MULTIDRUG-RESISTANT AND EXTREMELY DRUG-RESISTANT BACTERIA: ARE WE FACING THE END OF THE ANTIBIOTIC ERA?

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Abstract. Antibiotics are one of the most significant advancements of modern medicine. They have changed the prognosis of several bacterial infections, and made possible advanced medical practices associated with a high risk of infectious complications. Unfortunately, antibiotics are affected by the phenomenon of antibiotic resistance, which jeopardizes their efficacy. In recent years, antibiotic discovery and development has been lagging, due to a lower appeal of this sector for the pharmaceutical industry, while antibiotic resistance has continued to evolve with the eventual emergence and dissemination of bacterial strains which are resistant to most available antibiotics and pose a major challenge to antimicrobial chemotherapy. This worrisome scenario, indicated as the "antibiotic resistance crisis", has been acknowledged by Scientific Societies and Public Health Agencies, and is now gathering an increasing attention from the Media and Governments. This article reviews the antibiotic-resistant pathogens which currently pose major problems in terms of clinical and epidemiological impact, and briefly discuss future perspective in this field.

Key words: Antibiotics; antibiotic resistance; multidrug resistance.

INTRODUCTION

Antibiotics have represented one of the most significant discoveries of the twentieth century. They have dramatically changed the prognosis of bacterial infections (*e.g.* pneumonia, sepsis, meningitis, wound infections, cellulitis, typhoid fever, syphilis) by reducing morbidity and mortality due to these infections. Antibiotics have also made possible a number of medical practices that would otherwise be precluded by an exceedingly high risk of infectious complications, such as "contaminated" surgery, implantation of prosthetic devices, solid organ and stem cell transplantation, deep immune suppression treatments. Losing antibiotics due to bacterial resistance, therefore, is a very serious issue that can revert the morbidity and mortality by infectious diseases to that typical of the pre-antibiotic era, and jeopardize the success of several advanced medical practices. This has recently been acknowledged by the WHO, in a report on the global impact of antimicrobial resistance [1]. The growing and challenging menace of antibiotic resistance to public health has also been underscored in a recent report, commissioned by the government of the United Kingdom, where it has been estimated that by year 2050, antibiotic resistance could cause approximately 10 millions of deaths annually [2].

The phenomenon of antibiotic resistance reflects the ability of bacteria to evolve resistance mechanisms by which the bacterial cell can escape the lethal action of antibiotics. Well known examples of antibiotic resistance mechanisms include: i) production of enzymes that inactivate antibiotics (*e.g.* β-lactamases, which inactivate β-lactam antibiotics); ii) modification of the molecular target of the antibiotic, so that it is no longer inhibited by the drug while retaining its function; iii) reduction of permeability of the cell envelope to antibiotics, with impaired access of drugs to their intracellular targets; and iv) extrusion of the antibiotic from the bacterial cell by efflux pumps. Evolution of resistance mechanisms in the bacterial cell can be due either to chromosomal mutations or to horizontal acquisition of new resistance genes that encode the resistance mechanisms [3].

The evolution of resistance mechanisms among pathogenic bacteria is part of the normal evolution process, and thus is unavoidable. However, the dynamics of this evolution are strongly influenced by several factors among which the most relevant is the use of antibiotics in clinical, veterinary and agricultural practices. An overuse and misuse of these drugs accelerate the evolution process by increasing the selective pressure for strains that have acquire resistance mechanisms.

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THE ANTIBIOTIC RESISTANCE CRISIS

The phenomenon of antibiotic resistance has been evident since the introduction of antibiotics in clinical practice, and has affected every new antibiotic developed for clinical use. However, until a few years ago, the evolution of antibiotics resistance among pathogenic bacteria was steadily counterbalanced by the discovery and development of new antibiotics active against resistant bacteria. Examples of this saga, occurred between bacteria and humans and ongoing since the 1950s, are represented by the development of penicillinase-stable penicillins (*e.g.* oxacillin, cloxacillin) to address penicillin resistance due to penicillinase production in *Staphylococcus aureus*, of expanded-spectrum cephalosporins (ESC) (*e.g.* cefotaxime, ceftriaxone) to address ampicillin resistance due to broad spectrum β-lactamase production in *Escherichia coli* and other Enterobacteriaceae, and of carbapenems (*e.g.* imipenem, meropenem) to address ESC resistance due to production of extended-spectrum β-lactamases (ESBLs) in Enterobacteriaceae [4].

In recent years, antibiotic discovery and development programs have been lagging behind due to a lower appeal of this sector for pharmaceutical industry [5], while antibiotic resistance has continued to evolve in a relentless manner among clinical pathogens with the emergence and dissemination of bacterial strains which have acquired resistance determinants to several antibiotics and exhibit multidrug-resistant (MDR) or extensively drug-resistant (XDR) phenotypes [6]. These strains remain susceptible to only a few antibiotics and pose a major challenge to antimicrobial chemotherapy. Some strains can end up with being resistant to all the available antibiotics (pan drug-resistant strains) [6], causing untreatable infections.

Such a worrisome scenario, in which the clock is turned back to the pre-antibiotic era and antibiotics are lost to modern medicine, has also been indicated as the "antibiotic resistance crisis" [7]. In the following section, the antibiotic-resistant pathogens which pose major problems and of their clinical and epidemiological impact are briefly summarized and discussed.

THE MAJOR PLAYERS IN THE ANTIBIOTIC RESISTANCE CRISIS

Antibiotic resistance involves most bacterial pathogens. Nevertheless, some species are more prone than others to accrue resistance determinants, and eventually more challenging for antimicrobial chemotherapy. These include *Staphylococcus aureus* and *Enterococcus faecium* among Gram-positives, and Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* among Gram-negatives.

Methicillin-resistant Staphylococcus aureus, *the first challenging MDR pathogen*

S. aureus is a major pathogen, causing a number of community-acquired and healthcare-associated infections (HAI) that can range from minor cutaneous infections (*e.g.* furuncles) to severe life-threatening infections (*e.g.* pneumonia, severe cellulitis, septicemia and surgical site infections).

Methicillin-resistant *S. aureus* (MRSA), which is resistant to all β-lactams due to the acquisition of a new penicillin-binding protein (PBP) that can take over the functions of all other PBPs and is not inhibited by β-lactam drugs, has been the first challenging antibiotic-resistant pathogen and remains one of the most important in terms of epidemiological and clinical impact [8]. Infections caused by MRSA are associated with increased mortality and healthcare-associated costs, as compared to infections caused by methicillin-susceptible *S. aureus* [9], and the prevalence of MRSA has achieved very high rates in several countries worldwide [10]. In Italy, approximately one third of all *S. aureus* bloodstream infections are caused by MRSA strains according to the data of the European EARS-NET surveillance system (Figure 1) [11], and this proportion has remained overall stable during the past decade.

MRSA dissemination can be effectively controlled by the implementation of rigorous infection control practices in combination with antibiotic stewardship programs. In the Netherlands, where infection control for MRSA has always been aggressive, the prevalence of this resistant pathogen has remained very low, unlike in many other European countries (Figure 1). In England, where in the early 2000s the MRSA proportions were very high, a nationwide campaign to control MRSA has proved to be very effective at reducing MRSA [12], showing that similar campaigns can be effective at curbing the dissemination of this resistant pathogen even in settings of high prevalence.

Vancomycin-resistant enterococci: a serious problem in some settings and with some patients

Vancomycin-resistant enterococci (VRE), mostly belonging to the species *E. faecium*, are another group of problem resistant pathogens. VRE strains have developed a complex mechanism to escape vancomycin activity, by modification of their peptidoglycan structure. As such, they remain susceptible to only a few antimicrobial agents (linezolid, tigecycline, quinupristin-dalfopristin) and cause difficult-to-treat infections [13]. Severe VRE infections, including sepsis, are typical of immunocompromised patients, and VRE infections can be an important cause of febrile neutropenia episodes in oncohematology wards [14].

In Europe, the prevalence of VRE is overall lower than that of MRSA, and Italy is not among the most affected countries (Figure 2) [11]. However, a recent increase has been documented by the most recent data from the

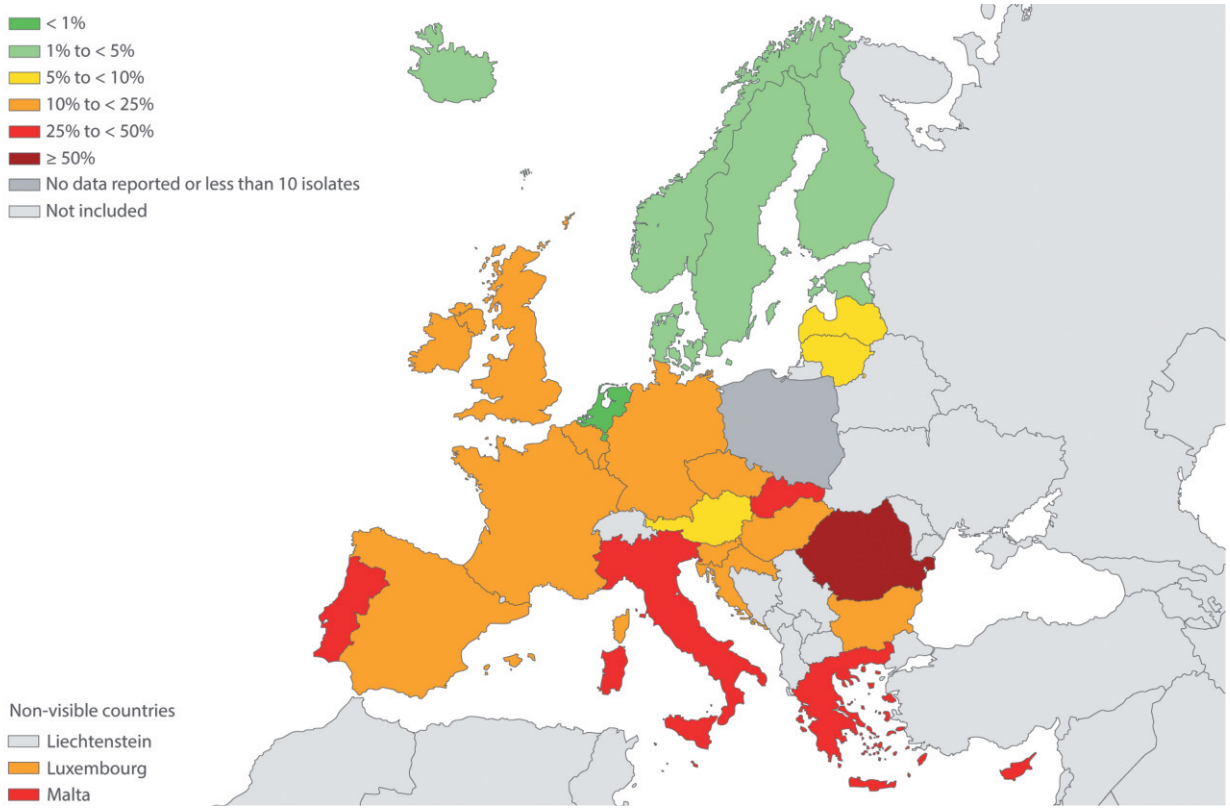

Figure 1. Percentage of methicillin-resistant Staphylococcus aureus among invasive *S. aureus* infections in different European countries from the EARS-NET surveillance system. *Reproduced from: European Centre for Disease Prevention and Control, 2015* [11].

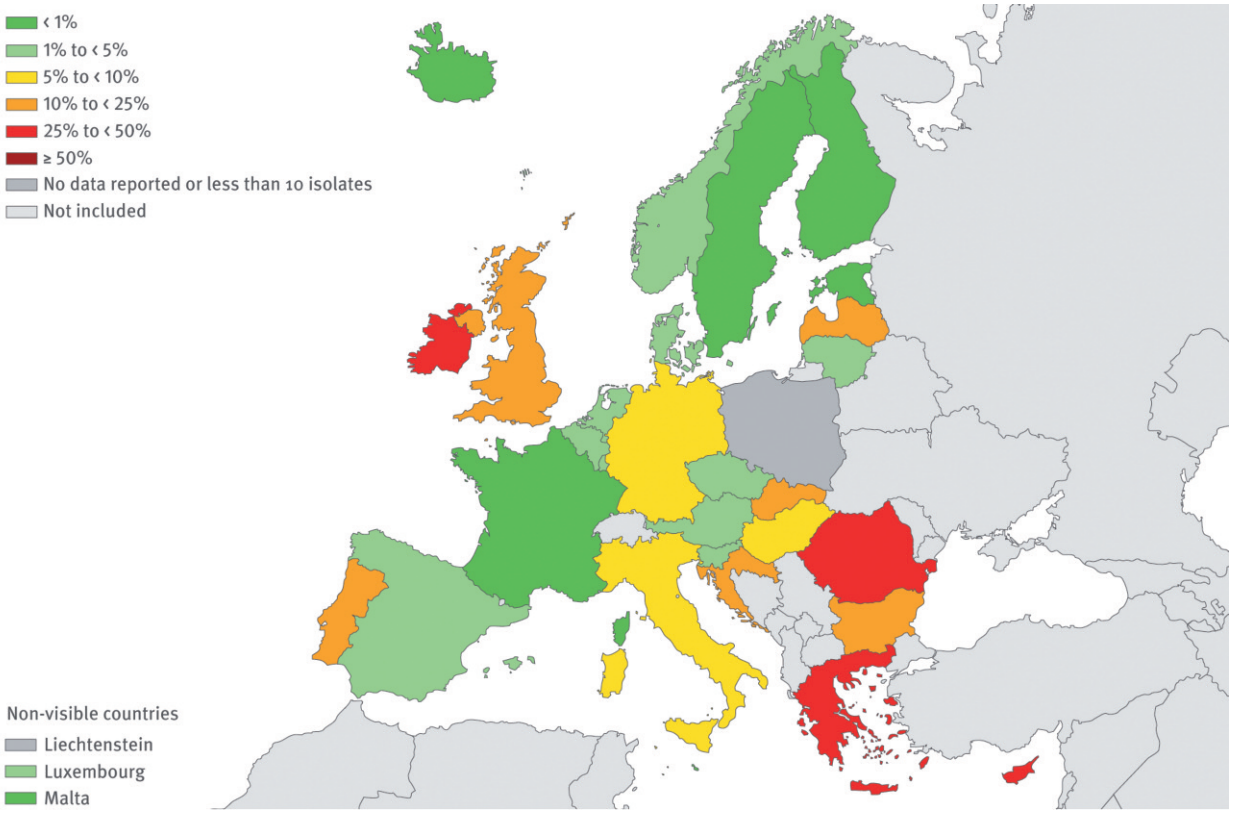

Figure 2. Percentage of vancomycin-resistant *Enterococcus faecium* among invasive E. faecium infections in different European countries from the EARS-NET surveillance system. *Reproduced from: European Centre for Disease Prevention and Control, 2015* [11].

EARS-NET surveillance system [11], suggesting that the importance of VRE should not be discounted.

Pseudomonas and Acinetobacter: the first XDR Gram-negatives causing clinical problems

Pseudomonas aeruginosa is a Gram-negative bacillus living in moist environments, which behaves as an opportunistic pathogen causing infections mostly in hospitalized patients with some impairment of the host defenses. *P. aeruginosa* is a common cause of hospital-acquired pneumonia, including ventilator-associated pneumonia, bacteremia, and catheter-related urinary tract infections occurring in intensive care units (ICU). It is also an important cause of bacteremia in neutropenic patients, and a major cause of burn infections and of chronic respiratory tract infections in cystic fibrosis patients.

P. aeruginosa is naturally resistant to several antibiotics, and exhibits a remarkable propensity to acquire resistance to the various anti-*Pseudomonas* agents. Among Gram-negatives it has been the first species to present serious problems of multi drug resistance [15]. Of particular concerns are strains exhibiting an XDR phenotype including all anti-*Pseudomonas* agents except colistin (the so-called colistin-only susceptible strains), which are very difficult to treat and dangerously close to pan-drug resistance [15]. The prevalence of these strains is not negligible (5-10% in recent surveillance studies) [16]. Some of these strains are now treatable with ceftolozane-tazobactam, a new anti-*Pseudomonas* β-lactam antibiotic that has been very recently approved for clinical use [17]. However, this new drug is not active against strains producing metallo-β-lactamases, which are often susceptible only to colistin and remain a serious clinical problem [17].

Acinetobacter baumannii is a Gram-negative coccobacillus living in the environment, which also behaves as an opportunistic pathogen in hospitalized patients with impaired host defenses. *A. baumannii* can be an important cause of ventilator-associated pneumonia in ICU patients, and can also be responsible of healthcare-associated bacteremia and skin and soft tissue infections [18].

Similar to *P. aeruginosa*, *Acinetobacter* is naturally resistant to several antibiotics and prone to acquire resistance to the relatively few agents that can be used for treating *Acinetobacter* infections. The major issue in this case is represented by resistance to carbapenems, since these drugs are the front-line agents for treating severe *Acinetobacter* infections and carbapenem-resistant *A. baumannii* (CRAB) strains have usually acquired resistance also to the other anti-*Acinetobacter* agents except colistin and, in some cases, tigecycline [19].

In CRAB strains, resistance is usually due to the production of carbapenemases, *i.e.* β-lactamases capable of degrading carbapenems. Successful clones of CRAB producing the carbapenemases OXA-23 or OXA-58 have recently disseminated globally and, in some settings, have largely replaced carbapenem-susceptible *Acinetobacter* strains. In Italy and other Mediterranean countries, according to the most recent data from the European EARS-NET surveillance systems, the majority of *Acinetobacter* strains isolates from invasive infections are CRAB [11], which are causing serious problems in several hospitals.

Carbapenem-resistant Enterobacteriaceae: the ultimate challenge of antibiotic resistance

The family Enterobacteriaceae includes several important pathogens (*e.g*. *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Serratia marcescens*, *Citrobacter* spp., *Salmonella enterica*, *Providencia* spp., etc.) which are overall a common cause of bacterial infections, either in the community and in the hospital setting. A recent point-prevalence survey on healthcare-associated infections (HAIs) in Europe, promoted by the ECDC, revealed that Enterobacteriaceae are the most common cause of HAIs in European hospitals, accounting for more than one third of cases, with *E. coli* and *Klebsiella* being the most important species [20].

Evolution of antibiotic resistance in these species during the past decade has been very rapid and challenging. Originally susceptible to fluoroquinolones and ESC, which were the front-line agents for enterobacterial infections, *E. coli* and *Klebsiella* have rapidly developed resistance to these agents. The global-scale dissemination of ESBLs, in particular, has played a major role in the evolution of resistance to ESC in *E. coli* and *Klebsiella*, with a steady increase of resistance rates that have achieved remarkably high values [11]. These epidemiological circumstances have caused an increased use of carbapenems, given the efficacy of these drugs for the treatment of severe infections caused by ESBL-producing Enterobacteriaceae, followed by a "falling dominoes" effect leading to the emergence and dissemination of carbapenem-resistant Enterobacteriaceae (CRE). CRE are mostly contributed by carbapenem-resistant strains of *Klebsiella pneumoniae* which have acquired carbapenemases of the KPC, VIM, NDM, or OXA-48 type. These strains have a remarkable ability to disseminate in healthcare settings, and have rapidly attained a high-level endemicity in several areas, including Italy [11,21]. This high propensity for dissemination, along with the invariably MDR and often XDR phenotype of CRE strains and the high morbidity and mortality associated with CRE infections, account for the remarkable clinical and epidemiological impact of these strains [22]. Colistin remains one of the few antibiotics active against CRE, and this old drug that had virtually been abandoned since the 1970s due to toxicity issues has now been "rediscovered" as a last-resort agent for treatment of infections caused by XDR strains of CRE and other Gram-negatives [23]. The increased use of colistin has rapidly been followed in turn by a "falling dominoes" effect leading to the emergence and dissemination of colistin-resistant strains of CRE [24,25], further narrowing

the treatment options that remain available for CRE.

The mechanisms of resistance to colistin in CRE strains has recently been investigated by means of whole genome sequencing technologies, and it has been shown that several chromosomal mutations can be responsible for upregulating the endogenous bacterial systems that modify the lipid A colistin target reducing its affinity for polymyxins [26,27]. More recently, a transferable plasmid-mediated gene encoding an enzyme responsible for colistin resistance by lipid A modification has also been discovered [28], raising a considerable concern.

CONCLUSIONS AND FUTURE PERSPECTIVES

The emergence and dissemination of XDR pathogens which remain susceptible to only a few antibiotics, with the occasional detection of PDR strains, has definitely led modern medicine dangerously close to face the end of the antibiotic era. Awareness of this antibiotic resistance crisis has emerged gradually, and the responses have initially been rather slow and partial. However, the call to action eventually prompted several initiatives to address this problem, including a new thrust to programs for discovery and development of new antibiotics.

The fact that MRSA has emerged first as a resistant pathogen of clinical and epidemiological relevance explains the earlier efforts at finding new drugs active against this pathogen. These efforts, started since the mid 1990s, have led to the relative large repertoire of anti-MRSA antibiotics that recently became available for clinical use against this pathogen (*e.g.* linezolid, tigecycline and daptomycin in the 2000s, and more recently ceftaroline, ceftobiprole, telavancin, dalbavancin, oritavancin and tedizolid) [29].

The efforts at discovery and development of new drugs against resistant Gram-negatives have started later, and the anti-Gram-negative pipeline is still much thinner and less advanced. A few agents active against XDR *P. aeruginosa* (*e.g.* ceftolozane-tazobactam) or against several CRE strains (*e.g.* ceftazidime-avibactam, imipenem-relebactam, plazomicin) have recently been approved for clinical use or are in the late stages of clinical development, and represent a major breakthrough in the struggle against resistant Gram-negatives. However, even these new agents will not cover all types of resistant Gram-negatives, leaving out *P. aeruginosa* and Enterobacteriaceae producing metallo-beta-lactamases, and CRAB [30].

Under these circumstances, it is evident that to address the antibiotic resistance crisis it is not possible to rely solely upon new drugs, but an integrated strategy is essential, including strict infection control practices and antimicrobial stewardship policies.

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STATISTICAL ERRORS IN MEDICINE

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Abstract. In this paper we deal with the statistical errors in medicine, analyzing the way they should hide in several phases of experimental process; particularly, we underlined relevant errors in which the researchers can stumble in every steps of the experimental research.

Key words: Statistical errors; medicine.

INTRODUCTION

"Statistics is the mathematics for experimental sciences"; this sentence, often quoted, underlines the relevance of the statistical thought, although it probably undervalues the deductive basis of math, compared to the inductive basis of statistics.

Anywhere, statistical error in medicine should hide in several phases of experimental process; in this paper we will consider the errors in which the researchers can stumble in experimental research; the examples will be drawn primarily from Phase 3 clinical trials [1].

Probably, most relevant statistical errors should be avoided if a statistician is involved in the first of these two steps; regarding this point, sir Ronald A. Fisher declared: "To consult a statistician after a project is finished, is often merely to ask him to conduct a post-mortem examination". Thus, statisticians not only make computations, but they chiefly plan the study design and actively participate in the development of the study protocol [2].

In fact, the most important statistical errors occur in planning stage of an experiment and, *sensu lato* in writing the protocol. In this document, fundamental for both the reliability and the validity of an experimental study, mistakes can hide in many different places; we will analyzed all them, generally following the points of an experimental protocol, as they were described and classified by Pocock [3].

Rationale and general objective of the study

It is important that the rationale (*i.e.* the formulation of the reasons which led to the experiment), as well as the general objective of the study were adequately described and specified.

Although this point seems not involving any statistical issues, nevertheless it represents the basis to correctly define the following protocol point (*i.e.* the definition of the specific objectives).

Relevant errors: to provide a not up-to-date bibliography; to not specify the rationale.

Specific objectives of the study

Once the rationale and the overall goal of the research has been clarified, it is necessary to identify the hypothesis to assess, a process involving the strict definition of both a main objective (which will be the one which will be used to calculate the sample size) and few secondary objectives (related to the main objective, limited in number and defined *a priori*).

The definition of the main objective is necessary in order to establish clearly and in advance which are the basis on which it will be determined the therapeutic efficacy of the treatment, so to avoid methodologically negative phenomena such as post-hoc analysis and data dredging.

We define post-hoc analyses those that are carried out after viewing the data, with a high risk of distortion of results (in particular a high risk of committing false positive errors).

Data dredging (sometimes also defined as fishing expedition) is the phenomenon in which the statistical analysis to be carried out are not fixed *a priori* (*i.e.* in the protocol), in search of significant results (also in this case, with a high risk of committing false positive errors) [4].

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These remarks, obviously, don't imply that post-hoc statistical analyses could not be executed, but that they only should have an exploratory interpretation; they can be a good starting point for new research, to be planned with new protocols.

Relevant errors: to specify more than one main objective; to indicate too many secondary objectives; to commit "data dredging"; to perform "post-hoc" analyses.

Criteria for patient selection

Establishing the criteria for selection of patients is not simply indicate the characteristics that patients should have to be enrolled in the clinical trial, but - most importantly - means to establish the population of future patients who potentially will benefit from treatment.

From a statistical point of view, this coincides with the idea of inference from a representative sample of a population to the same population from which the sample was extracted. The sample is a population in miniature and, in order to allow to draw conclusions on the population from which it is extracted, it shall be representative of that population.

This consideration may seem trivial and obvious, but the experience of many experiments warns us from this hasty judgment.

Regarding the selection criteria of the patients, we can choose between two opposing strategies (possibly opting for a reasonable compromise between the two) each of which has, of course, advantages and disadvantages; see for example [5].

In the first case, we can use very restrictive criteria: this strategy has the advantage of a more accurate comparison between the two treatments, because some variables, potentially confounding, will be kept under control. Moreover, following this strategy, the results will be moderately affected by the variability of the population. On the opposite hand, the disadvantages are represented by the increase in costs (time, money and human resources) for the recruitment of patients, but above all by the limited generalizability of results (because a sample selected with very stringent criteria corresponds to a limited population on which to infer).

Relevant errors: to indicate either too large or too restrictive criteria for the selection of patients; to infer towards a population different the population from which the sample was extracted.

Methods of assessing the patient's response

The principle which is the basis of this point is that all the procedures of evaluation of the patient should be determined and standardized in the protocol, for both the baseline evaluation and the outcomes.

The outcomes are represented by one or a few variables, which should be consistent with the objectives of the trial [6, 7].

In the choice of endpoints the use of surrogate endpoints (*i.e.* a criterion of evaluation of the patient who does not represent the real benefit of the patient, but - in fact - a surrogate) should be avoided, because a theoretical benefit may sometimes not reflect a real clinical benefit; for example, the decrease of cholesterol level does not necessarily correspond to a lower risk of heart attack.

Another important point of these aspects is the need to use assessment tools (*e.g.* clinimetrical tests) which have been validated according to the criteria of reliability, validity, responsiveness and definition of the least clinically relevant difference.

Relevant errors: to indicate outcomes not consistent with the objectives; to indicate surrogate endpoints; to use not validated clinimetrical tools.

Experimental design

We must therefore emphasize that the Phase III studies, in ideal conditions, should be:

- *Controlled*: that is, it must provide for the presence of a control group which can, depending on the experiments , assume placebo or a standard drug for the disease that is intended to cure;
- *Randomized*: that is, the allocation to one of the treatment arms must be based on criteria of randomness;
- *Blinded*: that is, as far as possible, both patients and experimenters must be unaware of the treatment that each patient actually take (*i.e*, if they assume experimental drug, the standard drug, or placebo).

Despite these ideal conditions are very clear, in practice different aspects of the experimental design will have nuanced connotations.

Regarding the experimental design, a Phase III study could be planned according either to a between patients or a within patients design [8, 9].

In "between patients design" the experiment is usually done by comparing two groups of patients, one subjected to the experimental treatment (experimental arm), and the other assuming a standard drug or a placebo (control arm).

It is however also possible to provide more than two experimental arms, for example in the case that there are two separate control groups (each of which assumes a different standard drug for the disease to be treated), or in the case that you want to experience two new therapeutic approaches comparing them with a control group.

In "within patients design" a single group of patients, assuming first the one and then the other treatment, is considered [10, 11]. Randomization, in this case, determines the order of intake of the two treatments.

The main advantage of these experiments is related to the fact that each patient is, in some way, control of himself; this implies that there will be fewer confounding variables to limit the interpretations of the results, because all the variables pertaining to the characteris-

tics of the patient will remain substantially unchanged in the two stages. Ultimately, this will result in a more accurate comparison between the treatments.

On the other hand, being dual participation request from the same patient in the study, this technique can only be achieved in the case of stable disease. In order to prove the stability of the pathology, it is usually foreseen before the start of the experiment a run-in of the patients, during which all assume only placebo.

Moreover, between the first and the second treatment, the patients have a wash-out period, during which they assume no treatment to avoid phenomena of carry-over, (*i.e.* residual effects of the first treatment during the period of second treatment).

We will neglect, in this paper, other less frequent designs, such as factorial e n-of-1 plans.

Relevant errors: to choose not suitable experimental design.

Randomization

Randomization is one of the fundamental aspects for the success of an experiment, and it represents one of the largest commitments for statisticians involved [12, 13].

Randomization is the random assignment of a patient enrolled in a trial to one of the experimental arms. This is one of the focal points of clinical trials because this procedure allows to avoid any form of selection in the allocation of patients, which, if happened, could have biasing effects, with serious repercussions the reliability of the outcome of the trial. Randomization ensures a certain degree of homogeneity in the two groups of patients.

In this paper we only consider the most relevant and used techniques of randomization.

Simple (or complete) randomization: it ideally corresponds to a coin toss, the result of which assigned the patients to arm A or arm B. Of course, from the operational point of view, the process of launching the coin is replaced by the random number generation (procedure now also achievable with the most common spreadsheets): for example, if the value is between 0 and 0.5, the patient is assigned to arm A, while between 0.5 and 1 is allocated to arm B .

The main advantage of this method is that each patient has the same probability of being assigned to arm A or arm B. In other words, there is no other method that allocates patients with as much randomness.

As for the disadvantages, however, this method presents a risk - usually however modest - to obtain unbalanced allocations (meaning by this term a different number of the two groups). Of course, due to the law of large numbers, the risk of imbalance becomes small when the sample size becomes large.

Block permutation randomization: this method, one of the most used in scientific literature, it has been proposed to avoid the risks of unbalance that the simple randomization presents. The idea is to establish some blocks characterized by an even number of patients, for example 4. Of these 4 patients, 2 will have to be assigned to the group A and 2 to the group B. It is clear that there are different orders (technically we define them as permutations) with two elements assigned to group A and two elements assigned to group B. Therefore it will be necessary to consider all the possible permutations of 4 patients. In the case of blocks of 4, the possible permutations will be the following 6:

AABB, ABAB, BBAA, BABA, ABBA, BAAB

In this case, to each random number (or range of random numbers) it will not be associated to a single patient, but to a block of 4 patients.

It is clear that the number of patients for each block may be also larger than 4, but always equal in number to ensure the balance. In fact, at the end of each block, this technique ensures the balance between the groups A and B.

A modest limit is represented by the predictability of the allocation of the last patient of each block (for example, if the first three patients are ABB, the fourth can only be A); in extreme cases, the predictability may also cover half of the patients of each block (for example, is the case of the blocks AABB and BBAA).

Stratified randomization: stratification is a procedure to be applied in conjunction with some techniques of randomization (simple, randomized block, and so on) in order to obtain a homogeneity between groups A and B with respect o the prognostic variables, i.e. variables that potentially could influence the therapeutic effect of the drug or at least the natural course of the disease [14].

Once you have identified prognostic factors with respect to which stratify, the procedure consists of building separate randomization lists for each level (or combination of levels) of prognostic variables.

For example, let's consider the following situation: we know the prognostic role of variables gender (male *vs* female) and age (<50 *vs* > 50). So, there are 4 combinations of levels (strata) of the two variables, namely:

males <50, males > 50, females <50, females> 50.

The stratification will consist in assigning to groups A and B patients belonging to the 4 strata described above, using 4 different randomization lists.

Another case that often uses stratification is represented by multi-center trials, that is where the enrollment takes place in different recruitment centers (sometimes located in different countries). In this case, to avoid distortions related to procedures (evaluations, clinical examinations, etc.) that could be different from center to center and from country to country, we decide to stratify with respect to recruit-

ment centers (that is, each center will have its own list of randomization).

In general, it is considered good practice to operate the stratification only in cases where there is no uncertainty about possible confounding factors. It is also not advisable when the size of the research is very large (in this case, in fact, the law of large numbers alone guarantees a good homogeneity between the groups).

Relevant errors: to not randomize; to choose not suitable techniques of randomization with respect to the experimental situation (*i.e.* simple randomization for small sample size, choice of too many confounding variables for the stratification).

Blindness

Blindness or masking is the way by which patients and clinicians remain unaware of which treatment each patient is assigned. A study conducted in the absence of blindness is defined as "open study".

Blindness should include:

- Patients, to avoid that the improvement or absence of improvement (or simply a public stake) are due simply to psychological effects;
- Clinicians providing treatment, to prevent them from transmitting, with words or nonverbal behavior, greater or lesser enthusiasm to the patient; also to prevent tend to care more closely of the patients assigned to the experimental therapy;
- Clinicians involved in patients evaluation, to ensure maximum objectivity of judgment;
- Statisticians, to avoid any form of manipulation.

Studies can be conducted in single blind when blindness concerns only the patients, and in double blind when blindness concerns both patients and clinicians providing treatment; finally, studies can be conducted in triple blind study, when blindness concerns even the clinicians that evaluate the patients, if they are different from the clinicians providing treatment.

Although triple blindness is the methodologically the ideal model, there are situations in which it is reasonable to use double or single blindness. The following considerations appear valid, according to the scheme formulated by Pocock [3]:

- Ethical aspects: sometimes blindness is not ethically correct (*e.g.* when blindness of patients requires not necessary invasive methods, as a placebo to be administered repeatedly by injection);
- Concrete feasibility: for example, there are cases in which the comparison is made with non-drug therapies (surgery, psychoanalysis, physiotherapy, and so on), with respect to which the conditions of blindness are not feasible;

Anyway, in cases where it is impossible to obtain the condition of blindness for patients and/or for the clinicians providing treatment, we should use a form of partial blindness, concerning at least the clinicians that assess the final evaluation of patients.

Relevant errors: to not perform double blind studies when they are feasible; to not guarantee blindness of clinicians that assess the final evaluation of patients, in case of single blind and open study.

Placebo and active control

Depending on the case, the control group of a trial may assume either a standard therapy (usually, the therapy normally recognized as the best one for the disease) or a placebo.

Thus, trials can be either active-controlled or placebo-controlled.

The placebo is a pharmaceutical formulation that is completely identical to that of the experimental drug for what concerns each organoleptic character and appearance (flavor, color, smell, packaging), except that in the active principle, completely absent in the placebo.

The purpose of the placebo is to evaluate the effect of the experimental drug actually attributable to the drug itself, and therefore net of the placebo effect. We define "placebo effect" the improvement that the patient simply shows because of the belief of assuming therapy [15].

Placebo effect occurs together to the pharmacologic effect during any therapy; in other words, a certain amount of improvement related to the placebo effect occurs during any therapy, and is therefore net of this dimension that the experimental drug should be evaluated.

From an ethical point of view, it is clear that we should use a placebo control only if a drug considered valid for the disease is not available.

Finally, there is a case where placebo is not used to take under control the placebo effect, but it has the purpose of ensuring the blindness to treatment. Suppose that an experimental drug is compared with a standard drug, and that the two drugs have different formulations and routes of administration: in this case, we can use the double-dummy technique, so that each patient takes the medication given to him along with a placebo formulated for the route of administration of the other active drug. In other words, a patient assigned to the experimental drug also will take a placebo quite similar (even for the route of administration) to the standard drug; *vice versa*, a patient assigned to standard drug also will take a placebo quite similar (even for the route of administration) to the experimental drug.

Relevant errors: to plan a placebo-controlled trial when a standard therapy exists.

Sample size

The sample size of patients to be enrolled in a clinical trial is the result of an *a priori* analysis. The basic principle is that the size should result from a compromise between two opposing views:

- the first is that research with too few patients have a high risk of producing false negative results, *i.e.* not

to highlight differences between the comparing treatments, for simple effect of "lack of data";
- the second is that too large sample size should provide positive results from the statistical point of view (*i.e.* statistically significant results), but not relevant from a clinical point of view.

Nowadays, methodologists suggest to limit the use of the two terms to particular sections of the clinical reports; they recommend that the term "statistical significance" should be reserved to the "Results" section and the term "clinical relevance" to the "Discussion and conclusions" section [16].

Practically, the definition of the sample size of a search is calculated first by means of statistical methods [17, 18, 19] and further basing on feasibility considerations.

Statistical methods

In order to correctly use of the statistical formulas for the computing of sample size, we have to answers to five key questions:
- What is the main criterion for measuring the outcome? From a statistical standpoint, this question implies that the classification of the response variable (*i.e.* qualitative: nominal or ordinal; quantitative: discrete or continuous) is known.
- Which statistical test should be used to analyze the data? The choice of the test obviously depend on the type of the considered response variable.
- What results are expected from the control group? The answer to this question can be derived for example from the literature, by any pilot studies of the trial, from the personal experience of researchers, and so on.
- What is the minimum clinically relevant difference? That is, what may be the minimum difference in response between the control and the experimental group to be considered relevant from a clinical standpoint?
- What is the degree of statistical safety that must be achieved? Here, the term "degree of safety" refers to the acceptable risk of making errors of the type: false positive (or Type I errors) and false negatives (or Type II errors). These risks are indicated by α (or significance level of the statistical test) and β (though usually in the protocols you prefer indicate 1-β, that the power of the test), respectively. Usually, in clinical trials, these risks of errors are fixed in $\alpha = 0.05$ (5%) and $\beta = 0.10$ or 0.20 (10% or 20%, corresponding to a power of 90% or 80%) [20].

Once the answers to these questions are given, we use suitable formulas that provide the number of patients required for the experimental trial.

Feasibility considerations

Once the statistical calculation is performed, it will be necessary to assess the feasibility of the trial; in particular, we have to establish whether the calculated number of patients is compatible with the expected rate of recruitment, if the economic funds are sufficient, and so on.

If such a realistic assessment is unfavorable, it is possible to operate - depending on the case - in different ways, such as:
- to increase the number of involved centers and investigators (in order to increase the rate of enrollment);
- to decrease the scientific safety degree (*i.e.* increase the risks a and b, but not exceeding the standard level of 5% and 20%, respectively).

Finally, if the previous strategies were not viable, it will be necessary to give up the search, because it would be unethical to start a research not leading to conclusive and reliable results [17].

Relevant errors: to not establish sample size; to not properly use the suitable formulas; to not consider the feasibility of the trial; to confound statistical significance and clinical relevance.

Monitoring

In case of long duration trial, we can perform some "interim" statistical analyses [21].

The purpose of these analyses can be schematically described in three following fundamental points

Monitoring the quality of research: it consists in evaluating the adherence of the researchers to the protocol. Different indicators of "loss of quality":
- Changes in the rate of enrollment: an increase rate could point out an easing of the eligibility criteria; a decrease rate could indicate a lack of enthusiasm of the investigators;
- Changes in the distribution of patient characteristics: if the change takes place in the time and manner equals between the two arms, it may indicate an easing in the eligibility criteria; if it differentially concerns the two arms, it may indicate the breaking of blindness conditions;
- Changes in the level of response to treatment: if it concern both the arms (experimental and control) it may indicate a modification in the patient assessment; if it only concerns the experimental arm, it may be the signal of breaking of blindness conditions.

Monitoring side effects: usually, an independent monitoring committee evaluates the possible side effects (both expected and unexpected).

Monitoring the efficacy of the treatment: the purpose of these statistical analyses is to assess whether, before the experiments end, there is evidence about the greater or lesser effectiveness of one treatment.

In other words, the question can be asked by an ethical point of view: is it appropriate to continue to enroll patients in one arm of the trial if there are already significant evidence that the corresponding treatment is less effective than the other?

The problem, in this type of monitoring, is that each statistical analysis performed goes to raise the risk of false positives or errors of type I (remember that with the level of significance, normally fixed at 5%, the experimenter assumes the commitment to maintain this level within the risk of making a false positive, which is a type I error) [22].

The protocol should therefore fix the number, the manner and timing in which these interim analyses have to be performed during the trial. However, there are two main approaches to the problem [23, 24, 25]:

Group sequential design: it is based on the idea of performing repeated significance tests. In this case, to avoid exceeding the overall significance level fixed (usually 5%), and once that the required power (usually 80% or 90%) are known, a nominal significance level (lower than the global one) is fixed by means of special tables, in order to maintain the overall level within the preset limits.

Continuous sequential design: it is based on the idea to perform an analysis for each patients that ends the trial. It is obvious that the problems of increased risk of type I error result amplified. From a practical point, a purpose-built chart is used for this approach; see for example [26].

Relevant errors: to perform interim analyses not planned in the protocol; to use incorrect methods to control the global risk of type I error.

Deviation from protocol

A protocol must contain the way we deal with deviations from the protocol itself. Notwithstanding the fact that a protocol should be respected as much as possible, it should be provided for any violations that may occur and establish standard procedures to address them [27, 28].

It is clear that when deviations assume catastrophic proportions (for example, the drug proves to be unstable, some data were invented, and so on) there is no other solution but to close the trial. But when deviations involve individual patients, there are some possible approaches.

The main possible violations are the following:

- enrollment of not eligible patients, in which case the opinions are varied, but usually the idea of excluding them from the final statistical analysis prevails;
- incomplete adherence to therapy: this situation may be due to a lack of cooperation of the patient, or sometimes to a change of therapy determined by the attending physician; in this case the possible approaches are different and they will be described below;
- withdrawal of patients (dropouts): the patient may withdraw from the trial at any time, by choice or even in the judgment of the treating physician who believes having to transfer him to other therapy. Notwithstanding the need to continue the patient evaluation until the end of the study (if possible), also in this case the possible approaches are different and they will be described below.

Thus, the problem is the following: patients who did not adhere fully to the protocol or who have retired, have or not to be included in the final data? There are two possible approaches to the problem:

Per protocol analysis (also named drug efficacy approach, or explanatory approach): in this his approach we take into account only patients who strongly adhere to the protocol. This approach is characteristic of phase II trials, *i.e.* studies aiming to an initial assessment of the therapeutic effects of the experimental drug evaluation that can be obtained only under a perfect adherence to the treatment protocol, as well as phase I trials, *i.e.* studies that aiming to determine the dosages based on the toxicity and the kinetics of the drug; in both cases, we need information that can be obtained only under conditions of perfect compliance.

Intention to treat analysis (also named pragmatic approach): in this approach we taken into account all the patients who participated in the study, whether or not they adhered to the protocol and whether or not they completed the study itself (of course provided we obtain the final evaluation). The only exception is represented by patients who have withdrawn before starting treatment. The idea is that if we exclude patients withdrawn or with poor adherence to the protocol, we would overestimate the effect of treatment (in fact, it is logical to think that withdrawals and no adhesions to the protocol are mainly caused by a dissatisfaction with the treatment). Furthermore, this approach tends to evaluate the effectiveness of the drug in an environment similar to standard clinical practice, where it is the norm that a patient tends to adjust the dose, change the timing of intake, discontinue therapy for short periods, and so on. For all these reasons, this is the approach characteristic of the phase III trials. However, we have to point out that, even at this stage, it is usual to combine the intention to treat analysis and the per protocol analysis, to get more information on the effectiveness of the drug.

Relevant errors: to not provide a way to deal with deviations from protocol; to choose incorrect approach with respect to the phase of the trial.

Plan for statistical analyses

In this section, we will list briefly some points that should be taken into account in the drafting and analysis of a protocol [29].

Descriptive indices (arithmetic mean, geometric, harmonic, median and mode, range, variance, standard deviation, coefficient of variation, interquartile range) should be chosen in an appropriate manner, taking into account the scale of measurement of the variables, the presence of censored data, the asymmetry of the distributions.

Plots should be chosen as appropriate in relation to the phenomenon being described, honest in choosing the axis scales, and if possible they should consider the individual observations rather than the values grouped into classes.

Measures of treatment efficacy should be always provided, and they should be specified in the protocol (*e.g.* ARR, or Absolute Risk Reduction; RR, or Relative Risk; RRR, or Relative Risk Reduction, NNT, or Number Needed to Treat; OR, or Odds Ratio).

Outliers (values strongly extreme into the distribution) should be excluded from the statistical analysis only if there are strong doubts about their credibility; specific statistical tests (such as the Dixon test) have to be used to support this decision.

Each estimate should be accompanied by a confidence interval, that takes into account uncertainty of the estimate. The technique of calculation of the confidence interval should take account of the distribution of the variable (for example, if the distribution is not normal Gaussian, one should make use of non-parametric techniques, such as the quantile method or bootstrap technique).

Statistical tests should only respond to questions submitted in advance (*i.e.* in the protocol) to avoid data dredging phenomena [30]. Other tests (post-hoc tests) should have a pure exploratory value.

Statistical tests should be selected properly based on the scale of measurement of the variables.

Statistical tests should always be performed in two-tailed version, unless there are reasonable reasons to choose the one-tailed form.

For each statistical test we have to verify the so-called parametric assumptions (such as normality and homoscedasticity for the execution of the Student's t test and of Anova). If these conditions are not satisfied, we can proceed to appropriate data transformations [31] and, in case of further failure in the basic assumptions, we have to perform the corresponding nonparametric test [32].

In case of multiple endpoints, we should proceed in order to avoid an excess risk of false positive error; we can choose either to apply Bonferroni's criterion (subdividing the overall significance level for the number of endpoints considered) or to apply multivariate analysis techniques (which consider simultaneously all endpoints) or, finally, to construct an overall score from single endpoints (thus obtaining a single response variable summarizing and takes account of all endpoints) [33].

Relevant errors: to use inappropriate descriptive indices; to use inappropriate or not-honest plots; to not provide measures of treatment efficacy; to exclude outliers without a valid justification; to not provide a confidence interval for the estimated values; to perform data dredging; to choose not suitable statistical tests; to perform one-tailed tests without a valid justification; to perform parametric statistical test without verification of the assumptions; to not use corrective methods in case of multiple endpoints.

CONCLUSIONS

In this short review, we have tried to show that statistical error in medicine should hide in several phases of experimental process, and not only in the execution of statistical tests.

Thus, statisticians should be also involved in the experimental planning, in order to avoid many types of error.

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STATISTICS, BIOMEDICINE AND SCIENTIFIC FRAUD

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Abstract. A consistent fraction of published data on scientific journals is not reproducible mainly due to insufficient knowledge of statistical methods. Here, we discuss on the use of proper statistical tools in biomedical research and statistical pitfalls potentially undermining the scientific validity of published data. Apart from unaware errors, a growing concern exists regarding data fabrication and scientific misconduct. Indeed, the social impact of false scientific data can be largely unpredictable and devastating, as shown by the worldwide dramatic effects on vaccinations coverage following a retracted paper published on a highly authoritative medical journal. Unfortunately, statistics shows a quite limited power in detecting false science, although a few statistical tools, such as the Benford's law, are known. Taken together, statistics in biomedical sciences i) is a powerful tool to interpret experimental data; ii) has limited power in detecting false science; and iii) first and foremost, is not the result of a simple "click of a mouse", but should be the result of accurate research planning by experienced and knowledgeable users.

Key words: Biomedical sciences; scientific fraud; scientific misconduct; statistics; statistical errors; statistical inference.

BACKGROUND

Statistics can be defined as a "battle against variability" (Prof. Claudio Scala). A consistent fraction of published data on scientific journals is not reproducible [1]. This could be mainly due to insufficient knowledge of statistical methods. On the other hand, there is a concept, largely attributed to the physicist Ernest Rutherford, supposedly saying, "if your experiment needs statistics, you ought to have done a better experiment." There is a lot of truth to this statement when working in a field with high signal-to-noise ratios. Nevertheless, statistical analyses are needed in all fields with a lower signal-to-noise ratio to properly quantify confidence in the study conclusions [2]. Indeed, in the absence of variability there would be little need for data analysis. Variability is avoidable in experiments due to both biological and technical effects [3]. Although biological variability needs to be maintained in order to allow generalization of the results to the population of interest, the tools that allow replicable results to be obtained despite the biological variability are experimental control, randomization, blocking and replication [3]. It is important to distinguish between sources of variation that are merely nuisance factors from those required in order to assess the variability of the effects in the population. The goal for every researcher should be to minimize the confounding factors of the experiment, as well as to sample and quantify the real biological variability in order to generalize conclusions and robustly determine uncertainty in estimates.

Of course, the concept that "there is no statistics without data" cannot be overstated. However, scientific intuition may start from a single case, a context where statistics is, by definition, not applicable. Nevertheless, a single anecdotal evidence can be a good starting point for interesting scientific discovery. For instance, the observation of a sudden drop in the pulse oximeter perfusion index during a transfer flight of a former premature male infant in a condition of severe clear air turbulence led one of the present authors (C.D.F.) to consider pulse oximeter perfusion index as an early marker of subclinical hypoxia. This finding was subsequently replicated on a large cohort study confirming early data (Figure 1) [4]. In a different context, fetal heart rate hypovariability in a singleton term pregnancy led the same author to interpret the finding as a potential marker of a prenatal inflammatory process [5].

On the opposite, the emerging "omics" sciences (*i.e.*,

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genomics, transcriptomics, proteomics, metabolomics) are facing researchers with important challenges to one of the main principles of statistics *i.e.*, the assumption of data independence (Figure 1).

In the present lecture, we discuss on the emerging need for using adequate statistical tools in biomedical research and the chance to unveil unaware statistical errors possibly in undermining the scientific validity of published data, as well as a mention to the, quite limited, statistical tools to unveil possible scientific frauds.

UNAWARE STATISTICAL ERRORS

"Experience has taught statisticians that data can be misleading and, even worse, wrongly give the semblance of objectivity" [6]. This sentence from Prof. David Rossell (Department of Statistics. University of Warwick, United Kingdom) efficaciously expresses all the concerns of statisticians towards current statistical standards. An important wake-up call in the literature is the alarming rate of non reproducible scientific published findings. In a study of 2011, only 20-25% of preclinical studies were found to be reproducible [7], as well as only 11.3% of basic cancer biology papers [8]. The problem of the poor reproducibility of scientific studies has attracted the attention of the National Institutes of Health [9], as well as non-experts [10]. One of the reasons behind this lack of reproducibility certainly lies in a poor understanding of statistical tools and concepts.

In our own experience, one of the most common mistakes is involving statisticians at the end of research instead that at its beginning. A certainly non-exhaustive list of common statistical errors from our personal experience is reported in Table 1.

Indeed, accurate experimental design, randomization, bias control should intervene much before the experimental procedures are carried out. It has even been suggested that, in genetic association studies, there is a positive relationship between individual study bias and journal impact factor [11]. Therefore, journal prestige and influence are not mandatorily indicators of high quality in research [12].

Unexperienced investigators are likely to make common mistakes, such as "*P*-hacking" [13], overuse of statistical hypothesis testing, and overreliance on the standard error of the mean (S.E.M.) (Table 1) [2]. In particular, there is abundance of confusion and criticism about the meaning of *P* value [14], and understanding of the word "significant", which is often misunderstood. Significant has two distinctive meanings in science: one is that a *P* value is less than a preset threshold (usually 0.05); the other is that an effect is large enough to have

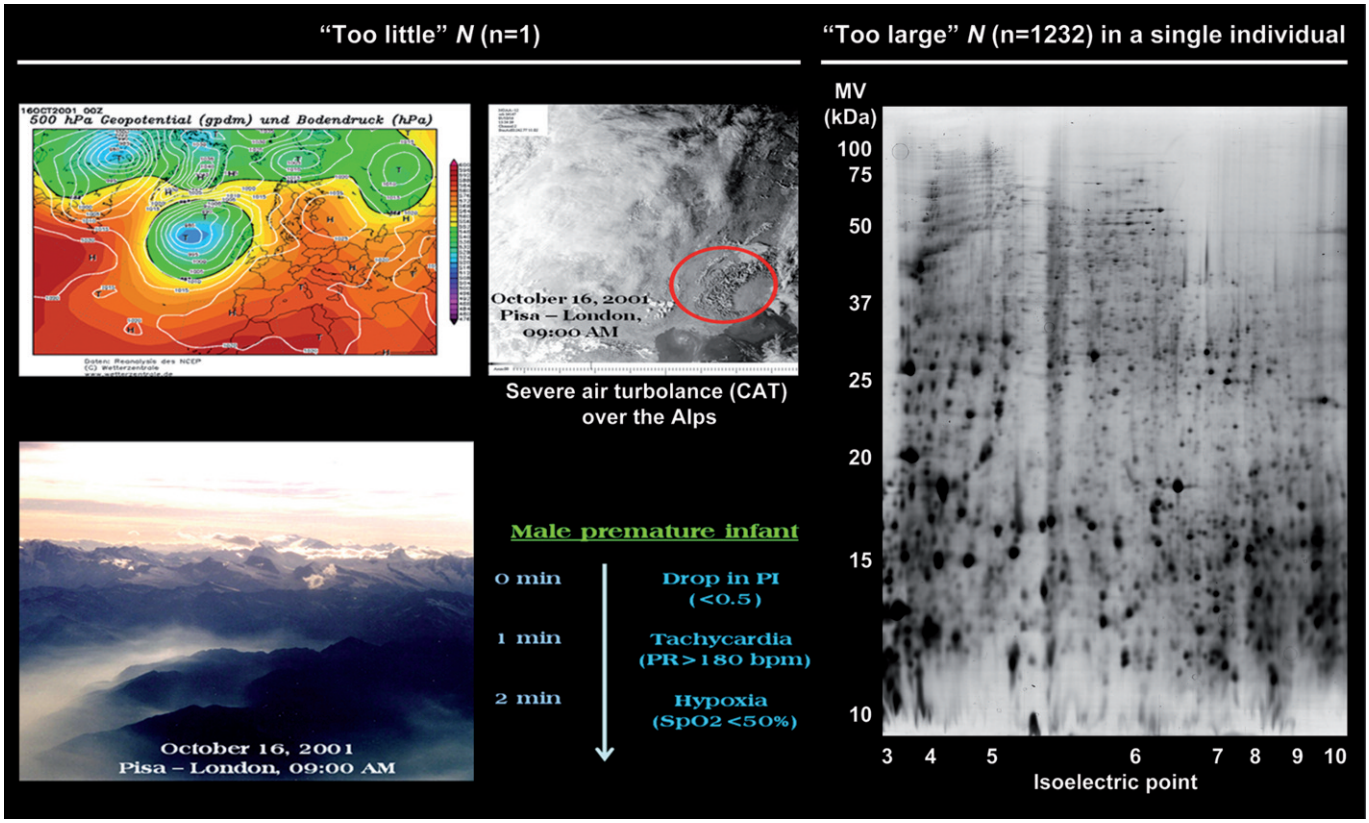


Figure 1. Common challenges in biomedical statistics.

a substantial biological physiologic or clinical impact. These meanings, completely different, are much too often confused. Indeed, the *P* value was never meant to be used the way it is used today [15]. The *P* value could be defined as the probability of seeing an effect as large as or larger that observed in the current experiment if the null hypothesis is true. Nevertheless, the *P* value gives no information about how large the difference (or effect) is. Historically, when the United Kingdom statistician Ronald Fisher introduced the *P* value in the 1920s, he did not mean it to be a definitive test. Fisher intended it simply as an informal way to judge whether evidence was significant in the old-fashioned sense of "worthy of a second look" [15]. Even at that time, famous mathematicians and statisticians Jerzy Neyman and Egon Pearson heavily criticized the *P* value as "worse than useless" [15]. In the meantime, other researchers, mostly non-statisticians, have created a hybrid system that squeezed Fisher's *P* value into Neyman and Pearson's reassuringly rigorous rule-based system, thus giving birth to the famous (or "infamous", according to several statisticians) preset value of 0.05 considered as "statistically significant". Actually, the only thing a *P* value could do is to summarize the data assuming a specific null hypothesis. A *P* value of 0.01 actually corresponds to a false-positive rate (type I error) of at least 11%, depending on the underlying probability that there is a true effect, while a *P* value of 0.05 increases this chance to at least 29% [16]. The underlying concept is that significance is no indicator of practical or biological relevance. The term *P*-hacking has been popularized by psychologist Uri Simonsohn and colleagues, intending data-dredging, snooping, fishing, significance-chasing and double-dipping [15]. In different words, *P*-hacking is a

Table 1. Most common errors encountered in statistics applied to life sciences.

Critical issues (personal experience)
Poor research design
Involving statisticians at the beginning of research, not at its end
Lack of a priori sample size calculation/effect-size estimation (statistical power)
Use of wrong statistical tests
Tested null-hypothesis (H_0) not rigorously stated
Study aims and primary outcome measures not clearly stated or unclear
Numerical information given to an unrealistic level of precision
Use of mean ± S.D. to describe non-normal data
Giving S.E.M. instead of S.D.
Lack of reporting on confidence intervals
Failure to prove test assumptions (*i.e.*, normal distribution)
Poor understanding of *P* values ("*P*-hacking" effect)
Significance unsupported by data analysis
"Non-significant" ≠ "no effect" ("non-significant" ≠ "negative")
Statistical significance ≠ biological or clinical relevance
Disregard for Type II error for non-significant results and multiple testing problem
Missing data issue
Inappropriate control group
Failure to use and report randomization
Too many variables involved
Association ≠ cause-effect relationship
Failure to discuss sources of potential bias/confounding factors
Understanding that statistics is not the simple result of a "click of a mouse"

way "to torture the data until it appears to support some pre-conceived idea" [15].

While standard deviation (S.D.) quantifies variation among a set of values, (*i.e.*, S.E.M., computed by dividing the S.D. by the square root of the sample size) does not. Indeed, the range mean ± S.E.M. is a confidence interval, depending on the sample size. This means that range is a 68% confidence interval of the mean when applied to large samples, but can become a 58% confidence interval with N=3 [2].

Doubtless, one of the most common statistic pitfalls in medical research is a failure to prove test assumptions (Figure 2). "Association is not causation" is a quite critical concept that must be kept in mind when drawing conclusions from statistical inference. Besides that (and prior to that), it is critical to test assumptions for any correlations. For instance, homoscedasticity is one of the critical assumptions in linear regression analysis. Figure 2 exemplifies a possible pitfall in the relationship between neonatal birth weight and gestational age at birth. As it can be seen from the scattergram plot (Figure 2), the principle of homoscedasticity is clearly not matched. Further analyses show a non-normal residual errors distribution (Figure 2). The other fundamental requirements for linear regression are the following: continuous variables; a linear relationship between variables; lack of outliers far removed from the mass of data; and independence of the observations. Therefore, the common linear regression analysis cannot be applied in this case. Incidentally, alike the incidence of coronary heart disease and cerebrovascular disease [17], birth weight does not follow a gaussian (*i.e.*, normal) distribution, but is more likely to follow a Weibull hazard model, as birth weight could be considered the final outcome of the "battle for survival" of the fetus.

For any study, the relevance of the research design cannot be overstated. In particular, it should be kept in mind that no statistics - and no statisticians - could ever remedy a poor study design. The example in Figure 3 tries to illustrate this concept by a statistical "divertissement" based on a recent news regarding the lack of natural hibernation in hedgehogs supposedly related to climate change [18].

If we simply test the difference between average temperature in earlier periods (*i.e.*, from 1943 to 1957 in Siena, Italy) *vs.* current data (years 2007-2015), a difference could hardly be detected (Figure 3). However, a more accurate research on the biological signal for the hedgehog natural hibernation appears to be the winter to spring (October to March) temperature. When this information is applied to the same database, the difference between historical and current temperature becomes highly evident (Figure 3).

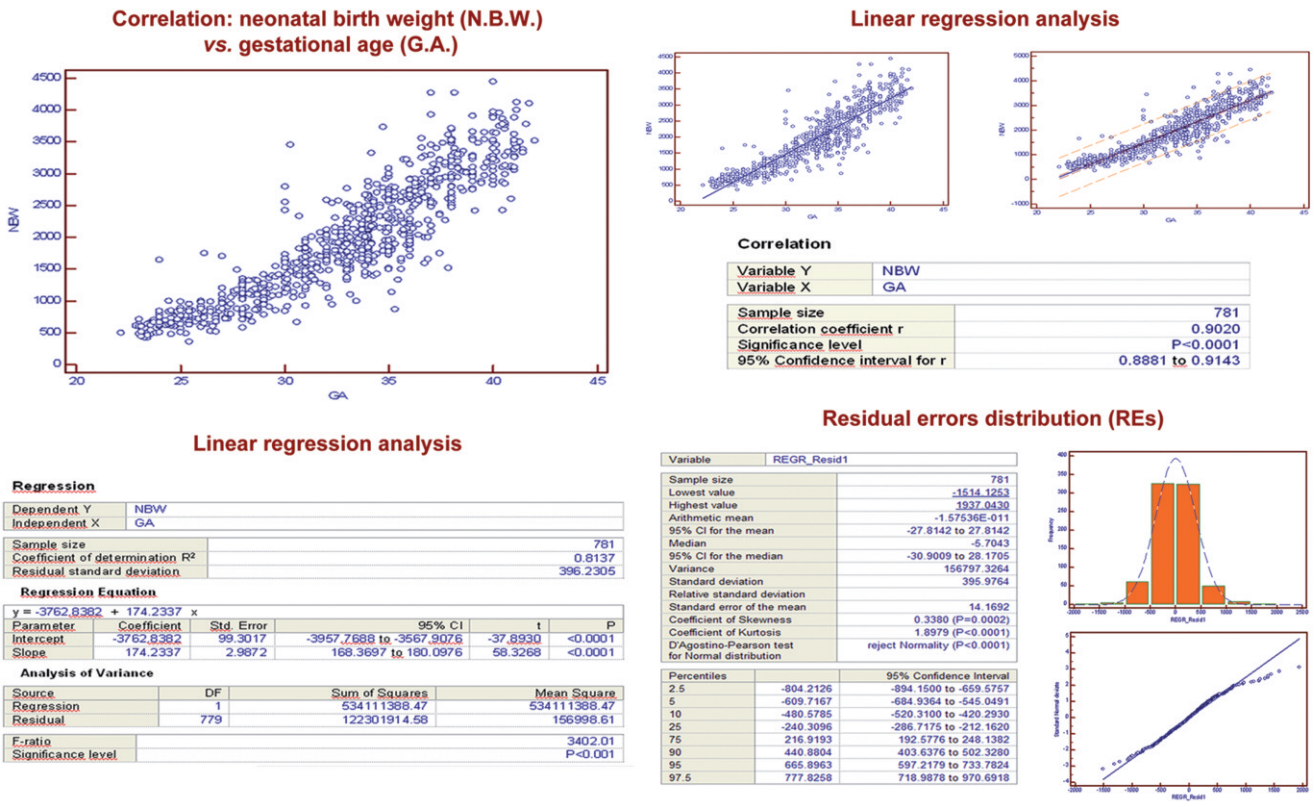


Figure 2. Pitfalls in linear regression analysis.

Another important, often misunderstood, concept is how large N (*i.e.,* sample size) should be in order to allow a meaningful statistical analysis. Of course, this is an ill-posed question. Actually, sample size critically depends on data variance. Nevertheless, Figure 4 illustrates the variation of statistical precision as a function of sample size. The data set refers to N=1457 temperature data from the Siena meteorological archive. A random selection from the whole data set was made in order to have a progressively increasing N (eight groups with sample size ranging from N=5 to N=1457) (Figure 4). Subsequently, deviations from the whole data set were calculated in terms of variance, variance ratio, error mean percentage and error median percentage. Surprisingly, deviation from the real median or mean values largely and unpredictably fluctuates with N ranging from 5 to 100 (mean error: from 2.11% to 35.84%; median error: from 8.2% to 68.6%). Only about a third of the total sample size is evaluated, deviations became negligible (mean error: 0.28%; median error: 2.98%; variance ratio: 0.99). A current challenge to present time statistics is represented by the so called "big data". Indeed, the world's capacity to collect, store and share data has hugely raised in recent times if it is true that 90% of the data in the world has been generated in just a couple of years [19]. Matching with such extremely large data sets will require active methodological research, as well as training a new generation of scientists to develop and deploy the resulting strategies [6].

Moreover, the fractal nature of life [20], represents a further challenge to statistics [21]. Although, it is difficult to understand the real underlying reasons of fractality of nature, the current explanations include the following: i) likely an evolutionary imperative; ii) critical for optimal substrate distribution and metabolic efficiency; iii) robustness and resistance to random errors [22-24].

SCIENTIFIC FRAUD AND SCIENTIFIC MISCONDUCT

The misuse of statistics in medical research can be considered both unethical and having serious clinical consequences [25, 26]. As a result, valuable efforts have been made to enhance the quality of statistics in medical journals [27, 28]. Despite several efforts, little evidence exists that statistical standards have improved over time [29].

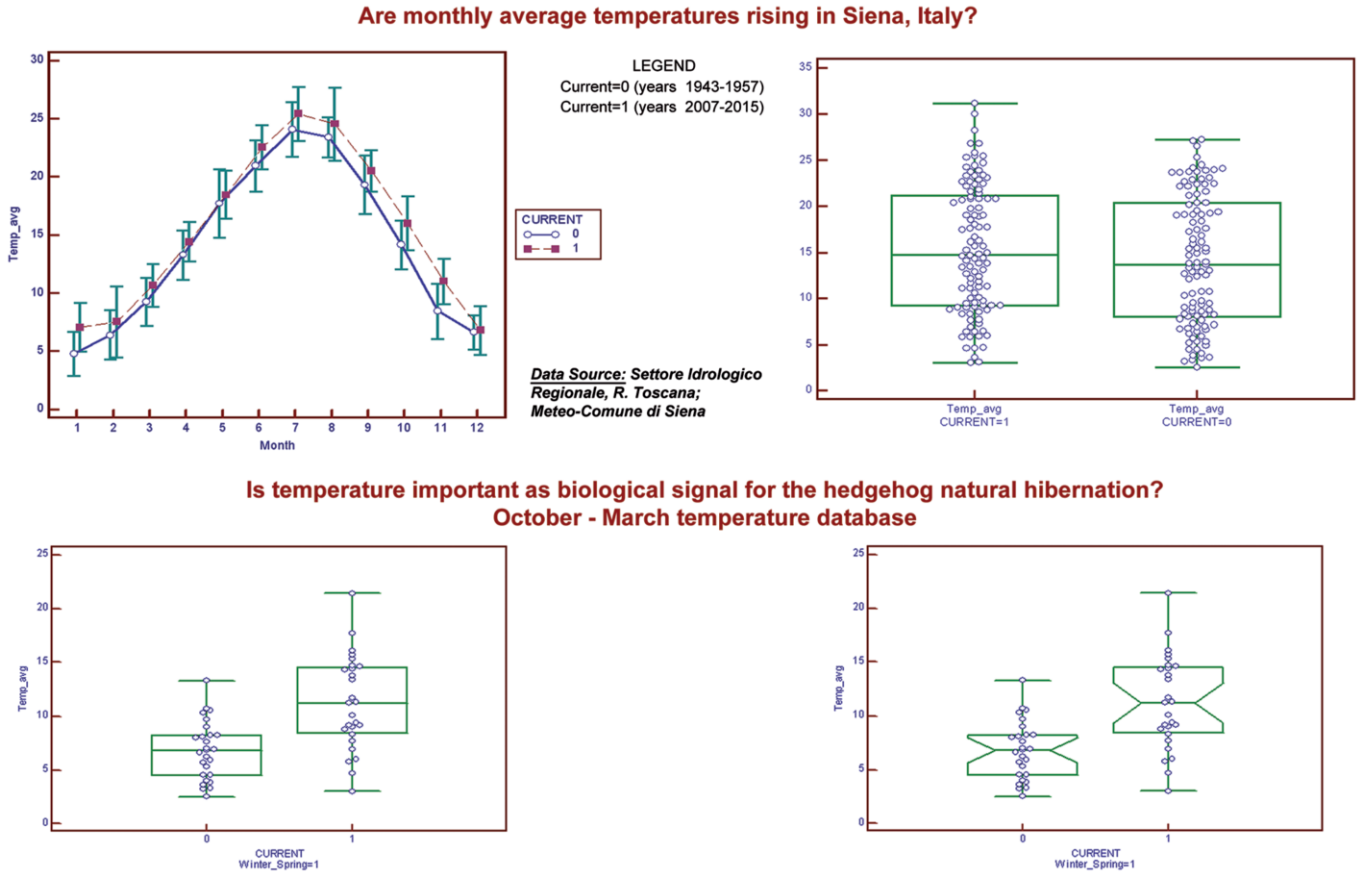


Figure 3. Average monthly temperature and missing of natural hibernation in hedgehogs (Siena, Italy, historical archives).

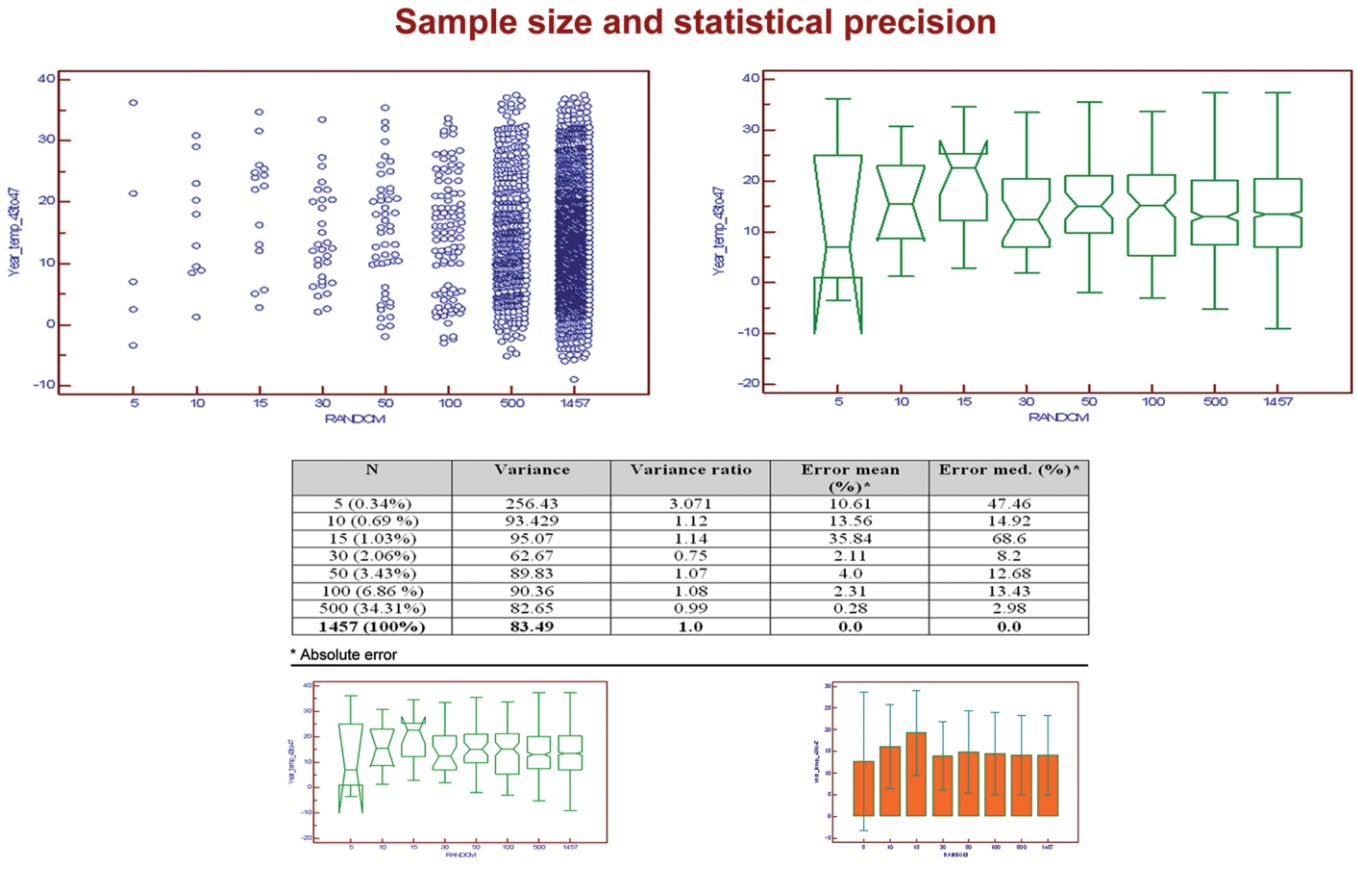
Data fabrication is another very relevant issue in scientific publishing. A meta-analysis of surveys asking scientists about their experiences of misconduct found that, on average, about 2% of scientists admitted to have fabricated, falsified or modified data or results at least [30]. Considering that these surveys ask sensitive questions and have other limitations, it appears likely that this is a conservative estimate of the true prevalence of scientific misconduct [30].

Despite the discovery and development of immunization has been a singular improvement in the health of mankind [31], confidence in vaccination has been declining in recent years. The current global anti-vaccine movement can be linked to a single retracted paper [32] published on February 28, 1998 in the highly respected Lancet journal. Sample size on the original paper was N=12. Further investigations on the leading author uncovered dishonest and unethical medical practices, resulting in losing his medical license. Although a careful review of publicly available information makes it clear that Wakefield's claims regarding vaccine safety are wrong [33, 34], vaccination rates plummeted in the United Kingdom from 92% in 1996/1997 to 80% in 2003/2004 [35], and outbreaks of vaccine preventable diseases followed [36, 37]. Measles remains of high clinical importance given that: i) infection leads to long-lasting immune suppression; ii) complications are of high frequency and severity; iii) there is no specific antiviral treatment; iv) vaccination is effective, cost-effective, and safe, with no demonstrated link between the measles vaccination and autism; v) can be eliminated from a population requiring a coverage with 2 doses of vaccine at rates of 93% to 95%; and vi) endemic transmission can be reestablished if rates of vaccination fall below the elimination threshold [34].

Although the Wakefield's controversy is a very good example of the negative impact of false science on real life, unfortunately, statistical review could do very little against publication of fabricated data. Nevertheless, some hope may originate from the so called Benford's law, *i.e.*, the form of logarithmic distribution of digits in statistical data when are produced by natural or social processes [38]. Indeed, Benford's law has been successfully applied to detect fabricated or falsified data [39] in tax or other financial reports [40-42].

CONCLUSIONS

Statistics is widely accepted as a powerful tool in the scientific research process, with a huge increase in the



N	Variance	Variance ratio	Error mean (%)*	Error med. (%)*
5 (0.34%)	256.43	3.071	10.61	47.46
10 (0.69 %)	93.429	1.12	13.56	14.92
15 (1.03%)	95.07	1.14	35.84	68.6
30 (2.06%)	62.67	0.75	2.11	8.2
50 (3.43%)	89.83	1.07	4.0	12.68
100 (6.86 %)	90.36	1.08	2.31	13.43
500 (34.31%)	82.65	0.99	0.28	2.98
1457 (100%)	**83.49**	**1.0**	**0.0**	**0.0**

* Absolute error

Figure 4. Data numbers and statistical precision.

use of statistical methods for a wide range of medical journals over the past four decades [43-45]. On the other hand, there is also wide consensus on generally low standards resulting in a large proportion of published medical research containing statistical errors [46].

Overall, statistics in biomedical sciences: i) is a powerful tool to interpret experimental data; ii) has little efficacy in detecting false science; and iii) is not the result of a simple "click of a mouse", but should rather be the final result of accurate research planning by experienced and knowledgeable users.

ACKNOWLEDGEMENTS

This *Lectio Magistralis* is dedicated *in memoriam* of Prof. Mario Comporti (1935-2014), an international pioneer in the exploration of oxidative stress in disease, co-editor-in-chief of the Journal of the Siena Academy of Sciences since 2009. He strongly believed in the key importance of scientific data and the critical importance of unveiling methodological influences that could negatively affect data reproducibility.

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MINI-INVASIVE SURGERY IN THE FIRST THREE YEARS OF LIFE

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Abstract. *Background:* In the last 30 years mini-invasive surgery (MIS) has been widely used becoming an important and irreplaceable method, safe and highly reliable, in both the diagnostic and therapeutic treatment, for a variety of conditions in both the child and the newborn. This has led to a significant increase of the use of this approach in the pediatric population under the age of 3 years, with results similar to the open techniques. *Materials and Methods:* At the Department of Surgery, Medicine and Neuroscience at the University of Siena we conducted a retrospective study of patients aged less than three years of life underwent minimally invasive procedures. The clinical information was extrapolated from a prospective database. They included all patients treated from June 2003 to October 2014. Were considered for each patient demographics, associated diseases, surgical procedure, MIS approach, the instruments used, the duration of surgery and hospitalization, intraoperative and postoperative complications (early and late). *Results:* From a total of 933 minimally invasive procedures, 150 (16%) were performed under the age of 3 years. The 76% of patients were males, 24% were females. Twenty-two patients (15%) presented associated diseases. 53 (35%) were diagnostic procedures, 97 (65%) were therapeutic procedures. We treated: 1 3 esophageal atresia, 1 diaphragmatic hernia, 1 congenital cystic adenomatoid malformation (CCAM), 8 stenosis of the ureteropelvic junction , 2 disorders of sexual differentiation (DSD), 20 inguinal hernias, 75 cases of non-palpable testis, 15 cases of Hirschsprung disease, 10 cases of fundoplication, 4 cases of ovarian tumors, 6 cases of nephrectomy, 4 cases of eminefrectomy and 1 case of thymoma. In total we performed 6 (4%) thoracoscopic procedures, 18 (12%) in retroperitoneoscopy and 126 (84%) in laparoscopy. 55 procedures (37%) were performed in "one-trocar" technique. Among the remaining 95 (67%), in 62 (41%) we used a "3 mm" instruments, in 18 (12%) "5 mm" instruments and in 15 (14%) they were mixed (3/5 mm). The duration of the procedure was an average of 59 minutes (range: 20-135 minutes) in diagnostic procedures and 184 minutes (range: 40-370 minutes) in the therapeutic procedures. We converted to an open technique in 14/150 (9%). We reported no postoperative complication and incidentaloma no intraoperative complications. The following is 100% survival. *Conclusions:* MIS is probably the most important change that has taken place in the field of Pediatric Surgery in the last 30 years. The optimum benefits are to be found in the lesser surgical stress, the lower cavity contamination, the magnification of details, the reduced hospital stay, reduced morbidity or the best aesthetic result.

Key words: Mini-invasive surgery; thoracoscopy; retroperitoneoscopy.

INTRODUCTION

Since the end of '80s mini-invasive surgery (MIS) has been widely used becoming an important and irreplaceable method, safe and highly reliable, in both the diagnostic and therapeutic treatment, for a variety of conditions in both the child and the newborn [1-6].

The purpose of our work was to evaluate the feasibility of the MIS comparing children younger than three years with the population over the age of 3 years.

MATERIALS AND METHODS

At the Department of Surgery, Medicine and Neuroscience at the University of Siena we conducted a retrospective study of patients aged less than three years of life underwent minimally invasive procedures.

The clinical information was extrapolated from a prospective database. They included all patients treated from June 2003 to October 2014.

For each patients we considered demographics, associated pathologies, the surgical procedure, the minimally invasive approach, the instruments used, the duration of surgery and hospital stay, the intraoperative and postoperative complications (early and late).

In connection with the feasibility of MIS in children under 3 years of life we compared statistically aspects of the population selected for the study and the rest of the patients older than 3 years who have undergone at MIS our center.

The parameters taken into consideration were the

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conversion rate and the rate of early and late complications. The test used was the test of Fisher. We considered as significant a P value less than 0.05.

RESULTS

A total of 933 minimally invasive procedures were performed, of these 150 (16%) in children under three years. 114 (76%) were male and 36 (24%) females. 22 of 150 patients (15%) had associated diseases, such as: esophageal atresia in 3cases, 1 case of diaphragmatic hernia, 1 case of congenital cystic adenomatoid malformation (CCAM), 8 cases of stenosis of the ureteropelvic junction, 2 cases of disorders of sex development (DSD), 20 cases of inguinal hernia, 75 cases of non-palpable testis, 15 cases of Hirschsprung disease, 10 cases of fundoplication, 4 cases of ovarian tumors, 6 cases of nephrectomy, 4 cases of eminefrectomy and 1 case of thymoma. In Figure 1 we show an intraoperative picture of congenintal diaphragmatic hernia.

Tables 1-3 includes all the procedures performed by laparoscopy, thoracoscopy and retroperitoneoscopy.

Of the 150 MIS procedures under 3 years, 53 (35%) were diagnostic, 97 (65%) therapeutic.

In total we performed 6 (4%) thoracoscopic procedures, 18 (12%) in retroperitoneoscopy and 126 (84%) in laparoscopy.

55 procedures (37%) were performed with "one-trocar" technique. Among the remaining 95 (67%), in 62 (41%) we used a "3 mm" instruments, in 18 (12%) "5 mm" instruments and in 15 (14%) they were mixed (3/5 mm). The duration of the procedure was an average of 59 minutes (range: 20-135 minutes) in diagnostic procedures and 184 minutes (range: 40-370 minutes) in the therapeutic procedures. The mean length of stay in the diagnostic procedures was 3 days (range 2-4 days) and therapeutic procedures 4.5 days (range 2-7 days). Were performed conversions equal to 14/150 (9%). No intraoperative complication, no accident and no postoperative complication.

The survival rate was 100%.

Analgesic requirements was 24 hours (paracetamol + codeine) in diagnostic and 48 hours (tramadol + ketorolac continuous infusion) in the therapeutic procedures. Feeding started on average after 9.5 hours (range: 6-24 hours) diagnostic and 24 hours (range 12-36 hours) in the therapeutic procedures.

We analyzed statistically the following parameters (Table 4) with Fish test:

- Conversion rate: P value equal to 0.0096;

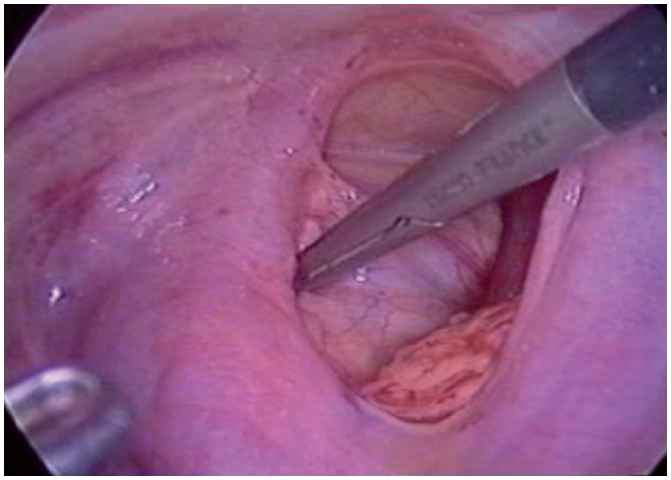

Figure 1. Congenital diaphragmatic intraoperative hernia picture.

Table 1. Laparoscopic procedures in children under 3 years.

Procedures	N.
Inguinal hernia	20
Non-palpable testis	75
Hirschsprung disease (surgery)	9
Hirschsprung disease (biopsy)	6
Ovarian tumors	4
DSD	1
Funduplicatio	10
Total	126 (84%)

Table 2. Toracoscopy procedures in children under 3 years.

Procedures	N.
Esophageal atresia	3
Congenital diaphragmatic hernia	1
CCAM	1
Thymoma	1
Total	6 (4%)

Table 3. Retroperitoneoscopic procedures in children under 3 years.

Procedures	N.
Stenosis of uretero-pelvic junction	8
Nephrectomy	6
Eminephrectomy	4
Total	18 (12%)

- Early complications: P value equal to 1.0000;
- Late complications: P value equal to 0.3687.

Feeding, analgesic requirements and the average time to process are basically the same so we did not run any statistical test.

DISCUSSION

Analyzing what are the results of our study it is clear that the MIS approach can be considered routine for a wide range of diseases that affect not only adolescents and pre-pubertal but also infants and children within the three years.

What looked like a problem until a few years ago, such as minimally invasive procedures in children, particularly in children under 36 months, today it has become an approach feasible without additional risks of particular relevance to the traditional approach in like age or minimally invasive procedures in older patients.

The evidence for this view comes from the observation of our series, where the total of 900 procedures performed in the last 11 years, 16% (150 procedures) were performed in patients by the third year of age.

This was made possible in particular by the miniaturization of the instruments. They are in fact now in use 3 mm trocar, with lengths reduced compared to the adult, which allow a movement within the operating cavity and ergonomics for the operator that guarantees a complete reliability to the MIS procedures in the small patient.

Laparoscopy is certainly the MIS approach that of choice in different type of disease. However even thoracoscopy and retroperitoneoscopy, as demonstrated by our series, can be applied without much difficulty.

The diagnosis is most important in the population of less than 3 years of life and many authors emphasize the merits of laparoscopic surgery especially for the accuracy of the diagnosis, the vision of the anatomical structures, for reduced hospitalization with a rapid return to the routine activity as reported in the work of Higashimoto, Cohen, Waldschmidt, Van der Zee *et al* [7-11].

Also the analysis of the type of procedures performed shows that now almost all of the laparoscopic surgery, thoracoscopic and retroperitoneoscopic that are performed in patients with more than three years of age is perfectly reproducible in individuals under three years, although the difference pathologies encountered in the two age groups [12-16].

Statistical analysis of our population (<3 years) compared to more than 3 years has given different results based on the aspects considered.

The mean duration of the intervention, the average hospital stay, analgesic requirements and early postoperative nutrition are equivalent results.

The conversion rate in "open" surgery for laparoscopic procedures was higher in young patients (9% *versus* 4%) with a statistically significant P value.

The reasons for this difference are to be found in the characteristics of the patients themselves: the volume of the cavity surgery in patients under three years of age decreases exponentially compared to that of older patients; in these conditions the possibility of a movement, of a difficult or incomplete view of the problem to be treated are more frequent for the surgeon and more easily require conversion to "open" technique [17-19].

This is why, however, on the basis of all these considerations the conversion in patients younger should not be considered a "failure" of the MIS but simply a potential problem that does not undermine the effectiveness of minimally invasive in children less than three years of life.

The other parameter that we compared statistically is the rate of early and late complications.

Our results have shown a "P value" not statistically significant, underlying how the MIS does not increase the risk of complications in young patients, and this allows us to conclude that the MIS in children currently is an optimal solution for the patient with age under 3 years.

Table 4. Statistical analysis: conversion to open, early and late complications.

Analysis	N./ Tot	P-value
Conversion in "open"		
<3 years	14/150	0.0096
>3 years	30/783	
Early complications		
<3 years	0/150	1.0000
>3 years	5/783	
Late complications		
<3 years	0/150	0.3687
>3 years	7/783	

CONCLUSIONS

The miniaturization of instruments, the development of sophisticated new techniques and a greater consensus on the MIS has allowed the widespread use of this procedure in the pediatric age with further specific applications in newborns and infants.

It is necessary in this type of surgery an adequate learning curve, especially in pediatric patients, even more in newborns and infants.

The optimum benefits are to be found in the lesser surgical stress, the lower cavity contamination, the magnification of details, the reduced hospital stay, reduced morbidity or the best aesthetic result. All these aspects have helped to improve the surgical and anesthetic techniques also stubbornly and always trying to get a lower rate of complications.

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HYPOSPADIAS: LONG TERM FOLLOW-UP IN A SINGLE CENTER

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Abstract. *Introduction:* Hypospadias is one of the most common birth defects that affect the male urogenital tract. It can present as isolated anomaly, but sometimes can arise in the context of complex disorders of sex development (DSD). These malformations are characterized by a great variety of clinical manifestations and compromise the aesthetic appearance, but also functional and psychological impact that the malformation can determine the patient. *Materials and Methods:* We conducted a retrospective study of patients undergoing surgery for hypospadias from March 2000 to January 2015. The data was extrapolated from a prospective database. It was considered for each patient: demographics; type of hypospadias; surgical technique; average age for surgery; intraoperative and postoperative complications (early and late). Duckett's classification was used. *Results:* 343 urethroplasties were performed. 320 (93%) were primary urethroplasties and 23 (7%) reoperations in patients who had performed many other surgical procedures. 7 patients with megameatus were excluded. The hypospadias have been ranked according to Duckett's classification, 35 patients had associated diseases. In total were performed: 186 (55%) Snodgrass, 71(21%) Duckett,10 (3%) augmented Duckett, 42 (13%) Magpi, 16 (5%) Duplay, 1 (0,3%) Bracka, 1 (0,3%) was a Bianchi'technique and 5 (1,4%) were Standoli. In 4 patients (1%) were used mixed technique. There were no intraoperative complications. The mean age at surgery was 15 months (range 12-22 months). Postoperative complications were 12%. Long term follow up was done with uroflussimetrie at 3 and 6 months in those who had reached the continence and possible urethral calibrations in those who had submitted a stenosis in post-op. *Conclusions:* The improvement of surgical techniques, the use of optical amplification tools, the use of suture material (PDS) and the experience gained in recent years have enabled us to optimized the results. Though aware of the potential and actual complications that this type of microsurgical correction can lead to the results we have obtained are comparable to those of major international series and can be considered satisfactory, both from an aesthetic and functional.

Key words: Hypospadias; disorders of sex development; microsurgical technique.

INTRODUCTION

Hypospadias is one of the most common birth defects that affect the male urogenital tract. It can present as isolated anomaly, but sometimes can arise in the context of complex disorders of sex development (DSD) [1,2].

These malformations are characterized by a great variety of clinical manifestations and compromise the aesthetic appearance, but also functional and psychological impact that the malformation can determine the patient [3].

Despite the frequent finding of the disease and the large number of surgical techniques described, it is not yet possible to indicate which of these are the best in terms of efficiency, reduction of complications, improved appearance and functional final.

The purpose of this article is, therefore, provide a review of the cases obtained in Pediatric Surgery Clinic of Siena and compare with the literature.

MATERIALS AND METHODS

We conducted a retrospective study of patients undergoing surgery for hypospadias from March 2000 to January 2015.

The data was extrapolated from a prospective database.

It was considered for each patient: demographics; type of hypospadias; surgical technique; average age for surgery; intraoperative and postoperative complications (early and late).

The Duckett's classification was used.

Preoperative management included: preoperative ultrasound of the urinary tract; psychological consultation in patients more than six years.

The postoperative follow-up included: clinical evaluation one week after discharge, 3 months and one year; uroflowmetry after 1 year from the achievement of continence and then depending on the patient.

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RESULTS

343 urethroplasties were performed. 320 (93%) were urethroplasties primary and 23 (7%) reoperations in patients who had performed many other surgical procedures. 7 patients with megameatus were excluded. The hypospadias have been ranked according to Duckett's classification (Table 1), 35 patients had associated diseases (Table 2).

In total were performed: 186 (55%) were Snodgrass, 71 (21%) were Duckett, 10 (3%) were Augmented Duckett, 42 (13%) were Magpi, 16 (5%) were Duplay, 1 (0,3%) Bracka, 1 (0,3%) was a White and 5 (1.4%) were Standoli. In 4 patients (1%) were used mixed media (4-Duckett/Nesbit and 1-Snodgrass/Duplay) (Table 3).

There were no intraoperative complications.

The mean age at surgery was 15 months (range 12-22 months).

Postoperative complications were 12%. 24 patients (7%) had one or more fistulas. Of these 13 they had had surgery for anterior hypospadias and 10 posterior (Table 4).

The average time of diagnosis/fistula/urethral and was 6 months (2 weeks-24 months).

In all it was performed the closure of the fistula, 6 months after surgery. 1 patient (treated in an another hospital) needed two operations and two patients of 3 interventions.

Ten patients (3%) had a urethral stricture. The symptoms occurred in an average of 7 weeks of surgery. The 10 patients underwent preoperative uroflowmetry and showed a pattern uroflussimetric

Table 1. Percentual of patient operated according to the Duckett's classification of the hypospadias.

Patient	N. (%)
Anterior	200 (58)
Glandular	42 (21)
Balanopreputial sulcus	103 (51.5)
Penile anterior	55 (27.5)
Medium	45 (13)
With cordhee	14 (31)
Without cordhee	31 (69)
Posterior	68 (22)
Posterior	22 (32.3)
Penoscrotal	36 (53)
Perineal	10 (5 DSD + vagina) (14.7)
Multisurgical	23 (7)

Table 2. Associates disease.

Disease	N.	Hypospadia anterior	posterior
Unilateral cryptorchidism	9	6	3
Bilateral cryptorchidism	7	4	3
Epileptic encephalopathy	2	-	2
Anorectal malformation	3	3	
Renal agenesis	5	2	3
Vescicoureteral reflux	3	1	2
Esophageal atresia	1	1	-
Scrotum vulva's type	2	-	2
Sketch vaginal residue	5	-	5

Table 3. Surgical techniques used.

Technique	N. (%)
Anterior/medium without cordhee	231 (67.3)
Snodgrass	173 (50.4)
Magpi	42 (12.2)
Bianchi	1 (0.3)
Duplay	15 (4.4)
Medium with cordhee/posterior	82 (23.9)
Duckett	64 (18.6)
Augmented Duckett	9 (2.6)
Duckett + Nesbitt	3 (0.9)
Bracka	1 (0.3)
Standoli	4 (1.2)
Snodgrass + Duplay	1 (0.3)
Multisurgical	23 (6.7)
Snodgrass	13 (3.8)
Duplay	1 (0.3)
Duckett	7 (2)
Augmented Duckett	1 (0.3)
Standoli	1 (0.3)

Table 4. Post-surgical complications.

Complication	N. (%)
Fistulas	24 (6)
Hypospadia anterior	14 (6)
Hypospadia posterior	10 (12)
Stenosis	10 (2.9)
Subtotal dehiscence	5 (1.4)
Urethral diverticulum	1 (0.3)

with an average Qmax <10 mL (range = 8-12 mL). 6 patients underwent a single calibration to a month after surgery urethroplasty, two were required with multiple calibrations and 2 of meatus plastic [2-4]. 3 of these have had a final resolution, 1 continues to present a stenosis, for which we set from about 2 months a program of home care expansion thanks to the compliance of the parents and the small with Nelaton catheters Ch [6-8] three times a day. All patients treated for urethral stricture finally performed a calibration uroflowmetry post about three months and then six months with normalization of urinary stream. 5 patients (12.5%) presented a subtotal of urethroplasty dehiscence around after 10 weeks of surgery (1-24 weeks), necessitating an intervention in two stages with a flat opening of the urethra and subsequent "Redu according to Snodgrass" at a distance of at least 6-8 months after the first half.

Only one patient presented at 12 months after surgery a urethral diverticulum diagnosed with HCM and a cystoscopy, surgically removed.

DISCUSSION

Treatment of hypospadias is essentially surgical and the last 15 years has undergone major changes.

A surgical correction of hypospadias performed successfully can get proper erection of the penis, a new meatus at the top of the glans, a urination in standing and normal sexual relations.

Although it is a very common disease it is not yet clear what the best surgical technique.

Our study was retrospective which recruited 343 patients.

The study population included patients with both proximal and distal hypospadias, is subject multi operated from another hospital. According to the literature patients undergoing surgery showed a mean age of about 15 months, which is the best age for 'surgery [4]. Tekgül S et al in the Guidelines of Pediatric Urology indicates the best age for surgical treatment from 6 to 18 months.

Over the years we have been used several techniques of correction. In the literature, it is not mentioned any technique as a gold standard, for both forms anterior or posterior and the choice seems to be dictated by the experience of the single center, surgeon, rather than from the comparative data. In our department for anterior hypospadias surgery of choice is urethroplasty sec. Snodgrass, with preputial flap sec. Retik. For distal hypospadias, the technique used was the urethroplasty as the Duckett's technique.

The postoperative complications appeared in 12% of our patients, of which 6% of fistulas, stenosis of 2.9%, 1.4% subtotal anastomotic leakage of the urethroplasty and 0.3% of the formation of urethral diverticulum. Fistula in our series is the complication most represented, especially in the posterior hypospadias (12%) than the anterior (6%), and the diagnosis was made at 6 months after surgery (2 weeks-24 months).

The patients performed a follow-up post-operative one week after discharge, three months and one year [5-6].

LQ Huang *et al.*, presented a case study of 167 patients with hypospadias front and back underwent surgery urethroplasty appearance of the fistula 12% of posterior hypospadias and 30% of anterior hypospadias. In terms of post-operative complications in our data, compared with the more recent literature, its show excellent results comply with the relevant international case studies.

Functional assessment in children not yet continents is difficult, they may be asked questions to patients and parents to get information about urination and urinary stream, but more accurate data are obtained through the uroflowmetry, according to the parameters of the International Children's Continence Society (ICCS) [4-8]. Some studies recommend performing a uroflowmetry after the child has reached the continence and the follow-up of patients showing parameters of obstruction to urine flow or borderline values [8-11]. There are papers showing that the location of the meatus and/or the surgical technique used questionnaire predict functional outcome, rather it seems to be related to the degree of severity of the chorda and the emptying of urine. Neither the location nor the meatus the surgical technique are predictors of outcome functional. You can distinguish symptomatic patients, for example, those exhibiting poor urinary stream, incontinence, drip, hesitation during urination and patients with subclinical symptoms as dysfunction or bladder hyperreactivity, whose diagnosis is sometimes late because of the difficulty in highlighting these aspects.

In our series all patients underwent uroflowmetry one year after achieving continence and then were evaluated according to the clinical application; in particular those with stenosis were subjected to repeated uroflussimetrie in the follow-up at a distance.

CONCLUSIONS

The analysis of our study found no substantial differences from the International Literature in terms of timing of the intervention, to choose the most appropriate surgical technique, duration of follow-up and incidence of complications.

Improving operating techniques, the use of instruments of optical amplification, of suture material and the experience gained in recent years has allowed the Pediatric Surgery Clinic of Siena to optimize their results. Although we are aware of the possible complications that this type of correction may involve

microsurgical, the results obtained by us are comparable to those of the major international case studies and can be considered satisfactory from both an aesthetic and functional point of view. The data collected in this study, according to the literature, demonstrate the importance of a correct timing of the intervention that is being pursued performing correction of the malformation at a time to allow the small patient does not remember the operation, of an adequate choice of the type of intervention between the various techniques used, a proper follow-up post-operative, aimed not only to an early recognition of complications but also to the long-term outcome evaluation in these patients. It is noted today a 'growing interest, not only for the correction of the functional defect, but also to the psychological consequences that such congenital malformation may result in the patient, in his life of relationship and in the parents.

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MORPHOMETRY AND LASER SCANNER IMAGING: A REVOLUTION IN ANATOMY

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Abstract. Anatomy is a visual science, even if the visual quality of anatomy can be declined in different ways, as we will show. Anatomy holds its own main feature in the etymology of the name itself: it is through cutting (ανά, *anà*) by lancet (τέμνω, tè*mno*) that preparations are yet prepared. This operation is aimed to make evident and well visible anatomical structures. Vesalius is the first modern anatomist and his main heuristic principle consists in the equivalence between seeing and knowing. The possibility of analyzing fresh anatomical preparations is made short by the unavoidable decomposition processes of the cadaver. Due to this reason beautiful and precious tables have always been drawn, painted and printed. We remember the ones by the Fisiocritic Paolo Mascagni, whose double centenary of death is celebrated this year. Watercolor tables are yet much realized and used. However they are bi-dimensional schematizations and, even if well done, they remain far from reality. Photography allows to fix the image of anatomical preparations with high fidelity of particulars. However these images are static. The graphic synthesis allows to realize four-dimensional human virtual models. They can be rotated according to the three spatial axes and, thanks to this, they can be observed from every point of view. Due to the fact that these are schematizations, they are very far from reality. For this reason CT three-dimensional reconstruction, that rebuilds anatomy in three dimensions, allows to obtain results with very superior quality and fidelity. However these reproductions lack color and real light. The gap between the iconographic representation and the existing thing is and will always be not fully eliminable. However our use of laser scanner technology allows to reduce this gap to minimal levels, with a quick and easy acquisition process. Laser scanner generates a cloud of points of the examined object. Each point is identified through exact coordinates. Besides, the photos of the same object can be over-placed to the cloud. The result is a virtual model that reconstructs the real object, highly corresponding as in morphology and as in colors. This virtual model allows us to interact and we can rotate it, watch at it from every perspective and especially we can measure it. The scanner we have used allowed us to reach an accuracy of ±25 µm. The anatomical preparation is literally "immortalized", up to under-millimetric details, where the naked eye is ineffective. The so obtained image allows to re-observe and to measure the object forever. We can imagine a lot of very innovative, if not revolutionary applications. We realized our four-dimensional models aiming to attach them to this project. These scanning are of two skulls and of a heart. They are the concrete proof of the possibility of obtaining surprising results in many areas, from normal anatomy to pathological anatomy, from legal medicine to biology. This way of obtaining anatomical images is marking a turning point from a taxonomic, serial and verbal conception of Anatomy to a visual, spatial and mathematical one. Instead of lists of nominal labels we have now coordinates and quantitative/structural references. This makes Anatomy more treatable through digital methods. The visual approach has far origins: since XIII Century, when real (not formal) Renaissance of figurative arts begins, and during following ages, visual paradigms gain more and more importance in human knowledge. Once more we are dwarves on the shoulders of giants. Besides, detecting morphology by laser scanner pushes us to re-configure the relationship between nomothetic and ideographic approach in building scientific models.

Key words: Anatomy; atlas; biology; immersive anatomy; laser scanner; legal medicine; morphometry.

INTRODUCTION

Anatomy is a visual science that holds its own main feature in the etymology of the name itself: it is through (ανά, anà) cutting by lancet (τέμνω, tèmno) that preparations are yet prepared. This operation is aimed to make evident and well visible anatomical structures. Andreas Vesalius is the first modern anatomist and his main heuristic principle consists in the equivalence between seeing and knowing.

Nothing is as effective as the execution of a dissection; this is a big truth that will never fade. However, since ancient times, anatomists had to deal with the unavoidable decomposition processes of the cadavers, which make short the possibility of analyzing fresh anatomical preparations. For this reason beautiful and precious tables have always been drawn, painted and printed. We remember the ones by Leonardo da Vinci, by Andreas Vesalius and by the Fisiocritic Paolo Mascagni, whose double centenary of death is celebrated this year. Water-

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color tables are yet much realized and used, however they are bi-dimensional schematizations. Even if well done, they remain far from the existing object, since they are different from reality both under the epistemological and the cognitive point of view. We have always to remember that only the anatomical structures portrayed by the author (and in the way they have been portrayed by the author) are observable in the paintings of a common watercolor atlas.

Photography allows to fix the image of anatomical preparations with high fidelity of particulars, bypassing the artistic representation by the author. Even if photos can be layered and arranged in sequence, they remain static, so they offer a limited possibility of interaction with the observer.

The advent of the IT and of the graphic synthesis has allowed to realize four-dimensional human virtual models. They can be rotated according to the three spatial axes and, thanks to this, they can be observed from every point of view. They allow to visualize the whole body or to stress the particular structures we are interested in: we can zoom in on muscles, nerves, vessels, on their topographic relation, we can observe the organs in situ or isolated. It is possible to observe cavities, organs or the whole body dissected according to sagittal, frontal or transverse planes. It is also given the possibility of resecting superficial anatomical structures, in order to reach and to make evident deeper planes and vice versa. For sure the possibility of observing the examined object both intact and dissected and from every point of view, according to the rotation perspective, represents a big help. However, since these are schematizations, they are very far from reality, apart from the accuracy. For this reason CT three-dimensional reconstruction, that rebuilds Anatomy in three dimensions, allows to obtain results with very superior quality and fidelity. It is fundamental to emphasize the fact that these reproductions lack color and real light.

The gap between the iconographic representation and the existing thing is and will always be not fully eliminable. However our use of laser scanner technology allows to reduce this gap to minimal levels, with a quick and easy acquisition process. Laser scanner generates a cloud of points of the examined object. Each point is identified through exact coordinates. Besides, the photos of the same object can be layered to the cloud. The result is a virtual model that reconstructs the real object, highly corresponding as in morphology and as in colors. This virtual model allows us to interact and we can rotate it, watch at it from every perspective and especially we can measure it. The laser scanner was born as a measuring instrument in industry and it maintains such feature in this new field, allowing to quantify with high precision the area of the organs, the diameters, the volumes and so on. The EDGE ScanArm HD® we have used allowed us to reach an accuracy of ±25 μm. The anatomical preparation is literally "immortalized", up to under-millimetric details, where the naked eye is ineffective. The so obtained image allows to re-observe and to measure the object forever.

We can imagine a lot of very innovative, if not revolutionary applications. We realized our four-dimensional models aiming to attach them to this project. These scanning are of two skulls and of a heart and they are the concrete proof of the possibility of obtaining surprising results in many areas, from Normal Anatomy to Pathological Anatomy, from Legal Medicine to Biology.

This way of obtaining anatomical images is marking a turning point from a taxonomic, serial and verbal conception of Anatomy to a visual, spatial and mathematical one. Instead of lists of nominal labels we have now coordinates and quantitative/structural references. This makes Anatomy more treatable through digital methods. The visual approach has far origins: since XIII Century, when real (not formal) Renaissance of figurative arts begins, and during following ages, visual paradigms gain more and more importance in human knowledge. Once more we are dwarves on the shoulders of giants.

Besides, detecting morphology by Laser Scanner pushes us to re-configure the relationship between nomothetic and ideographic approach in building scientific models.

APPLICATIONS IN NORMAL ANATOMY AND IN PATHOLOGICAL ANATOMY

For physicians, non-medical staff and for any researcher in the field of body studies, Human Anatomy represents the most part of the basis of the whole knowledge and it reveals to be the key element for the comprehension of many circumstances, as in the period of the studies, as in the profession. For this reason an accurate and organic background is an absolute duty. In the phase of the university education the dissection of a fresh cadaver represents the most effective and preferable approach, however the use of an atlas cannot be renounced during the "domestic" study on the anatomical treatise. We have to remember that fresh preparations are not always easily accessible (because of many reasons: strict law code, religion, small number of body donors, etc.) and that they can be analysed only for a short period of time.

The laser scanner is the perfect instrument to be employed in the realization of a completely new Human Anatomy Atlas (and not necessarily only human…) no more based on static tables, but on a four-dimensional model, that is to say a three-dimensional model which can be rotated and observed from every perspective, with an absolute fidelity to reality, closely linked to the existing object and analyzable as in a real dissection. Many scanning, realized in different moments, can be layered and processed with a specific software, allowing

to obtain the same features of the virtual atlases formerly described, with the new possibility of measuring the anatomical model with high precision and authenticity. Moreover this will be the first Human Anatomy Atlas in which the anatomical preparation is inserted in a virtual sphere, that represents a reference system made up of meridians and parallels. This will make the virtual handling of the model itself easier and more accurate.

It is important to stress a key point. In this case the observer has the opportunity of analyzing no more a schematization, but a model made through an objective instrumental data acquisition process, highly corresponding in colors, in light and in morphology, from the macroscopic aspect to the under-millimetric details, where the naked eye is ineffective, visible only after enlargement. Video clips from laparoscopic or robotic surgical operations may be attached to the atlas and thanks to this multimedia equipment it would be possible to visualize the anatomical planes while the surgeon is moving across them. This approach would be very helpful in the comprehension of the way the morphogenesis shapes the topographic relations between the organs.

Thanks to a specific software it is possible to convert the 3D textured model so that it can be watched with 3D glasses. In this way the anatomical preparation literally gets out of the monitor screen and matches the observer, maintaining a very high resolution which is fully noticeable only after strong enlargement: this is an experience that for medical staff, students, scholars, health-care workers and science lovers is really useful from a practical point of view, but also exciting and without precedent.

In the end a set of digital histological slides may be attached to the atlas and maybe a set of radiological images too. A so conceived and realized product has the highest completeness and it represents the perfect instrument to study Anatomy in an organic, accurate and detailed way.

This technology and the way of application we have described can be employed both in normal anatomy, as in pathological anatomy. Every day new clinical cases, characterized by new pathological features, or by novelty elements of already known pathologies, are published in literature, showing the problem from all the different perspectives: from the biochemical point of view, to the histopathological one, from the clinic features to the Anatomical aspects revealed by autopsy. Laser scanner data acquisition gives the possibility of producing a completely new Pathological Anatomy Atlas and thanks to the quickness and easiness of the scanning process itself, it would be very simple to update the literature iconography, despite the billions of coordinates that are at the basis of the models: very high quality in a very simple way.

This completely new approach represents the beginning of the 3.0 anatomical imaging era, which is preceded by 1.0 era of watercolor tables, by 1.2 era of photographic atlases and by 2.0 era of virtual graphic synthesis three-dimensional pictures.

Moreover, [for the first time] anatomy is hit by the so called *"data deluge"* and enters the *Big Data Age*. There is a very big dimensional/informational gap between the world of Laser Scanning 3D Models and the world of watercolor tables, of photos and of virtual graphic synthesis three-dimensional models. The digital anatomical preparations, obtained with laser scanning techniques, can be observed and measured with an accuracy of ±25 µm, that is to say with a resolution which is much higher than human eye's potentialities. As a consequence, the informational content of these pictures is beyond the physiological limits of our naked eye: only after enlargement the under-millimetric structures become analyzable. This is made possible by the billions of points identified by specific coordinates- the models are made of, which can be processed and managed only through the powerful computers existing today.

Each point and all points are not a bare offer of viewable points, surfaces or volumes: each one contains the quantitative information about its position in the structure, exactly matching with the real object. So our operating on the model (measuring 1/2/3 dimensional extensions, etc.) can replace the operating on reality, but in an easier and more warranted way. Besides, our operation can be directly saved and treated digitally.

Let us cast our mind into the future, maybe into a not very distant future, and let us imagine how one day Anatomy will be studied and taught, with high probability. When the technology of 3D holographic projectors will be developed and will be within everybody's reach, the same scanning that today can be observed on a screen will be projected in the three-dimensional space, giving to every student the possibility of executing and of observing virtual dissections of real bodies, at home or in any other place. The Human Atlas we have talked about will become literally three-dimensional and four-dimensional. In the lecture halls the dissections of the cadavers will be accompanied by the projection of 3D holographic models of real organs at high enlargement and maybe, through the use of a powerful 3D holographic projector, students will walk inside body cavities, such as the thoracic cavity, or the abdominal cavity, they will explore on foot the cranial fossae and they will walk inside the lumen of hollow organs. Anatomy will become *immersive*.

APPLICATIONS IN LEGAL MEDICINE

The laser scanner, thanks to its unique and inimitable capacity, can survey the least particulars of the analyzed objects and it can assign specific coordinates to each point. Consequently it gives the possibility of measuring with high precision lengths, angles, areas or volumes even after days, months, years and, virtually, forever. It is very important to underline that these three-dimensional models are the product of an objec-

tive instrumental data acquisition process, not falsifiable, absolutely accurate in morphology, both in the macroscopic aspect as in the under-millimetric details.

Such morphologic and morphometric precision and the possibility of analyzing without any limit of time these objective instrumental data, match the requirements of legal medicine in a perfect way.

At this point we introduce another laser scanner model that we have used in the realization of this project: the DPI-8 Handheld Scanner®. This instrument is analogous to the EDGE ScanArm HD® that we have already showed, but it has a lower accuracy, suitable for the different range and for the different use. It is characterized by small dimensions, quick data acquisition capacity, simplicity in use and in transport. This scanner, since it has an integrated camera, is able to generate the 3D model from the cloud of points and to layer the photographic texture in real time. It is the perfect instrument to survey a crime scene, to scan and to photograph at the same time wide spaces such as rooms or outdoors areas. Each object in the field is "immortalized" in its parts and reciprocal positions with the environment. We have attached to this project the survey of a table and of a heart lying on it, realized with the DPI-8 Handheld Scanner® in order to show the way this instrument works and the offered potentialities.

When we focus the attention on the details of a cadaver or on any other object whose dimensions are in the order of magnitude of 10^{-2} m, a higher level of accuracy is required and the use of the EDGE ScanArm HD® becomes essential. However both instruments are fundamental since they are complementary and they work synergetically: the choice for the use of one the two depends on the needs of the operator in accuracy and range.

The revolutionary element consists in the fact that a survey realized with this technology represents an absolutely objective instrumental source of data, which can be analyzed without any limit of time. Consequently everything can be reviewed. No detail is missed by laser scanner, thanks to which it is possible to eliminate every kind of human error and omission in surveying and the changes of the examined object in time: from the decomposition processes of a cadaver, to the changes in a crime scene, to the tampering with evidence and so on.

APPLICATIONS IN BIOLOGY

On this point we mention as an example of application another project of ours, introduced together with this one at the Academy of the Fisiocritici and to be published soon: *"Possible Contributions For The Development Of An Exaptive Theory: The Example Of Rachis"*. The morphologic and morphometric analysis of vertebras, realized through laser scanner technology, allowed to reach a comparative valuation of rachidian exaptive transformations in different typologies of animals: chamois, common fin whale, bottlenose dolphin and Man.

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POSSIBLE CONTRIBUTIONS FOR THE DEVELOPMENT OF EXAPTIVE THEORY: THE EXAMPLE OF RACHIS

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Abstract. Aiming at finding an objective method to define as exaptive (or as not exaptive) an evolutionary transformation, we can use a morphological and morphometric analysis of vertebras that can be realized through laser scanner technology. So we can reach a comparative valuation of rhachidian exaptive transformations from a typology of animals to another. In our work we referred to vertebras of a mammal tetrapod (chamois), of aquatic mammals (common fin whale, bottlenose dolphin) and of Man. We can suppose that the evolutionary transformation of the examined organ, system or structure is exaptive if: i) it remains the same under the phylogenetic/genetic profile, in other words it is the homologous; ii) it remains the same (exactly the same or however within certain limits that must be quantified by opportune indicators/descriptors) according to the morphometric/morphologic aspect; iii) its successive function changes with discontinuity if compared with the previous one; this aspects can be studied by modal analysis and by the model of *Eigenvectors* and *Eigenvalues*; iv) the new function is exclusive or however is prevailing on the original one. In particular, regarding rachis: i) we know the phylogenetic/genetic correspondence of this structure in all vertebrates; ii) we could choose some morphometric parameters as indicators of the maintaining/changing of structure; iii) we compare the previous and successive function under static/dynamic aspects. We refer to last year report, when we proposed that the evolutionary transformations of rachis static/dynamic function can be considered as exaptive. The functional transformation pushed us to rename human rachis as "*vertebral shock absorber*", due to its new vertical set-up, very different from the horizontal one that is proper of central axis (fishes and, later, of Cetaceous, even if in a different way), of beam (of tetrapods) and of shelf (of "partial" bipeds).

Key words: Exaptation; vertebral shock absorber; rachis; mechanical function; evolution.

"Evolution is an exaptive combinatory play in which new tricks are always teached to old genes."
(Francois Jacob, Exaptation, 1982)

Gutenberg took advantage of the already existing technologies: wine press, and movable type, combining them together, so to obtain the printing press.

Babbage "created" his Analytical Engine by "recycling" Jaquard's invention of punched cards that 30 years before were realized for managing mechanical looms automatically.

INTRODUCTION

Evolution is functional to expansion-maintaining of life. According to circumstances, expansion can be the same of maintaining or one between them can prevail on the other, or they can even be contradicting one another.

Life trend is maintaining-expanding itself. It owns both active and passive devices and sources functional to persistence of individual as much as of the species. From surviving instinct to capability of restoring structures or functions that are altered by pathological or traumatic processes, from reproduction instinct to enzymatic mechanisms that "correct" errors of DNA duplications, from homeostatic processes to negative feedbacks.

However there is a background noise that is never extinguished (genomic and epigenetic mutations, phenotypic plasticity) allows life to explore the space of state of adaptive and evolutionary possibilities in a random way.

In both cases of the single individual that adapts to context of the moment and of an evolutionary drift of long term, however it is changing capability. This capability must not be meant as possibility that remain silent, if nothing actives it. Moreover it is a connatural condition of life, an inextinguishable motion trend.

We notice two dialectical relations: i) between the trend to persist and the one to changing, and ii) between transformation factors and environment.

Persisting devices and variation ones are in a continuous dialectic relation, no one between them acts in an absolute way, but it is always somehow balanced by

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its counterpart. background noise does not allow immobility stops everything. Stability trend slows down, limits, filters trendy changing, it avoids that they become exaggerate and dangerous drifts, that they become uncontrollable and can beneficiate of feedback.

The dialectic of this mobility with environmental, orients it toward directions that *a posteriori* become recognizable, interpretable, explainable.

This dialectic is made by continuous feedbacks. Each feedbacks are independent one from another. They can be considered as point-like?. The direction of changing (both adaptive and evolutionary) emerges only from the fact that many of these feedbacks compose with a certain level of convergence. No change is without consequences and only very few of them are neuter and indifferent. Somehow they always bring advantages or handicaps. Some variations of these ones obtain advantages that are the feedback that confirm themselves. Other variations are eliminated by selection. From this combination of confirming/denying an emerges the orientations of evolutionary transformations.

Implementation of this process is the continuous interlocution of living and of livings with environment and with the contingence, functionally to adapt itself to context itself, that is to say to make surviving chance maximum. Surviving is not the simple avoiding of death, but it is the warranty of affirming its own dominium, first within the already conquered limits, but also to expand them in each possible direction.

If expanding dominium is possible, this must imply the confirmation within existent limits. If this is not possible, the bare persistence of the existent dominium or, at least, of what can be saved, is the goal for the moment. The main factor is surviving and maintaining the reproduction capability, so that expansion process is suspended and it is postponed to more favorable moments.

In general this is an opportunistic behavior, that is decided step by step, without any straight directional line. Direction is calculated and re-calculated moment by moment. The geometry of this line shows no big scale regularity, except for the fact that some transformation of these ones are the pre-condition for other ones or they open a set of possibilities that were denied until that moment (metaevolution). However only the single ("atomic") is properly directed. Nothing strange if only the addiction of many transformation comes back to a point which evolution seems to be passed through. But there is not cyclic trend nor reversibility, also if somehow we notice a "loop", a closed circle. Mammals came back to water, as Cetaceous, but they are not fishes again. In water environment Cetaceous compete with fishes, but structural and functional solutions are different.

Provenience features (the fishes' ones, in this case) have been replaced by "vicariance" (if we name them according with Alain Berthoz) by different ones that are equally effective. This means only that, even if the circumstances request the same adaptation, the same evolutionary answer is never actuated: too many factors are implied…

Same adaptive exigency can be implemented by different solutions (structural or functional) and the same structure or function can satisfy different exigencies. There is no biunivocal correspondence between anatomical/physiological source and its role in adaptive play.

Therefore vicariance is a distinctive mark of life. We can name the over described cross correspondence just as vicariance. Exaptation itself can be defined as the capability of a structure of satisfying an exigency which it is not phylogenetically developed for.

The frequency, the effectiveness, the extremely wide range of ways of actuating exaptation make us recognize it as a fundamental evolutionary mechanism, dialectically related with the "classic" one of "creating" a new apposite organ/system aimed to satisfy the adaptive exigency.

Simply, we could speak of primary or secondary function. We define the function as primary, if it remains the same or it changes with continuity ("classic" adaptation). We define the function as secondary, if it became different through a discontinuity (exaptation).

Coherently, we can notice not cyclic processes nor reversibility: the apparent coincidence of two different moments of evolution does not mean that evolution goes back: evolution does not forget. Even if evolution does not walk according to "wonderful and progressive destinies", its direction is only forward. So, if they must implement the same adaptive exigencies, this origins different adaptive modifications, even if somehow they are equi-functional.

The so named "living fossils" themselves are not hypostasis of a life form that has never had reasons to change, but they represent the maintaining of some features thanks to globally neuter fluctuations, while other vary. Maintain is only apparent or relative.

This process of "fluidity" of life forms, related to maintain adequateness to environmental conditions, must not overcome the surviving possibility limit and is implemented in two different ways, often reciprocally contemned.

i. Creating *new* structures and transform them during time, according to the exigencies of the moment; this is possible if among mutations there is one of these always beyond the limit of being adapt; this is the *adaptation*, when we observe a new structure that request time to affirm itself, we must think that each provisory step is advantageous: it cannot be originated or tolerated "waiting for" the conclusion of the process.
ii. Co-opting *existing* structures that execute certain functions to convert them to "new" ones, that is to say they differ more than a discriminating limit (how can we determinate it under the qualitative and quantitative profile?) if compared with the original ones, there is "discontinuity of employing", it is the *exaptation*

In other words evolution uses two sources, or two proceeding ways: adaptive and exaptive. Now it uses one, now the other, according to which reacts before and better.

The central question of our work is: *basing our reasoning on these arguments, the human rachis can be considered a shock absorber that was produced by exaptation?*

In our opinion, during its history, rachis is the protagonist of two important exaptations. We hope science will give clarifications soon.

The first one between these two exaptation was the transformation from internal sustaining of the simple body of the proto-chordates (and moreover of fishes, almost without no fundamental functional variations) for their swimming and for a little more, to beam of tetrapods.

The second one is when the beam gains the vertical position, and due to this the rachis can be called (vertical) "shock absorber".

Both these passages was made possible by intermediate transitory conditions: between fishes and tetrapods with elevated trunk we find the slithering tetrapods. These have very limited problems of antigravitational sustaining, but the propulsive function of the tail fin (and of the whole body with the metachronal waves that generate lift in water) is replaced by ground reaction force (that are transmitted along the skeletal chain), generated by the action of the paws on the ground. When the trunk is no more leaned on the ground, the gravity is the main cause of transversal static solicitations, typical of tetrapods. This is the most relevant new function of rachis that makes us speak about "exaptative jump".

A brief parenthesis: when some terrestrial tetrapods come back to sea life(cetaceous) we have a new changement, but it is not symmetric to the previous. The meta-chronic waves are executed now on a different plane, perpendicular to the original one. The swimming movement has been reorganized and converge with the original ones regarding the function.

When the rachis becomes fully vertical, gravitational solicitations are mainly axial. The establishment of natural sagittal curves transforms the rachis in an effective shock absorber meant as a compression spring with a complex mechanical behavior.

The beam moves according to a wave-like pattern that makes it resemble to the rachis of fishes, but the resembling is cinematic rather than dynamic. This was the first exaptation. The second one is when the rachis underlies to axial static/dynamic charges and is made stable by a complex system of combined muscular tie rods, similarly to the stays of a mast.

Before the fully vertical position of the mechanical axis (that we can find in human structure), we can notice partially vertical positions, as in many bipods: kangaroos or apes, etc.

Their balance is less stable than the one of human body and needs compensative devices: the tail of kangaroo, the sporadic leaning of the knuckle on the ground (by apes).

The gravitational solicitations changes direction. From the transversal ones of the tetrapods these solicitations becomes oblique an reduce their angle until the direction of gravity coincides with the axis of the rachis. This new and different situation makes us propose the denomination of "exaptational jump" once more.

Aiming to maintain and to manage its balance, rachis uses tensegrity. This becomes the decisive factor when rachis is lacks of anterior/superior leaning. Meaningful examples are the neck of the giraffe or the rachis of bipeds, even with different inclinations of its mechanical and anatomical ax: kangaroo, apes, man...

A fleshy mass sustained by an internal semi-rigid axis, is a body plan that was established during the wonderful Cambrian explosion. This being is a protochordate. It lived in the water and nowadays Fishes' summary architecture is not very different from the one of this ancient and simple creature.

The wave-like movement on horizontal plane, moreover aimed to propulsion is furnished by a muscular motor that is a more or less continuous muscular mass: it is not organized in different and independent muscles as we find, for example, in Arthropods. Chordates' and Fishes' muscular mass has a metameric organization, whose elements are called "myomeres". These are strictly packed and surround the skeletal axis, acting on it and obtaining the simple wave-like movements. On the contrary, in the Arthropods a single muscle can act on a single articulation of external skeletal, obtaining a wide variety and combination of movements.

Proto-chordates can execute only the propulsive wave-like movements and some rough movements of avoiding an eventual predator, for example, and a little more.

No doubt bodily plan of Proto-chordates is less sophisticated than the Arthropods' one, but it allows to obtain a much more advantageous energetic efficiency. This will be more and more evident with the wide radiating differentiation of Vertebrates of later periods. If the external skeleton of Arthropods offer an energetic advantage on the soft body of worms (it is enough to compare the efficiency of a digger worm with the one of a Mole-Cricket), the internal skeleton allows a further jump of efficiency (now compare a Mole with a Mole-Cricket).

We can notice this superior efficiency also in the swimming: Fishes' speediness is incomparable with the Invertebrates' one, even if squids, with their "reaction" device, are very efficient.

The real difference will be very evident when Vertebrates will develop limbs and differentiated muscles instead of simple muscular masses as Fishes.

As we have already noticed, the bodily plan of internal axis remained similar to its original version until actual Fishes. A few of them (as Sole or Brill Fish) adapted their body to benthonic life turning it (and, together, the plane of movement) of 90°. A side takes the

place of the ventral side and the other side takes the place of dorsal side. Another group of Fishes, Rays and Mantas, use the movements of the "wings", that are still wave-like. These movements became prevailing on the body's ones and they are realized according to a vertical direction, no more to an horizontal one.

Among the three fundamental kinds of Fishes' swimming (strong acceleration, long distance and maneuver), only the last one is out of the schema and is realized thanks to pectoral fins that generate propulsion, moreover when the animal executes precision movements in small spaces, where irregular obstacles are present: the coral reef is the most typical example of this environment.

Strong acceleration swimming (see predators as the Pike), or the long distance one (see pelagic fishes as the Tuna) and even the swimming of the fishes that usually execute the manoeuvre one (when they swim for longer and more regular distances without obstacles) are very similar one another, and differ only for secondary parameters: however their base is the wave-like movement of the body on the horizontal plane.

Changing parameters are localization of junction points, wideness of angular/linear excursions of the tail fin (aimed to obtain the propulsive lift force), frequency of tail movements, but the essential swimming pattern remains the same. The unique interesting difference is the use of the pectoral fins to propulsive aims in swimming that is aimed to maneuvers, executing a rowing action. In this case pectoral fins have a function that is very different from the usual regulation of direction.

Anyway, in Fishes' swimming, gravitational factor can be considered as absent: we find only fluid and internal frictions, inertial reactions and muscular forces. These are the main mechanic factors that involve Fishes' rachis.

When first tetrapods go out from water, they lean the whole body on the ground to better contrast gravity. They must slitter and trail their body. Dry friction is strong, but it can be used through wavy movements in order to obtain locomotion. This movement pattern is directly inherited from their aquatic ancestors and now is supported by the contribute of the four limbs, that is synergic and coordinated with body waves.

The wavy movement is highly efficient and evident in snakes. Even if snakes have no limbs, they can be very quick thanks to wide flexibility of the body (their rachis is composed by a very high number of vertebras) and quickness of oscillation.

Later we find the elevation of the trunk, it is no more leaned on the ground. The weight of the animal is suspended on the two girdles: the shoulder (anterior) one and the pelvic (posterior) one. Only feet are leaned on the ground. Limbs become longer and stronger and each one is placed nearer the counter lateral one. walking is now quicker and running becomes possible. Running implies moments of full detachment from the ground!...

However the suspension of the trunk (it is now no more leaning on the ground) transforms the rachis from axial pillar to beam. Neck and head on the anterior pole and tail on the posterior one become a sort of shelves. Gravitational forces gain a new and different role both for shelves and for vertebral beam. They act on rachis according to a transversal direction. This mechanic condition is a very important factor (even if not the unique one) that contributes to limit the mass of terrestrial animals, differently from what happens in big Cetaceous.

The mass of the most gigantic herbivorous Dinosaurs is an exception, but maybe that at least they spent most of their time in a partial floating condition, with their body partially immerged in water.

It is a general rule that the scale factor limits animal's dimensions (moreover of the terrestrial ones) through different aspects, but the rachis static/dynamic charge is surely among the most important ones.

In fact, when linear measures grow, the two dimensional ones (as bones sections – for mechanical resistance- and muscles sections – for the absolute strength) grow according to the second power, while the volumetric ones (linked to mass, inertial reaction, weight) grow according to third power. This means that when an organism changes its linear measures, the ratio between surface and volumetric parameters becomes very different, with the obvious consequences for physic/biological features. When linear measures grow, for example, the absolute strength, that is linked to only surface parameters (muscular section), grows much more, while the relative strength (linked to the ratio l^2/l^3, between muscular section and mass and weight) becomes lower.

A further evolutionary modification was the raising of the anterior part of the body: the anterior limbs are no more leaned on the ground. The mass is balanced on the transversal axis that crosses the hips. It is the beginning of the more or less stable bipedal asset.

A much more extended part of the rachis (from pelvis to head, no more only neck and head) becomes a shelf: the balance on the hip joint axis is not complete: the anterior mass is prevalent and trends to lean on the ground again. Bipedal position is maintained thanks to erector muscles of the rachis and to the extensors of the hip joints. Due to necessity of resting or to balance safety, often the bipedal position is abandoned for provisory regaining of quadruped one.

The unique mass that balance the anterior one is the tail. This can reach a conspicuous mass or length, in order to this function. See the Kangaroo, or the Squirrel, or the Lemurs. The anthropomorphic monkeys, that often use the bipedal position, use moreover the support of the knuckles of the superior hands.

Bipedal position reduces the surface of the polygon of support very much. Walking we have a further reduction: an only foot is leaned on the ground alternatively with both them.

This condition requests quick and exact postural adaptations, aimed to maintain the dynamic and static

balance, even if, in extreme danger of falling, it is possible to use the quadruped support.

Homoeothermic regulation warranties quickness and efficiency of nervous and muscular systems and therefore it is indispensable for the bipedal balance and locomotion. For this reason Dinosauria, Aves, Mammalia were or are homoeothermic. This physiologic feature is even more important for big animals. Due to the already cited scale factor, if a small animal (of a few hundred grams or of a few centimeters) falls, it is very improbable that it receive an important trauma. If a big animal (as an elephant) falls, the traumatic consequences are very important and, probably, lethal. The decisive factors are the distance between barycenter and the ground (that determines acceleration and quickness of the impact), combined with the quantity of motion, that is widely due to the mass. The passive resistance to trauma is linked to the section of the structures, as the bones and other tissues. This ratio (between mechanic solicitations and resistance of organic structures) is less and less favorable when the bodily dimensions are bigger and bigger.

If Dinosaurs were not homoeothermic paleontologists should find the skeletons of the big bipods (*i.e.* the famous *T. rex*) with important traumas: it would be unavoidable for mechanic and statistic reasons. During its whole life, if it was not homoeothermic, such a big animal would have had many occasions of being bleary for the low environmental temperature and statistically it would have fallen. Paleontologists tell us that it did not happen, so this can be considered an even indirect proof of homoeothermic nature of bipod Dinosaurs.

The actual exception of "Lizard Jesus" (that is a Saur, not a Dinosaur) that runs on the water on only two limbs does not deny the rule. Bipod locomotion is realized in a few occasions and for a short time. Besides the animal has an exiguous mass and the water surface is not dangerous in case of impact…

In human beings the vertebral "shelf" of "occasional bipods" becomes a structure that till now has been named "vertebral column". The lug wrench toward the anterior direction is very reduced or almost eliminated. The barycenter of the trunk is almost fully balanced on the axis of the hip joints. The verticality is much more advanced and the balance is much more stable, even if there is a residual trend of imbalance toward the anterior direction. However the balance can be maintained and managed, *ceteris paribus*, by a smaller muscular effort.

The postural difference is not only quantitative (the complete or almost complete extension of the hip joint): it is also qualitative: the global concave forward curve of the rachis, in Man is transformed in a series of shorter alternated curves on the sagittal plane.

We find four curves (cervical convex forward curve, thoracic concave forward curve, lumbar lordotic convex forward curve, sacral concave forward curve). The first three are mobile, the fourth one is fixed. The mobile curves make the human rachis a composite shock absorber, that works as a spring, moreover to be pushed, seldom to be stretched. Its mechanic resistance, if compared to an hypothetic rachis without curves, is ten times higher, according to the formula

$$R = n^2 + 1$$

where "R" is the resistance and "n" the number of curves. "n" = 3 offer a resistance ten times higher if compared to "n" = zero.

When human position of rachis was established (however this fact is evident also in anthropomorphic monkeys and even in other "partial bipods"), we notice a rotation of the main movements of the trunk. In Fishes and in Proto-chordates (as much as in quadrupeds) we find a meta-chronic wavy movements on horizontal plane, aimed to swimming and later to walking. In bipods are more important sagittal movements. Besides in bipods another movement gains importance: linked to walking and to running, we find rotation movement around the main axis (the vertical one) of the body: the shoulder diameter turns in a contrary sense of the pelvic one. This is aimed to maintain the frontal and balanced position while we walk.

The human rachis works according to its axial direction, against gravity. It is a fasciculate structure that is composed by three substructures. If observed in a transversal section, they are disposed as a triangle. On the anterior side, along the medial plane, we find the bodies of the vertebras alternated to inter-vertebral discs. On the posterior side, symmetrically, we find the two lines of the articular processes. These are linked to vertebral bodies thanks to stems pedicles. These three substructures are parallel and form a unique functional system. According to our opinion, this system could be called "vertebral pilaster", obviously in the case of human rachis, that is unique among all animals. However the name of "pilaster" refers only to the static function. But due to the main function of our rachis the label of "shock absorber" is more proper.

The real novelty is the different distribution of the gravitational stress (meant both as punctual or distributed stress), if compared with the situation of the beam. Before bipedal (vertical) arrangement of the body, the function of shock absorber was carried out by limbs, not by rachis. Due to this rachis is deprived of the elastic-viscous component. Differently man entrusts the role of elastic-viscous shock absorber to the rachis itself. The stress is no more applied to the rachis perpendicularly (creating an angular momentum), but it is applied as co-axial. This causes a compression or traction (when the body is suspended or hung). However we always have pression mixed with flexion, due to physiological curves or to other causes of a never perfectly vertical direction of rachis. This indicates us that in mathematics and in engineering there is the

possibility of a modelization of human rachis as an harmonic oscillator with n degrees of freedom.

In the movements around longitudinal axis, each vertebral body make a rotation, related to the contiguous one, thanks to the torsion of the discs.

Articular surfaces slip reciprocally according to main vertebral movement. Amounting all the rotations/torsions of each metamere we have the global torsion of the rachis and the rotation of an extremity regarding the other.

In each other movement we have always the elastoplastic deformation of discs and the slipping of the articular surfaces.

This is a well known story. Starting from it we pose our question. Our observations are based on the exposed facts. If compared to the original proto-chord, the function of axial support has made some evolutionary jumps. From water (hydrodynamic propulsion) to land (leaned on the ground and later suspended as a beam), to elevation of anterior limbs (only pelvis sustains the charge of bodily weight, without the support of the shoulder girdle), to the more complete verticality of human rachis, including the peculiarity of the physiological curves.

Many factors have changed: i) mechanic solicitations (gravity instead of fluid friction and so on); ii) quantitative parameters (vector modules, their direction and sense…); iii) cinematic equations (mathematic models of different kinds of motion, to calculate speediness, accelerations, shakes…); iv) biomechanical functions (shock absorber, spring to be pushed/stretched, instead of pillar aimed to warranty swelling to bodily mass and efficiency to meta-chronic waves; v) main plane of movement (no more the coronal plane, but the sagittal and transversal ones).

No doubt, during this long evolutionary drift of rachis, there are also factors of functional continuity: generically rachis has always been an interior sustention, but the discontinuity cannot be ignored.

This ambivalence can be detected also in some widely demonstrated exaptations.

Swimming bladder is a cave organ whose gaseous content is controlled and regulated as much as in the lungs, even in these two cases, this function is differently aimed.

Feathers as thermal device interact with air, and so happens for the flight organs, even in a different way and for different aims.

And penguins' wings? Their use has converged with the one of the pectoral fins of Fishes. Due to this reason, is this transformation definable as exaptation? The same question can be posed for the limbs or ex-limbs of Cetaceous or Pinnipeds, or, *illo tempore*, of Ichthyosaurs. These structures have converged toward Fishes' fins.

When some snakes go forward through rectilinean motion (some author names it "caterpillar"), they create grip and propulsion, managing ventral scales as small crammed paws of myriapods, where flows metachronal waves. Scales evolved as mechanical defense of the reptilian body, not as locomotion appendixes.

The case of the elephant's trunk is a different case: would that nose become so long and so powerful, if it should not carry out tasks that are different from the respiratory ones?

The dentine is a further different case. It sprang in fishes as somatic protection. Later, in other vertebrates, it migrated on teeth's surface. Can we consider this repositioning as a transformation, so we do not name it as exaptation?

The prehensile tail of certain monkeys works as a fifth limb and it is a very important role in arboreal locomotion of the animal. the vertebrates' tail has the function of compensating for asymmetrical movements, overall the walking of tetrapods, and of balancing body in symmetrical movements or postures, for example in bipedalism of animals as kangaroo or squirrel. The grasp (that allows the animal to be suspended or to climb) is a function that is obtained through exaptation?

Was this evolutionary transformation an exaptation?

The first exaptation was the transformation of fins in limbs in the tetrapods, when vertebrates conquered the terrestrial environment. Sustaining the body's weight and pushing it forwards thanks to attrite and to vinculum reaction, is a fully different task if compared to swimming in a gravity free environment.

We know that evolution is an irreversible process: it doesn't come back to its origins erasing the transformations that happened in the meantime. Well: the passage from limbs to fins is not the reverse of the previous passage from fins to limbs. It is a further and different passage.

The same question could be legitimated about many other transformations.

No doubt these jumps imply quantitative and qualitative variations. The question "Exaptation or not exaptation?" can be posed each time.

Other hierarchically more general and more important questions can be posed: i) Can we establish a criterion according to which the change of function (and, even marginally, of structure) can be defined "functional cooptation", so that the definition of "exaptive" can be legitimated? ii) Can we build a detailed model of this protocol of examination and classification of the evolutionary transformation? iii) Can we structure a taxonomy of indicators and descriptors on which we can base our interpretations? iv) Can the phenomenon be quantified (not only qualified)?

We hope to have answers in a near future…

And, during the evolutionary drift, how many convergences and analogies could arise this question?

These jumps imply qualitative and quantitative variations in an absolute way. The remaining problem is if they can be named "exaptations" or not.

This is a difficult question for scholars in the future…

APPENDIX

Even if many things may be changed, the following suggestions can be useful.

We think that some factors can be meaningful in indicating the maintaining or the transformation of the rachidian structure. These factors must be defined with a more exact and articulated criteria, but nevertheless it can be important they are indicated, even if summarily:

- Regionalization. Number of regions (it is the same or it is different) and number of vertebras in each region (it is the same or it has an increase/decrease of a certain percentage, up to 20%, up to 40%, ...). Under the genetic profile it is important to compare the function of Hox genes.
- Index of flexibility.

$$= \frac{(\text{Iv D G})\text{^ } 2}{(\text{Iv D d})(\text{V G})}$$

If

If = Index of flexibility

Iv D G = Intervertebral Disc Gauge

Iv D d = Intervertebral Disc diameter (transverse or sagittal)

V G = Vertebral body Gauge

- Ratio between (transversal or sagittal) diameter and gauge of vertebral body
- Angulation of transverse processes in projection on transverse plan (within a certain value or wider)
- Angulation of transverse processes in projection on sagittal plan (within a certain value or wider)
- Angulation of spinous processes in projection on sagittal plan (within a certain value or wider)
- Presence/absence of the homologous processes
- Extension of muscular insertion surfaces on vertebras (especially on the processes)
- Ratio between vertebral volume and whole body volume
- Vertebral density
- The comparison is to be made between homologous vetebras (of the same segment).

We would like to focus that these parameters can be easily detected and compared thanks to laser scanner surveying.

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Choledochal cyst: our experience in a single case by laparoscopic approach

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Introduction

Choledochal cysts are disproportionate dilatations of the biliary system for the presence of a congenital malformation, the persistence of the common biliary pancreatic channel. Complete excision of the cyst is the best treatment strategy to avoid long-term complications especially malignant transformation, recurrent cholangitis and gallstones. We present a clinical case treated at our center with minimally invasive surgery.

Case Report

A female patient was admitted to our center at the age of 3 years, with right hypocondrial pain, followed by jaundice, vomiting and recurrent fever. No abdominal mass present. Abdominal ultrasound was performed and also Magnetic resonance cholangiopancreatography. According to the Todani classification modified by Alonso-Lej classification we identified a type I with three gallonstones. She underwent laparoscopic cyst excision and hepatico-jejunostomy Roux-en-Y with perianastomotic drainage positioned. No early and late postoperative complications after 1 year of follow-up.

Discussion and Conclusions

Choledochal cysts can present at different ages with variable symptoms. Common presentations include abdominal pain, jaundice, and right upper quadrant mass and are most common seen in pediatric patients. Associated congenital anomalies of biliary tract may be present. Most cases of choledochal cyst disease have type I and IV-A cysts. If left untreated, choledochal cysts have an increased risk of malignant transformation. Early surgical excision and restoration of biliary tract continuity is mandatory, whatever the symptom severity to avoid long term complications whenever possible. Currently the gold standard treatment is the mini invasive surgery, in fact the advantages of this technique is the intraoperative visualization of deeper structures, decreased postoperative pain, shorter hospital stay, improved cosmetic result and decreased postoperative ileus. However, these cases remain reserved for highly specialized surgeons with a thorough understanding of hepatobiliary anatomy and minimally invasive techniques. Finally, limited case series of robotic pediatric choledochal cysts resection and reconstruction have been reported with acceptable outcomes, although more studies are needed before widespread acceptance and implementation of this technique in pediatric age.

Partial congenital bowel obstruction by duodenal atresia windsocks type: case report

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Introduction

Duodenal atresia Windsocks type is a rare condition of congenital bowel obstruction. Thanks to recent technological advancements of prenatal diagnosis it is possible to make a diagnosis of duodenal atresia with high degree of certainty through the radiological sign of "double bubble", but up to date it is not yet possible to identify the type of duodenal atresia. We report the case of a patient with prenatal diagnosis of "double bubble". The patient had no other concomitant malformations.

Case Report

The patient came to our attention after prenatal ultrasound that showed a picture of double bubble. At the 27th week of gestation we performed fetal MRI that confirmed the US pattern of double bubble but it did not identify with certainty the type of duodenal atresia. At birth the patient underwent GI rx examination that showed a picture of partial duodenal obstruction compatible with the Windsocks type. On the following day, we performed endoscopy which showed the presence of duodenal membrane, so the patient underwent surgical treatment with a longitudinal duodenal incision in order to treat the wind-sock membrane. After one month a further Upper-GI rx examination showed a regular transit of the contrast. Four months after the first operation the patient underwent new surgical treatment for bowel obstruction by adhesions. The operation was successful and the patient had a complete recover.

Conclusions

Patients with prenatal diagnosis of "double bubble" require a multidisciplinary approach for proper clinical management. Unfortunately it is not currently possible to identify with certainty by prenatal ultrasound the type of duodenal atresia, but in case of incomplete bowel occlusion , the possibility of an atresia Windsocks type should always be considered, especially for setting the right surgical approach.

A case of ileoileal intussusceptions caused by Burkitt's lymphoma

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Introduction

Burkitt's lymphoma is a high grade B-cell tumor described for the first time by the Irish surgeon Dennis Burkitt in 1958 in Africa. The most frequent of the clinical variants, in which it is classified by the World Health Organization (sporadic, endemic, HIV-associated), is the sporadic one, which usually involves the abdomen, in particular the ileocecal tract. Thus, a common clinical presentation is that of a child suffering from abdominal pain with nausea and vomit, until the dramatic case of an intestinal occlusion by an intussusceptions. According to this, the surgeon is the first who diagnoses and treats this tumor, playing an important role for the treatment, in terms of reduction of the metabolic complications of the medical therapy and of improvement of survival rate. In this work we present a case of a child operated for intestinal occlusion by ileoileal intussusception, caused by a Burkitt's lymphoma, as it was diagnosed by histological examination few days after surgical intervention.

Case report

A 12-years boy reached the emergency department for abdominal pain and vomit. Two weeks before he had a surgical intervention for a suspected appendicitis at another hospital. An ultrasound examination was performed and it revealed the presence of a complex mass in the right iliac fossa. The day after the patient felt worse and he had an episode of bilious vomit. An x-ray examination of the abdomen was performed and showed the presence of an intestinal obstruction. The patient underwent to surgical intervention. The obstruction was caused by an ileoileal intussusceptions, and it needed to perform a resection followed by anastomosis. Few day after surgical intervention, the result of histological examination indicated the presence of a Burkitt's lymphoma within the tract resected. A CT scan was performed and showed the involvement of mesenteric lymph nodes. The bone marrow aspirate and the lumbar puncture showed no neoplastic presence. Then the patient started therapy according to protocol.

Discussion and Conclusions

Burkitt's lymphoma represents 3-5% of all non-Hodgkin lymphomas, and 40% in pediatric population. Children have an excellent prognosis with contemporary treatment regardless of the disease stage. Patients with limited stage disease are curable with limited treatment, avoiding complications associated with more intensive therapies. Nevertheless surgery is important in the management of this disease, the role of the surgeon has usually been controversial. A surgical intervention can be resolving in case of limited disease, or, in case of a high stage disease, it can be diagnostic or helpful, through the debulking of the mass. However, apart in case of intussusception, the diagnoses of Burkitt can be challenging and the presence of disease can revealed after a story of recurrent abdominal pain or after surgical interventions for appendicitis. In this work we show how a timely diagnosis can be difficult and how it can be an obstacle for treatment. In this case the sudden worsening of clinical conditions permitted an early diagnosis with a complete resection of the ileum involved by Burkitt's lymphoma has improved the prognosis and reduced therapy's complications.

La modulazione dei livelli di glutatione come strategia di attacco nelle interazioni ospite-parassita

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Abstract. Insect studies, dealing with parasitism of aphids, have shown that the disruption of host glutathione (GSH) pool and metabolisms significantly contributes to its physiological regulation and castration. The parasitic wasp *Aphidius ervi* injects into host aphids a venom containing large amounts of a gamma-glutamyltransferase (Ae-GGT) enzyme, which causes a depletion of GSH primarily involving ovarian tissue. Injected Ae-GGT in fact consumes substrate GSH, which ultimately triggers apoptosis. Studies on virulence factors of microrganisms have documented that the invasion strategies of selected pathogenic bacteria also target host GSH metabolism. Indeed, it has been shown that GGT activity of *Helicobacter pylori* and *H. suis*, the agents responsible of peptic ulcer, can exert antiproliferative and pro-apoptotic effects in gastric epithelial cells. By confocal microscopy, *H. suis* outer membrane vesicles (OMV) – submicroscopic structures 20-50 nm in diameter, budding from the cell surface – were identified as carriers of *H. suis* GGT, capable of delivering the enzyme to the deeper mucosal layers. In association with such membranous structures, active GGT from *H. suis* in fact translocates across the epithelial layers and can access lymphocytes residing in the gastric mucosa, resulting in the inhibition of lymphocyte proliferation, *i.e.*, a perturbation of host immunity and a facilitation of bacterial infection. Cellular GSH appears, thus, to represent a conserved target for parasitic (micro)organisms which aim at altering host redox homeostasis to weaken its immune defenses, using GGT as a key-element of a virulence strategy. Taking into account the "parasitic" behavior exhibited by malignant cells spreading across tissues and organs of the patient (the "host"). GGT activity is in fact expressed in a number of malignant tumors, and expression levels often increase along with progression to more invasive phenotypes. Now, active GGT can be released from cells, including cancer cells, in association with submicroscopic vesicles resembling exosomes. The similarity of such structures with GGT-rich OMV particles of *H. pylori* and *H. suis* is indeed obvious. GGT activity of cancer cells can affect intracellular redox equilibrium, and produces in addition significant extracellular effects, *e.g.* on the redox status and ligand binding affinity of cell surface receptors related with cell survival/apoptosis balance. Thus, GGT-rich exosomes shed by cancer cells can produce in host's surrounding tissues effects comparable to those reported for Ae-GGT or *Helicobacter* GGT, possibly resulting in facilitation of malignant cells survival and diffusion.

Key words: Host-parasite interactions, glutathione, gamma-glutamyltransferase, tumor metastases

I parassiti instaurano uno stato di simbiosi con individui di diverse specie – definiti "ospite" – in modo da accedere alle risorse metaboliche necessarie per sopravvivere e riprodursi. Queste associazioni di tipo antagonistico si caratterizzano per la presenza di complessi "dialoghi molecolari ospite-parassita," selezionatisi durante l'evoluzione per lo più al fine di rendere i tessuti dell'ospite più accessibili alla colonizzazione e sfruttamento da parte del parassita [1]. In un gran numero di esempi, l'azione del parassita non soltanto ostacola la fisiologia e la riproduzione dell'ospite, ma può addirittura culminare nella morte di quest'ultimo e/o la consumazione completa dei suoi tessuti. Questa è la norma con gli insetti, che comprendono il numero più elevato di specie con abitudini di vita parassitarie, e sono caratterizzati da sottili strategie di virulenza che spesso condividono con organismi appartenenti a gruppi filogeneticamente distanti [2]. L'incredibile diversità, e le inattese somiglianze con esseri viventi tassonomicamente non correlati, offrono l'opportunità di interessanti paragoni tra le strategie di virulenza che prendono di mira vie molecolari specifiche e conservate, come risultato di schemi evoluzionistici convergenti. In questa sede ci occuperemo dell'analisi comparativa di una peculiare strategia per la regolazione fisiologica dell'ospite, diretta al controllo dell'omeostasi redox, che sembra capace di modulare le interazioni parassitarie sia a livello cellulare che di organismo *in toto*.

Studi condotti sugli insetti, in particolare sulle paras-

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sitosi da afidi, hanno mostrato che l'alterazione nell'ospite delle riserve e del metabolismo del glutatione (GSH) può contribuire significativamente al controllo fisiologico e la castrazione dell'ospite [3]. La vespa parassita *Aphidius arvi* al momento di deporre le uova è capace di iniettare negli afidi-ospiti un veleno contenente grandi quantità dell'enzima gamma-glutamil transferasi (Ae-GGT), il che innesca l'apoptosi nella parte superiore degli ovarioli dell'afide, bloccando in questo modo l'oogenesi [4]. Come questa azione specifica resti limitata agli ovarioli, e come sia modulata a livello molecolare, resta da scoprire; tuttavia, dati preliminari indicano che nell'afide-ospite parassitato si verifica una deplezione del GSH, che interessa innanzitutto i tessuti ovarici (Masi e Pennacchio, risultati non pubblicati). Non richiede particolare fantasia immaginare che la Ae-GGT iniettata possa competere con la GGT endogena per il substrato GSH, in tal modo interferendo con il regolare riciclo del GSH ed esponendo le cellule dell'ospite, così deprivate di GSH, ad uno stress ossidativo che in ultimo scatena il processo di apoptosi.

Ad ogni modo, è ugualmente ragionevole supporre che gli effetti pro-ossidanti della GGT, dovuti all'azione metallo-riducente del metabolito cisteinil-glicina ed alla produzione in sede extracellulare di specie reattive dell'ossigeno (ROS) [5]; possano esitare in alterazioni del bilancio cellulare apoptosi/sopravvivenza. La presenza di una GGT anche nel genoma di un entomopoxvirus associato alla vespa parassita *Diachasmimorpha longicaudata* [6], inoculato con la deposizione delle uova e usato come un sistema di rilascio di fattori di virulenza, indica che questo enzima possa avere altre azioni regolatorie sulla fisiologia dell'ospite, per ora sconosciute. E' certamente plausibile l'ipotesi di effetti negativi sulle difese immunitarie dell'ospite [7]. Studi sui fattori di virulenza dei microrganismi hanno documentato che le strategie di invasione di alcuni batteri patogeni sono anch'esse dirette a colpire il metabolismo del GSH dell'ospite. E' stato infatti riportato che l'attività GGT di *Helicobacter pylori* e *H. suis,* i patogeni responsabili delle ulcere peptiche, può avere effetti antiproliferativi/proapoptotici sulle cellule epiteliali gastriche [8-10]. Tali effetti vengono innescati dal metabolismo GGT-dipendente del GSH e dalla conseguente produzione di H_2O_2, con conseguente attivazione del fattore NF-kB, aumentata espressione di interleukina-8 ed aumentato danno ossidativo al DNA [9,10]. Paradossalmente, l'aggiunta di glutatione alle cellule trattate con *H. pylori* aumentava marcatamente gli effetti [11,12]. Mediante microscopia confocale, è stato accertato che la GGT di *H. suis* si associa alle *outer membrane vesicles (OMV),* strutture submicroscopiche del diametro di ca. 20-50 nm che gemmano dalla superficie cellulare. Le OMV risultano capaci di veicolare l'enzima negli strati profondi della mucosa [12]. Trasportato da queste strutture vescicolari, l'enzima GGT di *H. suis* può attraversare in forma attiva gli strati epiteliali e può raggiungere i linfociti situati nella lamina propria della mucosa gastrica. Il risultato di questo curioso processo sembra essere un'inibizione della proliferazione linfocitaria, ossia un'alterazione delle difese immunitarie dell'ospite e una facilitazione dell'infezione batterica [13].

Gli studi futuri permetteranno probabilmente di osservare altri esempi di interazioni ospite-parassita basate sul GSH, come forse nel caso dell'attività proapoptotica della GGT rilasciata da *Campylobacter jejunii* [14]. Il GSH sembra dunque rappresentare un bersaglio conservato per i (micro)organismi parassiti che mirano ad alterare l'omeostasi redox dell'ospite per indebolire le sue difese immunitarie, e la GGT emerge come fattore-chiave di questa strategia di virulenza. Si può ulteriormente ragionare su questo concetto spostandosi nel campo della biologia dei tumori e prendendo in considerazione il comportamento "parassitario" che viene adottato dalle cellule maligne che si diffondono nei tessuti ed organi del paziente (in questo caso, l'"ospite"). L'attività GGT viene espressa infatti in un gran numero di neoplasie maligne, ed i livelli di espressione spesso aumentano durante la progressione della malattia e con la comparsa di fenotipi più invasivi (come rassegna, v. Pompella et al., 2006)[15]. A questo proposito, studi recenti hanno dimostrato che GGT attiva può venir liberata dalle cellule, comprese quelle tumorali [16], associata a vescicole sub-microscopiche del diametro di 20-40 nm, simili ad esosomi [17]. In effetti è evidente la somiglianza di queste strutture con le vescicole OMV ricche di GGT rilasciate da *H. suis*. L'attività GGT delle cellule tumorali può alterare gli equilibri redox intracellulari [18], e produce inoltre effetti significativi anche a livello extracellulare, sullo stato di S-tiolazione di proteine esterne alla cellula [19], e persino sullo stato redox e l'affinità di legame di recettori della superficie cellulare collegati al bilancio cellulare apoptosi/sopravvivenza [20]. Sorge pertanto spontanea la domanda, se gli esosomi ricchi di GGT dispersi dalle cellule tumorali non possano produrre sui tessuti circostanti dell'ospite effetti paragonabili a quelli descritti per la AeGGT o la GGT di *Helicobacter.* Tali effetti potrebbero risultare nella facilitazione della sopravvivenza e diffusione delle cellule tumorali nel corpo dell'ospite.

Nel loro insieme questi processi GSH/GGT-dipendenti, osservabili in organismi evolutivamente distanti e popolazioni cellulari diverse, rinforzano ulteriormente la notevole importanza che riveste l'omeostasi redox nella modulazione degli stati di salute e malattia. Far luce sugli aspetti regolatori di queste strategie parassitarie convergenti permetterà probabilmente di identificare nuovi potenziali bersagli terapeutici.

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