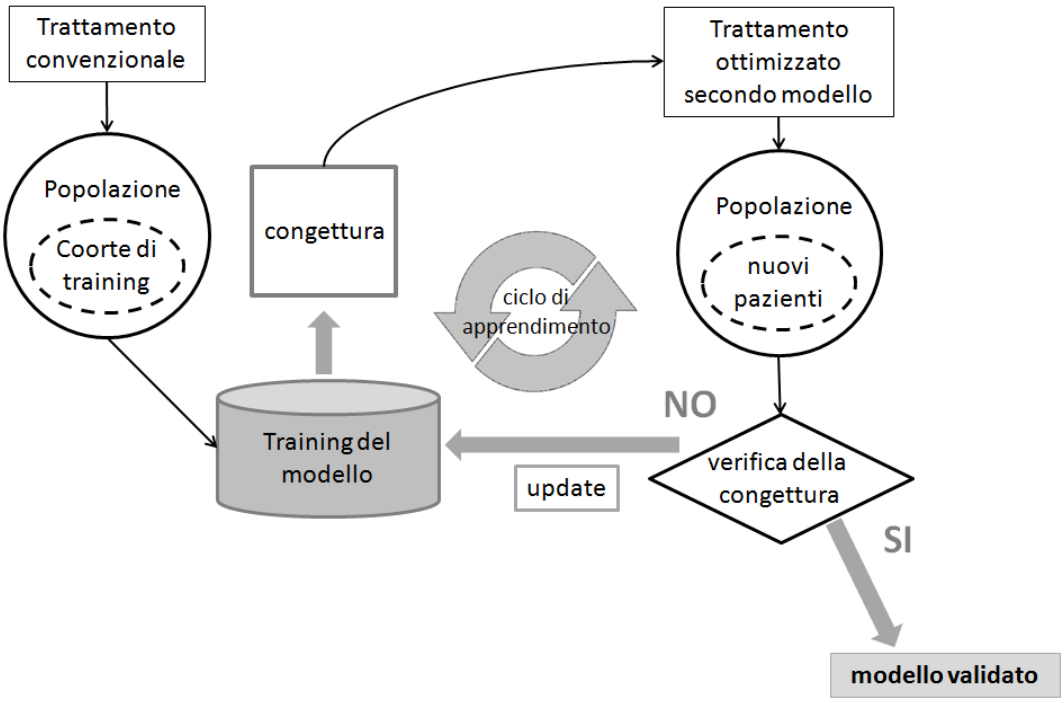

Figura 1: Possibile approccio pratico all'utilizzo di modelli fenomenologici per la predizione di tossicità radioindotta per l'ottimizzazione del trattamento. Un primo modello viene creato sulla base dei dati clinici derivati da pazienti trattati con trattamento convenzionale. Il modello viene considerato una congettura per l'ottimizzazione del trattamento, ma si raccolgono anche i dati clinici dei nuovi pazienti per verificare l'accordo tra la predizione di tossicità prevista dal modello e l'incidenza di tossicità osservata. Se l'accordo è soddisfacente, la congettura può essere considerata confermata e il modello può essere considerato validato. Se invece l'accordo tra previsioni del modello e realtà clinica non è soddisfacente, è necessaria una modifica del modello tramite aggiornamento del dataset di training, che prevede l'unione dei dati della prima popolazione con trattamento convenzionale e della nuova popolazione con trattamento modificato. Il primo modello viene quindi sostituito con il modello aggiornato e si riprende con la verifica delle sue predizioni e con il ciclo di apprendimento.

Questo approccio mantiene delle limitazioni. La prima è legata al fatto che ancora si sta considerando la validazione del modello all'interno di popolazioni cliniche abbastanza simili: tipicamente pazienti afferenti allo stesso centro, o allo stesso consorzio di centri partecipanti a trial multicentrici, dove la selezione dei pazienti e le tecniche di trattamento hanno una lenta evoluzione. Anche in presenza di un approccio iterativo, resta dunque ancora aperto il tema della validità del modello in contesti sostanzialmente diversi. Una seconda importante limitazione è legata al tempo necessario per seguire questo processo, dati i tempi di arruolamento dei pazienti, i tempi di follow-up necessari per valutare tossicità tardive (spesso di almeno 3 anni), a cui si aggiunge la non conoscenza a priori del numero di iterazioni necessarie per ottenere un modello soddisfacente.
Un requisito fondamentale, per realizzare questo approccio di apprendimento continuo dei modelli NTCP di tipo statistico, è inoltre la promozione di una cultura di raccolta dati standardizzata all'interno dei centri ospedalieri, riconoscendo contemporaneamente che queste raccolte dati producono un aumento del carico di lavoro e necessitano di personale tecnico dedicato, per esempio data manager o data scientist. D'altro canto l'approccio di apprendimento continuo è molto semplice dal punto di vista concettuale, diretto nella sua implementazione e assicura il raggiungimento di modelli fenomenologici affidabili, oltre a permettere, come effetto secondario, un accumulo e una registrazione automatica e facilmente accessibile dell'esperienza clinica.
In ambito europeo, sia la Società Europea per la Radioterapia Oncologica (ESTRO) [4], sia la Comunità Europea (attraverso i programmi di ricerca settimo programma quadro e Horizon 2020 nel pilastro società), sottolineano la necessità di creazione di database complessi di dati clinici per i test di modelli statistici di predizione di tossicità e di controllo del tumore, e incentivano questi aspetti in diversi bandi competitivi. Il progetto REQUITE (www.requite.eu, *Validating Predictive Models and Biomarkers of Radiotherapy Toxicity to Reduce Side-Effects and Improve Quality of Life in Cancer Survivors*) è un esempio di consorzio europeo che sta lavorando per raccogliere dati standardizzati per la validazione di modelli esistenti di predizione di tossicità conseguente a radioterapia per tumore della prostata, della mammella e del polmone, includendo anche validazione dei polimorfismi genetici indicati come predittori di aumentata radiosensibilità in studi già pubblicati [5]. Allo stesso tempo il progetto si propone di diffondere la cultura di raccolta standardizzata di dati, rendendo disponibili (già tradotti in diverse lingue europee) i form di raccolta dati elaborati all'interno della collaborazione.
Una diversa possibilità, per accelerare il processo di apprendimento continuo e correzione iterativa dei modelli, è quella di promuovere validazioni esterne su popolazioni ampie già esistenti, che sono state seguite con programmi di follow-up standardizzato e prospettico e che possono quindi essere utilizzate per verificare la generalizzabilità dei modelli. Un punto cruciale in questi casi è la definizione degli endpoint di tossicità che sono stati utilizzati nei diversi studi. Deve essere infatti possibile ottenere uno scoring condiviso; questo è più facilmente ottenibile quando gli studi utilizzano scoring proposti dalle linee guida internazionali (Subjective, Objective, Management, Analytic/ Late Effects of Normal Tissues – SOMA/LENT - scale or Common Terminology Criteria for Adverse Events -CTCAE – scale), o questionari validati somministrati ai pazienti o ancora quando vengono considerati gli endpoint di tossicità più severi, che sono più difficilmente misinterpretabili. Per modelli che considerano effetti tardivi, la possibilità di utilizzare popolazioni già esistenti riduce notevolmente il tempo necessario per chiudere il cerchio di apprendimento, guadagnare confidenza nella affidabilità del modello e poter beneficiare del suo utilizzo all'interno della pratica clinica [6,7].

L'approccio proposto può essere poi coadiuvato da metodi che si propongano di validare l'interpretazione causale dei modelli fenomenologici. Per esempio trial clinici randomizzati possono confrontare gli esiti in termini di incidenza di effetti collaterali di trattamenti ottimizzati utilizzando modelli NTCP alternativi.
E' anche estremamente positiva l'implementazione di studi pre-clinici che abbiano lo scopo di investigare i possibili meccanismi biologici alla base delle associazioni statistiche che sono state osservate. In questo caso i modelli fenomenologici forniscono un importante serbatoio di

informazioni in grado di generare ipotesi e di ispirare e guidare nuova ricerca di base. Un esempio in questo campo è dato dai trial pre-clinici dedicati allo studio dei livelli plasmatici di marcatori di infiammazione, alla determinazione delle loro variazioni durante la radioterapia e alla loro associazione con tossicità radioindotta, nati dalla osservazione fenomenologica di un aumentato rischio di insorgenza di tossicità rettale in pazienti con una pregressa chirurgia addominale [8]. Da questo punto di vista i modelli statistici e la radiobiologia restano due realtà separate ma complementari, espressione gli uni (modelli statistici) di approcci di conoscenza bottom-up e l'altra (radiobiologia) di approcci top-down.

In conclusione, i modelli NTCP fenomenologici non dovrebbero essere considerati come descrizioni generali della realtà, ma piuttosto come congetture che hanno origine dalla esperienza immagazzinata, e che raggiungono sempre maggior affidabilità al crescere di questa conoscenza accumulata. Quando applicati con attenzione, accompagnati da programmi di registrazione prospettica dei dati e combinati da validazioni esterne, risultano essere dei mezzi potenti di apprendimento dai pazienti del passato per ottimizzare i trattamenti dei pazienti futuri.

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ASPETTI CLINICI DELL'ADROTERAPIA

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Introduzione

L'Adroterapia è una forma di trattamento radiante di precisione che utilizza particelle pesanti (protoni, ioni, neutroni) per il trattamento radiante di pazienti affetti da patologie tumorali. La sua applicazione richiede una apparecchiatura pesante di produzione delle particelle (ciclotrone/sincrotrone) ed un sistema di trasporto del fascio e di rilascio sul paziente (gantry o linea fissa). In particolare i fasci di protoni, cui ci si riferirà in specifico nel resto del presente testo, rilasciano la dose con estrema precisione sul bersaglio risparmiando i tessuti sani, permettendo così di incrementare il controllo di malattia e/o di ridurre gli effetti collaterali (tossicità).
La peculiarità di questa terapia consiste principalmente nelle sue proprietà fisiche ed in particolare nella sua selettività spaziale. La dose in entrata della radiazione è bassa, raggiunge il suo culmine (rilascio di energia) nel target desiderato e praticamente si azzera una volta superato il bersaglio: la distribuzione è tipica e delineata nel cosiddetto picco di Bragg (vedi figura 1), con evidenti vantaggi nel risparmio dei tessuti sani circostanti che con la radioterapia tradizionale vengono inutilmente irradiati. Per questo motivo la protonterapia si propone quale trattamento di elezione nelle neoplasie localizzate in vicinanza di tessuti critici e dove è necessario un trattamento di alta precisione.

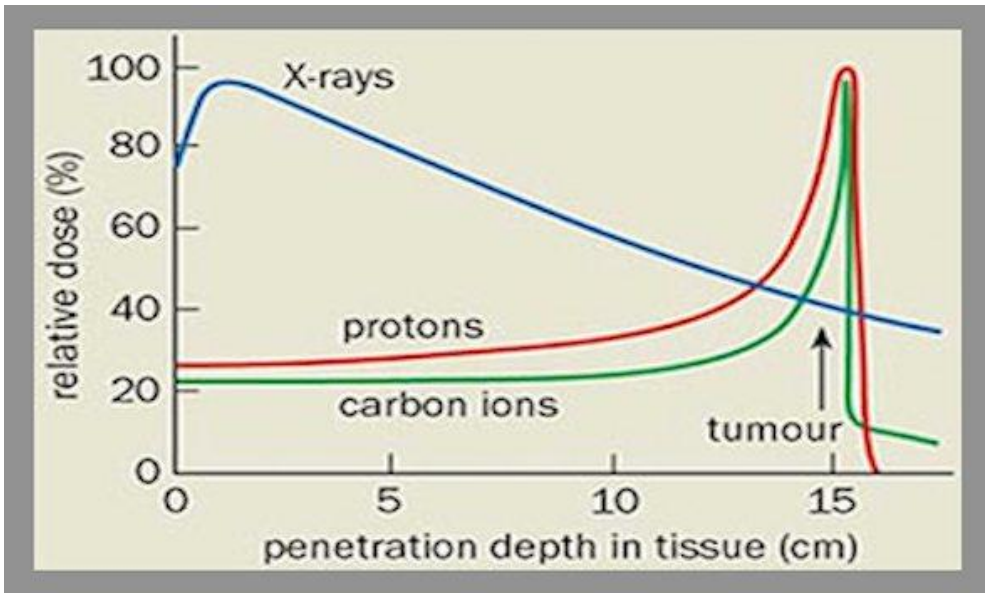

Figura 1: picco di Bragg per ioni carbonio (linea verde) e protoni (linea rossa) in confronto con un fascio di fotoni (linea blu)

Lo sfruttamento delle proprietà fisiche dei protoni in campo medico è reso possibile grazie anche alla sempre maggiore evoluzione tecnologica dei sistemi di imaging per l'individuazione del bersaglio, che permettono di sfruttarne le favorevoli proprietà balistiche.
La proposta di uso di tali caratteristiche in campo medico non è nuova: è iniziata negli anni '50 dopo che Robert Wilson (1) ne intuì l'applicabilità in clinica, resa però allora difficilmente

applicabile per le carenze tecnologiche dei sistemi allora utilizzabili che oggi sono state in gran parte superate.
I nuovi sviluppi tecnologici con il maggiore uso di tecniche attive di erogazione e ad intensità modulata sta migliorando ulteriormente le possibilità d'uso della metodica anche se va ancora più attentamente valutata e studiata la vulnerabilità delle particelle alle varie incertezze (di range, anatomiche, radiobiologiche, legate alla calibrazione TC ecc…) con la pianificazione robusta del trattamento.
L'uso delle particelle è ormai invalso in campo radioterapico: vi sono oltre 50 centri che utilizzano particelle attivi nel mondo, e oltre 150.000 sono i pazienti trattati in centri dedicati e commercialmente disponibili che hanno sostituito l'utilizzo, nelle fasi pionieristiche, di centri di fisica sperimentale adattati parzialmente all'uso clinico (2).
In Italia sono operativi clinicamente tre centri: i Laboratori nazionali del Sud dell'INFN di Catania dedicati al trattamento di neoplasie oculari, il CNAO di Pavia dotato di fasci fissi di protoni e ioni carbonio e il CPT dell'APSS di Trento dotato di gantry con protoni. Complessivamente le tre strutture hanno trattato sino alla fine del 2016 oltre 1500 pazienti con particolare attenzione a melanoma uveale (Catania), sarcomi, neoplasie del capo-collo e carcinomi adenoideo-cistici (Pavia) e neoplasie dell'encefalo e pediatriche (Trento).
La strutturazione di un centro di adroterapia deve tenere conto di un difficile equilibrio di diversi fattori che peraltro influenzano la pratica clinica: necessità di una tecnologia complessa, elevato costo di impianto e di esercizio, concetti di biologia non ancora completamente noti e definiti e incertezze nella erogazione e dosimetria dei fasci. L'insieme di questi fattori rende la terapia ancor oggi un trattamento che richiede investimenti non solo economici ma anche di tipo tecnico-industriale e di sviluppo delle competenze.

Indicazioni terapeutiche per l'uso clinico

Le finalità del trattamento, rispetto alla tradizionale terapia con fotoni, sono così riassumibili:

- per i protoni, come la possibilità di usare una dose equivalente ed avere un risultato analogo / minor tossicità oppure erogare una dose aumentata (dose escalation) con l'ipotesi di poter raggiungere un risultato migliore / pari tossicità.
- per gli ioni, l'aspettativa è usare una dose equivalente con il fine di avere un risultato migliore / pari tossicità sfruttando le caratteristiche radiobiologiche del fascio.

Ma in che condizioni cliniche si prevede che l'uso delle particelle possa essere particolarmente indicato? In linea di principio la tecnica è preferenzialmente applicabile in caso di volumi complessi, in prossimità di organi a rischio di particolare sensibilità, in pazienti giovani, in caso di re-irradiazione, per casi con particolare sensibilità genetica alla irradiazione e nei casi (in particolare per gli ioni) radioresistenti.
I protoni risultano di particolare interesse nei pazienti pediatrici, nel qual caso le caratteristiche del fascio consentono un potenziale vantaggio in termini di tossicità tardiva e di sviluppo di tumori secondari. Un esempio del risparmio di tessuti sani nel trattamento di ampi volumi come la irradiazione cranio-spinale è riportato in figura 2.
Le indicazioni al trattamento sono andate sviluppandosi (3) negli ultimi anni ben oltre i classici target storici rappresentati dal melanoma oculare (4,5), di cui sono stati trattati oltre 30.000 pazienti con dati che suggeriscono un migliore outcome rispetto alle altre terapie conservative disponibili, e dalle neoplasie della base cranica e spinali, in particolare cordomi e condrosarcomi (6), dove l'adroterapia è considerata attualmente un gold standard in fase post-chirurgica. Sono andati accumulandosi inoltre dati in molte altre patologie oggetto di studio e di pratica clinica nei diversi centri sparsi nel mondo, in particolare in ambito pediatrico (7) e nei tumori di polmone (8), prostata (9), apparato gastrointestinale (10) etc.

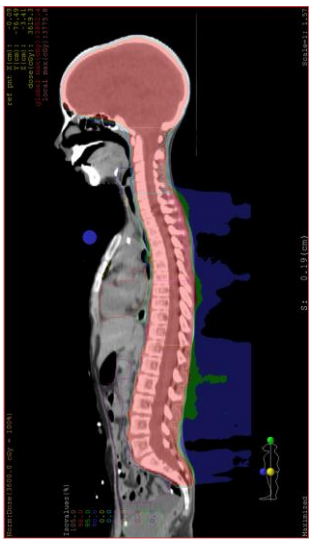

Figura 2: distribuzione di dose in un caso di irradiazione con protoni per un paziente affetto da medullo blastoma. Si osservi il sostanziale risparmio di dose degli organi anteriori addominali e toracici e l'omogenea irradiazione dell'asse craniospinale

È ormai generalmente riconosciuto che la terapia protonica risulti un trattamento oncologico sicuro, efficace e raccomandabile per molti tipi di tumori. Nonostante questi promettenti risultati continuino ad essere segnalati per nuove forme tumorali e vadano consolidandosi in quantità e qualità, essi si basano però su studi usualmente di modeste dimensioni e non randomizzati. Considerando l'elevato costo di costruzione e gestione dei centri di terapia adronica, sono state sollevate diverse questioni circa il loro rapporto costo/efficacia, e vi è un consenso generale sulla necessità di raccogliere prospetticamente i risultati in registri multi-istituzionali per dimostrare in modo inequivocabile il loro vantaggio nell'uso clinico.

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