REQUIREMENTS FOR THE ASSESSMENT OF PHARMACOKINETIC, PHARMACODYNAMIC AND MIXED POPULATION MODELS AND SOME TOPICAL CONSIDERATIONS: A SEMINAR

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Summary

The reader interested in a critical reflection on the issues related to the biomedical pharmaco-toxicokinetic and pharmaco-toxicodynamic models developed since the foundation of Ancona University, now Polytechnic University of Marche (UPM), is referred to the references.

KEY WORDS

Pharmacokinetic (PK), pharmacodynamic (PD), mixed PK-PD models

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"What is culture? The education of our attention" Simone Weil.

"My goal is to make myself useful" Giuseppe Prezzolini, Introduction to "Amici"(1922).

1. Foreword

The guidelines of the FDA (2004) and the EMEA (2006) lay down the rules for the presentation of studies of the potential greater stability of pharmacokinetic (PK) and pharmacodynamic (PD) population models, also with a view to identifying significant differences among subgroups, by simulating clinical trials. The aim, an important one for the progress of applied pharmaco-toxicological research, which has been found unsatisfactory also in phase I (Cf: Ref. 183 in [1]), II and III trials [2]), is to identify inter-individual covariates, assessing their difference from intra-individual ones. The difficulties of this approach have been analysed and demonstrated theoretically (pp 383-4, in [3]). However, it is hard to understand why these studies should not be conducted and regulated in the framework of preclinical trials [4]. Nonetheless, even when such investigations have been performed in human subjects - as in the 324 studies selected from the 482 conducted in 2002-4 reported in MEDLINE (PubMed), whose preliminary evaluation of adequacy, i.e. validation, qualification, appropriateness, performance and predictivity has been made available (360 different PK and 118 PD models) – the results they have obtained [5] prompt a number of further considerations.

In synthesis, the nine specialists [5] judged whether the models met the presentation criteria of data, which were always limited (descriptive interpolation approach), or else they inferred the results (predictive interpolation and extrapolation approaches) by adopting *internal*, *advanced* and *external* evaluation methods. Evaluation according to the internal methods ("*basic"*, overall 45% of PK models, accounting for 68% of those selected and for 62% of PD models, only 9% of those selected – various plots of goodness of fit (GOF), those most frequently adopted: 69% of PK models, 65% of PD models; the uncertainty of parameter estimation (detailing whether the standard error was calculated from the data matrix according to Fisher, using maximum likelihood or the bootstrap method), accounting overall for 45% of PK and 62% of PD models. They used *advanced* evaluation methods, i.e. data splitting; resampling methods derived from bootstrapping and cross-evaluations, examining the stratification of covariates; and various Montecarlo simulations, including sometimes original Bayesian ones, of *a posteriori* distributions [Cf: 6]: 28% PK, 16% PD). They finally used *external* methods of evaluation of the tiny remaining fraction of the models in the overview (7% PK and 8% PD). After analyzing metric appropriateness, they concluded with subjective syntheses and evaluations of individual reproducibility. Notably, the regulating authorities insist on the need for such evaluations, but have so far failed to reach a consensus on them; indeed the same FDA encourages their development. This situation reproduces the inconclusive situation of the lack of iso-receptor autoclassification, groupings of similar drugs, repeated converging optimizations that are never definitive and therefore the need for rapid completion [Cf: 7]. What is more, there is a patent gap, resulting not only from the lack of available data, but also of data obtained by pharmaco-toxicological research to support the soundness of recommendations when not of political-regulatory impositions, national and, even more disappointingly, regional and global constraints [8]. Here the prerequisite of the linear nature of the relationship between evidence and policy, rendered elusive, provides merely programmatic data [8; Cf: 9]. At the same time, it is becoming increasingly evident, as well as necessary, that traditional trials are substantially being supplanted without side-tracking delays, by those based on genetic investigations, which are potentially more patient-tailored [Cf: 10]. The use of advanced simulation systems for PK/PD models (e.g. *Simulink* R) and for metabolic networks (e.g. *SimBiology* R) [11] of metabolimic diagnostics may constitute useful simplifications to achieve these goals, where the sole exhaustive published investigation exhibits a disappointing incompleteness, as only about 50% of estimated parameters were provided with the respective, necessary standard errors and/or confidence intervals, and only about a third of the papers selected reported similar measures, essential

for any statistical analysis, for randomized effects e.g. non-linear non-independent residuals (e.g. *NONMEM* R, *PCNONLINE* R, etc), making the body of data as precarious as they are rationally unusable. The same goes for the other criteria, especially the *external* ones, certainly the most stringent in establishing the models' predictive ability. Thus, the deficiencies of the data and/or the rare application, added to their incomplete nature, undermine most of the data, while the one justification offered by the authors is the editorial and publishing process, a problem that is easily resolved by *on line* procedures, with sound evidence that is as exhaustive as possible.
It is to be hoped, and this hope warrants alone the present note and seminar, that web diffusion of scientific papers will soon be adopted not only by the regulatory bodies most directly involved, but also by international organizations in expressing complementary and supplementary recommendations, by the prestigious organizations that fund research and by industry-related Foundations, to which the authors not accidentally belong. This would make complete detailed examples of the processing developed by the mentioned software freely available and allow to facilitate and contribute effectively to achieving, finally, with acceptably satisfactory evidence [8] the necessary results of the analysis of deficient and inconclusive comparative applications in view of their increasingly rapid compatible/sustainable updating. This has been done [12].

2. Pharmacokinetic (PK) Models

Biomathematics and pharmacokinetics have been removed from the syllabus of Ancona University, now Polytechnic University of Marche (UPM) Medical School [Cf: 13], and the question remains of how the graduate students of the various courses, including residents and master holders, can ever learn and verify the theoretical-practical premises of dose optimization in drug administration for clinical and therapeutic purposes, at least in the framework of whole-body clinical pathophysiological pharmaco-toxicokinetic models [14], extended to mixed models incorporating mechanistically-based receptor theories from binding to activation and interactive transductional effects, up to those proper of disease processes and progressions [15], which have certainly not been supplanted by ignorant empiricism besides the adoption of international manuals, of initiatives and especially of the body of practical lessons conducted here systematically [Cf: 16-23]. The present, relevant and not only theoretical work, pursued with strong commitment (e.g.: [24]) despite the lack of funds, continues to address the problems that shall have to be recognized, not only where the ambiguities of problems relating to population studies mentioned above persist, but also, quoting the Master [3], where clinicians need to have rationalized in advance how they should proceed in individual patients exposed to basic validation of the 7 standardized parameters, at least selecting 4 to derive the others individually characterizing ones, independent but pooled for each molecule and dosage prescribed:

$$Clearance^{1} \times Turnover\ time^{2} = Volume\ of\ distribution^{3};\ Turnover\ number^{4} \times Turnover\ time = Permanence\ time^{5},\ Yield^{6} \times Permanence\ time = Occupancy^{7}.$$

This is particularly true given that the models subjected to the first overall evaluation performed are all "mixed non-linear"[5] in the absence of relevant comparative analytical-statistic studies of data, their arguable trends and "residual" models that cannot be simply reduced to isolated outliers.

Besides the application of linear models to highlight their looped factors in isolated models [24-25], we have attempted to extract new parameters in conditions that were or were not proved to be linear, by addressing and developing original issues of the revived systemic, basically holistic pharmaco-toxicology, in the time and frequency domains, as reported previously (3rd paragraph in [10]; [26]), reproposing also here parametrizations (such as, for the originally identified oscillations / fluctuations, amplitudes and their indices of instability / decay, phase shifts; etc) that are clearly necessary to define better structured and consensually more complete evidence without however any recognition having emerged locally to date, besides the contradictions of intervened decisions formalized without explicit motivations for initiatives that have remained anonymous and anyway damaging (°).

3. Pharmacodynamic (PD) Models

3.1. Incompleteness of the biophysical/biological-molecular demarcations of "receptor theories".

The comments of David Colquhoun, written with understandable and extraordinary grace, anyway surprising, deny validity to past and current "pharmacologic-therapeutic receptor theories", especially as regards the dose-effect/response relationships empirically postulated to describe physical and non-physical models, advocating and presenting their substitution expressed by physics-based equation polymaths [27]. While noting the curious implications of the adsorption isothermal equation for the binding of the nicotine agonist, attributed to Langmuir (1918) but proposed by Hill in 1909 for the initial phase of structural identification and of binding affinity presented for the antagonist by Gaddum (abstract of 1937), already written by Haldane (1930) but published by Michaelis and the Canadian Menten in 1913, the alternative hypothesis by Briggs and Haldane (1925) (Cf: p 34 in [28]) is totally ignored, a hypothesis that at least pools in the equilibrium the estimation of the relationship of the two (pseudo)-constants of velocity in opposite directions – k_1, forward, of association and k_{-1}, backward, of dissociation – including in it the one of the successive step –expressed today by the word "looped" – in the (pseudo)-k_2, constant of transformation into the product (and/or result, or effect, also pharmacological, as ordinarily measured), although the author of the recent historical overview (2006) is quite aware of the need for using it to avoid the most common imprecision which unfortunately too often continues to be practiced in the presentation of studies of the application of the mass action law to calculate the equilibrium (pseudo)-constants of initial bonds, in case of the emergence of the isomerization mechanism of Castillo and Katz (1957), which is an application of the seminal work of Wyman and Allen (1951)[29]:

$$\text{Drug} + \text{Receptor} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{FR} \overset{k_2}{\rightleftharpoons} \text{FR}^*$$

(unbound) (bound, inactive) (bound, active, gated)

The reader is referred to the Box, because, evidently especially in pharmacology, the analytical contributions dating back to Eigen (1968)(Cf: [30]) can no longer be ignored.

Colquhoun [27] denounces the delays, persistent errors and missed opportunities from inattention to major contributions from biophysics and molecular biology, citing the absolutely original contribution, which was neglected by the same eminent naturalized

Anglo-Saxon researchers, of Wyman (1951), who was subsequently a guest in Rome. The same goes for Clark, who was aware that "in the first place, there is no advantage in fitting curves by a formula unless this expresses some possible physico-chemical process, and it is undesirable to employ formulae that imply impossibilities". Given the impossibility of separating binding from its effects, Schild is acknowledged as the first to adopt the so-called "null method", based on evaluations with constant effect/response (but at what level? Certainly not only around the not further defined hemimaximal, an imprecision that is repeated in the formulation of "τ" by Black and Leff (1983)(Cf: [31]), see below) of different bonds, achieving quantification of the concentrations expressed as agonist to antagonist [B] "dose ratio" (1949), $r = 1 + [B] / K_b$, where K_b is an equilibrium pseudo-constant of bond dissociation of the same receptor, obtained by the equation of Arunlakshana and Schild (1959), at the time considered identical for all antagonists, for receptors thus classifiable in the various tissues independently of the agonists present and/or administered in the assays, something that has also been contradicted by our contributions [32]. We had to wait from 1909 (Hill) to 2001 (Venning and Dilger) to measure not the equilibrium constant but the inherent association and dissociation velocities of a first competitive antagonist (D-tubocurarine), despite the extant difficulties of separating the surroundings and/or the metabolic tissutal context also for the "traditional" agonists, where all attempts have been vain to distinguish binding ability (affinity) from the ability to activate responses not always linearly correlated after binding, with the shift from an action as full agonists to low *intrinsic affinity* according to Ariëns et al. (1954), or of different *effectiveness*, *partial* agonists according to Stephenson (1956), an author whose theoretical structure of the definitions has been declared erroneous, extending to the consequences of the operational model according to Black and Leff (1983) and to the proposals of Kenakin (1985), to mention but the most significant.

In fact, even after the measurement of the first step [of structural recognition and binding affinity; a radioisotope methodology introduced by Paton and Rang (1965) and subsequently extended by Mintun et al (1984) and Baron et al (1985)] using positron-emitting isotopes in *in vivo* positron emission tomography, it has proved impossible to define the exact parametrization of the dynamic mechanism of the second step, which may or may not be related to tissutal factors measured from the reserve of spare receptors [whether or not they participate in activation processes according to Furchgott (1955) and Nickerson (1956)], occupancy, later considered a "misnomer", and "elusive" the same intrinsic effectiveness [33], according to dynamic models similar to the complex models of population development growth [34], and even advocating that the evolution of the properties of ligands and receptors should never be neglected in view of their characterization [35]. In fact, occupancy values may prove misleading, like the more traditional measurements of binding performed according to Scatchard (1949) when the affinity of the receptor is found to be multiple, and different when this is bound or unbound: ever since the equation proposed by Hill for the Hb-O_2 bond (1910), it should be kept into account in relation to the dose-effect relationship that *the affinity of receiving macromolecular subunits can vary for each individual molecule of the substrate or of the modulator* (for instance: for Hb the number of hydrogen ions dissociated from globin following oxygenation of the 4 hemes - Bohr effect -; for red blood cells, the ratio of free/bound 2,3-diphosphoglycerate and the effect of propranolol [36]; etc), extensively of the agonist and/or antagonist subsequently, due to kinetic accumulation, errors of estimation of affinity that may have occurred in sub- and supra-cellular structures, in the traditional organ surviving *in vitro*, which is considered intact, native in standardized biological assays [37] and, consequently, *in vivo*. The same exponents of the equation proposed by Hill, similar if not identical to the logistic equation [38; 40], measured and identified both for traditional agonists and antagonists, may thus be

due or not to (Gaussian) symmetric probability distributions, integrated with slopes of logarithmic transformation to the x axis which are significantly different from 1, and are also different in individual traits at any reagent concentration, linearization slopes of the transforms which, by applying the least-squares method can present weighted coefficients of regression reasonably and usually considered as significant common resultants, but in fact merely on average of those of the two or more, only apparently identical iso-receptors or multiple bonds (Cf: [39]) that are found in different *densities* also in the same cell and even more in the same organ. At different equilibrium ratios corresponding to different substrate-receptor concentrations, or to different response levels even in the same preparation, and why not? to different "*r*" of the antagonists, the *intrinsic efficacy* can thus assume diverse significance and parameter values. Indeed, Furchgott has designated it as "unitary", a utopian entity of reduced dimension associated with a single receptor, or, better, bond (1966); these are errors related to the estimation of differences between receptor occupancy and activation, or estimation of bound and unbound receptors, but also potentially of whether they are or are not activated, structurally different or functionally non equivalent, which usually interact by cooperative modalities, neither identified nor recognized by the introduction of $\tau = R_o / K_e$, the ratio between total receptor concentration ($R_o = R_t$) and the concentration of the [AR] complex, producing a hemimaximal effect where, according to the cited operational model, $K_e = \log A_{50}$. Such facts and caveats, which can be read between the lines of the latest IUPHAR recommendations (1995) of quantitative receptor definitions, also in view of drug classification [40] – see also [41] -, possibly not yet comprehensively compared, are not kept in the least account even in high-level publishing practices and are ignored in the current verifications of propedeutic effectiveness, where avoidable imprecise definitions, like the definition of potency, may however acquire critical basic significance. In the presence of indeterminate intermediate complexities that still cannot be established, we have opted for a merely probabilistic [42], consciously abstract approach (see Box), which has nonetheless allowed to acquire quite valuable data (Cf: [43]), albeit gathering into a *unitary scheme* a large number of such phenomena. It should however be noted that, for the sake of completeness, the general stance of Pharmacology, like *Grenzgebiete* (Loewi, 1935), has not been neglected, with the contribution of one specific, analytical model of pharmaco-toxicological conformational transactions [44].

Quantitative mathematical definitions are still lacking for agonist dynamics on receptor metabotropic complexes, of which GPCRs are the most frequent whereas, to quote Colquhoun again [27], the only systems that have proved to be "so simple that it is possible to establish with reasonable probability the relation between quantity of drug and the action produced" are a few agonist-activated ion channels. Here research has progressed, in some cases actually achieving the measurement of "as many as 18 rate constants from a single set of ion-channel recordings" (Project to measure conformational changes before the glycine channel opens (2004)[45]) thanks to the development of biophysical technologies (Cf: Neher and Sakmann, 1976; etc) and of mathematical theory (whose merit is mainly ascribed to Hawkes (1990, 1992; etc), often with the collaboration of Colquhoun himself (Cf: 1977, 1981, 1982, 1985, 1986, 1987, 1998, 2003, 2005, 2006; etc).

We will not discuss further the problems of the mechanisms of ion channel opening, limiting citations to those which, for their analogies with enzyme complexes [46], also activated by endogenous transmitters [47], have achieved bewilderingly detailed data on overall selectivity not only of kinetic nature, but with reference to multiple functions, individually analyzed in all the gradual chemical details identified, topically localized and time-interdependent [48]. In fact we believe that the lucky electro-physiopharmacology student

who was accepted in the lab of Neher and Sakmann's group, can best meet the challenge, mentioned in the shared published work [49] included in the recent references of the most prestigious international literature [50] and reason for explicit satisfaction.
As with any subject approached within Ancona University, now UPM, after the initial opening, the author's work has always turned to other, different topics hoping that the collaborators would then autonomously pursue the potential developments. After the six regulatory months from hiring, individual researchers become the sole responsible to the senior researcher for their research subjects if these are still topical (°).

--(BOX)----------

The minimal model proposed in the sequence of pages 6-7 according to Eigen [30]:

$$E + S \underset{k_{-1}}{\overset{k_1}{=\!=\!=}} ES \underset{k_{-2}}{\overset{k_2}{=\!=\!=}} EP \underset{k_{-3}}{\overset{k_3}{=\!=\!=}} P + E$$

admits for each enzymatic reaction process a description that can be reduced to *6 microscopic velocity constants*, whose detailed analysis is however commonly considered illusory. The reader is referred to the discussion, mentioned in chapter "*Il controllo della velocita' della reazione enzimatica*" [51], which could well be included in the present paper given its very topical nature. In the model discussed in 1965 [52] the diversity of each pair of constants measured in opposite directions can be sufficient to understand the kinetics of cyclical metabolic fluctuations, whether or not involving sub- and supra-cellular structures up to the response cycles of chrono-pharmacology, which can no longer be neglected [53].
For the same equilibrium reaction of the first intermediate [substrate-enzyme] compound undergoing turnover, measured by Chance and found to be consistent in the cases that at the time were proved to be non allosteric with hyperbolic equilateral function, linearized by Lineweaver and Burk (1934) albeit inadequate and potentially misleading to the ends of the double reciprocal plot – which afford the advantage of a smaller number of errors in parameter transformation of variables, obviously including dynamic studies of antagonists, whether or not isolated, by applying the "most simplified" least-squares analysis (Cf: from the functions corresponding to the plots, Dixon (1953), Hofstee (1952), Eadie et al (1949), and/or Hunter and Downs (1945), and later [54], the reader is referred to Walter's contribution, *First Portonovo Conference* (1974), where even for enzymologists preferences tend to obscure the information found in their data, as noted by *J Biological Chemistry*, leading to the continuation of the practice of prejudice rather than of science [55]). Clearly, there has not been an absolute lack of contributions to resolve the inconsistencies of the analysis of ligand-receptor interactions [43], but their experimental application has been sporadic.

With these premises, the fundamental equations of the so-called reference basic binding theory, enzymatic [E] non-allosteric and receptor [R] are reported as in a)-e), corresponding, respectively for substrates [S] (including hemoglobin, which has proved to be a necessary reference), agonists [X] and antagonists [Y], their pseudo-constants of equilibrium estimated by the least squares method after forced linearization with logistic transformation as "positions" on the y axis and respective *n* and *m* slope coefficients, interpreted to be expressions of exponential interaction; lastly, the elementary premises of probability theory [42], and how it has been applied reaching corresponding parametrizations at least in the central positions of the characteristic dose-effect functions [43].
a)
We mention the evolution of the elementary prototype of Scatchard's equation (1949) of binding measures according to the mass law action: $v / c = K_n (n - v)$, where v is the molar units of bound receptor by molar units of protein, c is the concentration of free receptor in solution, K_n is the apparent association constant and n is apparent maximum of binding sites (Cf: [37, 39, 54, 75, 81]). Since c is practically constant, equalling fractions of 1, and/or 100%, it can be replaced with the version adopted in all subsequent equations, considering it equal to 1 in the equations reported in b) and d), Vmax in c), and finally p = 100% in e), also in view of logistic linearization.

b)
For the prototype Hb and O_2 bond, the contribution of Hill (1910), stated as fitting not explaining any mechanism (Cf: p 150, in [27]):

$$[Hb\text{-}O_2] / (1 - [Hb\text{-}O_2]) = K_a [O_2]^n, \text{ from which the function and diagram:}$$

$$\log [Hb\text{-}O_2] = \log K_a + n \log [O_2],$$

universally known for the implications that did not escape Wyman and Allen (1951).
c)
For the more exemplified and most commonly applied enzyme kinetics, according to Michaelis and Menten (1913):

$$v = V_{max} . [S]^n /([S]^n + K_m) = V_{max} / (K_m / [S]^n) + 1, \text{ or } Vmax / v = 1 + (K_m / [S]^n),$$

which in logarithmic transformation:

$$\log \{(V_{max} / v) -1)\} = \log K_m + n \log [S],$$

where [S] is the molar concentration of the substrate, K_m is the affinity pseudo-constant estimated at the hemimaximal when *n*, constant of Hill (1909, 1913) and/or of Langmuir (1918), is equal to 1.
Notably, (Vmax / v) -1, or (Vmax – v) / v, which in the logarithmic transformed reproduces the logistic equation according to Berkson (1944) as an integral of the Gaussian function, corresponds to the final probabilistic equation in e): (1-*p*) / *p*. We do not report further linearization equations, the most widely adopted of the "inverse doubles", quoted above, nor do we report the discussion of the same plots corresponding for the estimation of the equilibrium pseudo-constants and the similar indices to Hill's (1913) for inhibitors with competitive dynamics, irreversible non-competitive and acompetitive dynamics, for which the reader is referred to [32], equations according to Loftfield and Eigner (1969), Wilkinson (1961), Myers (1952), Goldstein (1944), Easson and Stedman (1936).
d)
For the antagonists, the contributions of Gaddum, Clark and Arunlakshana and Schild (1959) have been abundantly cited, equation AGCS: $r = 1 + [B] / K_b$, the latter a dissociation equilibrium pseudo-constant for antagonist B in the assay, from which similarly:

$$\log (r– 1) = \log K_b + m \log [B],$$

logarithmization that has allowed the application of the least-squares method to extract as usual with the index of significance of the slope (tg α) or the exponential index *m* of the interactions of the same antagonist, in addition to the estimation of the affinity of the position, that is the ordinate value at zero abscissa point. Finally,
e)
The *probabilistic* interpretation, which generalizes the common discussion of the previous sections considering especially the possible interaction not yet structurally verified, extending their consequent capacity both for the quantal and the gradual effects [42], with the premise that the ligand molecules (X, or Y) that act and/or are adsorbed to the receptors are proportional to their probability (*p*) of meeting them, and because *p* is the ratio of favourable to possible cases, i.e. *p* = % / 100 = receptors $[R_x]$ that participate in the reaction / total receptors $[R_t]$, and moreover if *n* is the index of a multiplicity of interactions, based on the mass law, at equilibrium:

$$K_x = ([X]^n [R_t]) / [R_x] = [X]^n([R_t] – [R_x]) / [R_x], \text{ therefore } K_x [R_x] = X^n ([R_t] – [R_x]), \text{ or } [X]^n[R_t] = [R_x] (K_x + [X]^n, [X]^n / (K_x + [X]^n) = R_x / R_t = p,$$

$$1 / p = (Kx + [X]^n) / [X]^n = K_x / ([X]^n + 1), \ (1 / p) – 1 = K_x / [X]^n \text{ , and}$$

$(1 – p) / p = K_x / [X]^n$, which transformed into logits, in the form of the "characteristic line" becomes:

$$\log (1 – p) / p = \log K_x - n \log [X],$$

where if $p = 0.5$, $\log K_x = n \log [X]$, with K_x corresponding to the affinity pseudo-constant and [X], if $= [X]^n_{50}$, corresponds to DE_{50} when $n = 1$. Notably, for quantal data, for Gaddum (1953) the linearized function represents the integral of that of the normal (Gaussian) distribution of the sensitivity of the individual reacting units, whereas the slope would indicate the precision of measurements, or dispersion of the trait.

The analogy of the functions, if not their actual homology, allows to accept the conclusions that the theories based on traditional methods have become obsolete: "It is sobering that receptor pharmacology can already provide several examples of fortuitous agreement between experimental observations and the prediction of hypotheses that we now know to be quite incorrect" (Cf: p 8, in [56]). It should be noted that for the same saturation function of Hb, or better oxyhemoglobin

dissociation, some authors propose that it be represented by "inverting the Cartesian axes", aiming particularly to achieve the benefit of immediacy [57]; whereas for unstable Hb another lesson has come from clinical practice, where the molecular biology approach to the globin phenotypes in conjunction with the genetic approach, to date of the best known models, has resulted to be inadequate to achieve diagnoses of pathophysiological susceptibility due the exploration deficits of the "notes" (Cf: consequences of the coexistence in the same Hb Jamaica Plain molecule of the double globin-ß $^{Glu6Val,\ Leu68Phe}$) mutation [58]. It is interesting to note how during the so-called deorphanization of receptors previously defined as *inverse* – e.g. $ß_3$, individualized by the characteristics of the prevalent gene and/or protein sequences before their functional identification with a procedure that is *inverted* compared with that of multidimensional phenotypic screenings of traditional serendipity -, objections may arise (Cf: [59], for G protein-coupled receptors, GPCR, 80/99, P2Y), and how there has never been a confirmation of the genetic risk of 85 possible factors of acute coronary syndrome [60], whereas it has been reported that a variant on chromosome 9p21 increases the risk for coronary disease and cardiac infarction [61], confirming the paucity of the definitively validated knowledge with high clinical relevance.

Going back to our theme, the references reported [43] describe the conditions to measure the application of original models, where each detail of the statistical tools applied allows to acquire the degrees of freedom of the confirmations of significance with P ≤5% of error both for regressions of parallel lines and of the pseudo-constants and the *n* and/or *m* exponents obtained. In [43] (1964, 1) the characteristic functions of the effects have been acquired for [X], Ach in this case, in 3 traditional receptor preparations and for l-norepinephrine and l-epinephrine also on two of the same (guinea pig vas deferens and seminal vesicle), obtaining effects of antagonism as well as of competitive synergism through dl-isoprenaline at only two concentrations, whereas in ref [43] (1964, 2) the competitive antagonism of the effects of Ach has been studied in guinea pig ileo-distal preparations in association with dl-dichloroisoprenaline, achieving by application of the equation $p = K_y[X]^n / (K_y[X]^n + K_x[Y]^m + K_xK_y)$, which for 50% response (p = 0.5) presents as $[X]^n_{50} = (K_x / K_y) [Y]^m + K_x$, and finally via *ad hoc* linearization:

$$\log ([X]^n_{50} – K_x) / K_x = - \log K_y + m \log [Y],$$

where the analysis of parabolic function significance results from the four exponential values *m* acquired and validated based on the respective assayed concentrations of the antagonist [Y].
The original body of complete evaluations has subsequently been repeated for a number of irreversible inhibitors (covalent block of "free" residues of primary aminoacid chains) in the same ileal receptor preparation [43](1969) and in the enzyme activities of its extraction Ach-esterase and MAO [43] (1971), where histamine is an agonist in addition to Ach, yielding the corresponding equation:

$p = (K_y[X]^n) / (K_y [X]^n + K_x [Y]^m + [X]^n[Y]^m + K_xK_y)$, solved with $p = 0.5$ ed $[X] = [X]_{50}$, and linearized in:

$$\log ([X]^n_{50} – K_x) / K_x = m \log [Y]\{(1 + [X]^n_{50}) / K_x\} – \log K_y,$$

the estimation with the usual level of significance (P ≤ 0.05) of the affinity constants as well as of the exponential values of agonists and antagonists (insurmountable), with the relevant estimation of their ratios in relation both to Ach and H, i.e. *achieving the first quantitative definition in the standardized experimental conditions of the two central issues of pharmacology, respectively the nature of the efficacy and of the appropriate taxonomy of analogous drugs and their iso-receptors.* In Ancona, applied to the definition of the spectra of ß-blocker antagonism ([43] (1980, 1979/1980; 1976/1977), this has led, so far uniquely in Italy, to the Editorial [98] as well as to more recent developments [102].
---(End of BOX)----------

3.2. Evolution of knowledge on constitutive G-protein-coupled receptors (GPCRs), activated or not activated.

In the initial 30 minutes of the cycle of lessons, devoted to the discussion of the material distributed to the students to verify their knowledge of the notions that are essential to understand the topics to be treated according to the schedule, as in the case of the brief note of Nickerson (1956) [62] of Manitoba University, interpreted at the time as occupancy correlated linearly to the single state (energetic, functional) of receptor activation, a similar bewilderment has followed the work of Cerione et al (1984), Costa and Herz (1989) and Costa et al (1990, 1992) and finally that of Lefkovitz et al (1993), Samama et al (1993) and the equally brief and seminal research of Bond et al (1995) [63], where the mutation of $ß_2$ adrenergic receptor and its alkylation, overexpressed (x 200) in transgenic mouse myocardium, have been found to be reconcilable only with the minimum model of two receptor states in equilibrium of, respectively, inactive and spontaneously active allosteric conformations, that can associate to protein G also in the absence of the modulating receptor, agonist or antagonist. Whereas *traditional agonists* bind to and increase the density of the activated form, *inverse agonists* bind to the inactive form and shift the equilibrium, reducing their molarity, presenting *negative intrinsic efficacies* and/or *activities*; *neutral antagonists* devoid of *intrinsic efficacies* and/or *activities*, whether they present equal affinity for the two conformations – or do not alter the equilibrium - or not, anyway behave as *inverse agonists*, reducing active receptor *density*; it is however expected that *agonists as well as antagonists* can in turn equally and independently act with *positive* as well as *negative intrinsic efficacies* and/or *activities*, disrupting their conventional classifications and moreover evidencing therapeutic consequences where the lowered *functional tone* (discussed by Zamboni (1971, 1958)[64]), with intense when not catastrophic consequences for dosages, alas not commonly evaluated when the normalizations to the ordinate are transformed to fractional, percent, probit, logistic, etc values, and consequent variations in slopes, whether or linearized (Cf: pp 2-3 in Cingolani et al [41]). Such *tone* can be an expression for example of mutations on which depend *inductions of constitutive receptors* with *high functional* rates, leading to possible adverse and even severe consequences (Cf: [66](1999), etc).

The above mentioned notes, at the time almost simultaneous with other contributions from the more advanced scientific community, as indeed natural and expected [65], represented but a new paradigm, still evolving and continuously debated, with a myriad theoretical contributions over time, here merely *referenced as presented and similarly discussed in the classroom* [66], also an expression of renewed applications developed by the global industrial research, which however still finds it hard to understand its revolutionary potential for innovation [67]. The *ad hoc* methods developed subsequently have without any doubt represented significant advances, leading to the verification of experimental models that have continued to evolve [68], particularly those of the dynamics and kinetics of the processes of desensitization and prolonged action [69], and of the introduction of the concept of dynamic *efficiency*, or effect per concentration unit [70], which cannot be addressed in detail here. Returning to the main Hb model, isomeric T-deoxy state, with low or negligible affinity *vs* the relaxed R state typical of Hb-O_2 with high-affinity equilibrium conformation, it has already been advocated by Birdsall et al (1978) for rodent brain muscarinic iso-receptor that GTP converts the bond of agonists to low-affinity forms; but even earlier Pert et al (1973) had identified the selective cation effect to discriminate agonist from antagonist affinity for opioid binding, whereas Snyder et al (1980) proved that GTP and Na^+ act synergistically in reducing the agonist affinity for the same receptor (Cf: [71]). However, what most needs to be highlighted here, in line with [27], is that besides the confirmation of the physical-chemical nature of the PK/PD models of the mentioned prototype of receptor-channel [45], "attempts to make similarly detailed studies on G-protein-coupled receptors have, so far, proved impossible. Although reaction mechanisms have been proposed that are based on similar considerations, the information is simply not there to identify even

equilibrium constants, never mind rate constants". Therefore, only the general model, independent of still unsolved physically and quantitatively groundless structural interpretations, has provided some data.

4. Requirements of Simultaneous PK/PD Trials and Model Implementation

It is maintained that experimental trials (and even more so clinical ones) should envisage maximum utilization potentials with minimization of risks and waste, both pharmaco(toxico)kinetic and dynamic; this has been stressed internationally for the former [72], and must obviously occur without exceptions for the latter. *Ever since the founding of this University, teaching in this field - devised and implemented together with practical tests* made obligatory by subsequent regulations and consistently considered essential to achieve minimum standards of verified professional competence - has been parsimonious, combining models with the practice of methods of concentration analysis *vs* times of sampling, as well as intensity of the effects *vs* times of development (with respect to the minimum, non-reducible third dimension of the experimental design), to detect differences in trends and relevant parametrizations, where possible simultaneously validated. At various levels of complexity (e.g. [15, 73-75]), therefore, we have certainly not been alone, nor have we remained behind subsequent contributions (Cf: [10, 24, 76-78], if we agree that there are also examples of dissociation between the kinetics of concentration ranges and of opposite functional metabolic effects, as well as prototypes of the ongoing hormetic dose-response revolution [79]). Simply, it was established and it has not been forgotten that the fundamental models, initially biophysical and subsequently molecular biological, ever since the earliest papers [Langley (1878); Hill (1909, 1910); Gaddum (1914, 1937); Clark (1926, 1937, 1959); Guarino and Bovet (1949); Arjëns (1954); Nickerson and Stephenson (1956); Schild (1949, 1959); etc] have not prevented the application of the law of mass action to derive the first relations between concentrations of an agonist and that of the intermediate compound with the receptor substance according to Langley, later absorbance isothermal according to Langmuir (1916), both in equilibrium and in kinetic form: Hill (1909) measured *agonist* (nicotine; frog abdominal rectus) response *vs time* (exponential), and Schild (1947) studied "*antagonist* activity and specificity and the *relationship with time of action*" (quoted in [56]). In reply to [80], the relative independence of the kinetic parameter models has been reiterated in the inadequate measurements of equilibrium, of the estimates of those of allosteric or non-allosteric association (binding), the earliest phases of pharmaco-receptor structural recognition [81]. *Kinetic* differences of effects that are noxious in the short term (acute effects) but favourable in the long term have emerged from the treatment of ingravescent cardiac insufficiency with *ß-antagonist blockers with the properties of inverse agonists* (carvedilol, metoprolol), but not with *neutral antagonists* (bucindolol), a paradigm that has been found sound in a murine asthma model, acute treatments with heightened methacholine bronchial sensitivity with *$ß_2$ inverse agonists*, which reduce resistance in prolonged tests, simultaneous overlapping PK/PD relationships that involve the amplification chain of signalling in opposite directions as a function of time (quoted in [67], pp 94-95).

It is thus once again necessary to ask why the proposal advanced at the WHO [19], and later at the interregional level at SIF [82], to *store all individual cohort data of treated/exposed subjects* (Cf: *"Sample size n of 1 randomized clinical trials"* [83]*) in current therapeutic practice, in the various phases of clinical trials – and earlier, in the experimental trials that precede them* -, enabling PK/PD reviews with the desired exhaustiveness of associated genomic/proteomic/metabolimic statistical analysis gradually practicable, *are not yet overall repeatedly validated, systematically predisposed/programmed nor applied.*

5. Concluding Remarks

There is a widely felt need to found a cohesive universal ethical code on "rigour, respect and responsibility" for researchers, which is acknowledged to be still lacking [84], while researchers are brilliantly discussing the eternal return of the *déja vu*, which is not felt to be solvable on the mere scientific level [85] and coherently continue to maintain, usefully at least for their Countries, that the ideal solution is to draw from the New Economic Policy, where science, education and healthcare, essential elements of the State, are not dependent on private capital, typical of trade, services and small industry (Cf: pp 248-252, 266-274, etc in [86]), where capitalism needs to be "saved from capitalists" [87], by promoting research and first and foremost pharmacological development, without however excluding Academia [88]. Maybe strong archetypes of the difference between science and myth that justify medicine [89] survive; therefore it would be useful to go back and reflect, i.e. stop ignoring that the general evolutionary Darwinian model typical of large scale biological organization is itself inapplicable in the case of selectively inaccessible molecular mechanisms [90], where environmental constraints are stronger than atomic and subatomic ones [90-91]. After *The structure of scientific revolutions* (1962) and at least the *post scriptum* to the 1970 edition [65], cultural relativism in human and social sciences is accepted beyond the same contribution to affirm it "to help better understand the scientific endeavour" (Cf: pp 79-87 in [92]; see also [93]), and yet, already in 1972, "receding into the mythical reimagining the difficult factual and historical situations from a different and more advantageous viewpoint" (p 17 in [94]), and recognizing that "when the dominant view that holds a period of culture together cracks, conscience regresses to more ancient containers, seeking survival sources that also offer sources of rebirth" (p 11 in [94]) has been addressed in ever greater depth. And, "understanding what we know is as important as knowing" (p 30 in [94]), if not more, but what one believes one knows is too often undervalued, as the intention expressed in this contribution has endeavoured to remind. Ultimately present, if a globally usable comprehension of precision is lacking, for instance at the same microcompartmented levels of the volumes associated to local concentrations supposed to be on average univocal, of causal recognition considered attributable to kinetic equilibria of mass actions of the selective iso-effects, which sometimes appear without evidence of specificity of major homogeneous receptor groups (Cf: [95-96]), if the same bipolarities of the more traditional clinical [97] and receptor [98-99] diagnostic simplifications are therefore considered as putatively identifiable, especially in their evolution, like off-label prescription practices (Cf: [100]), it might become necessary to go back – regress? – to the alternative models of "time and space" continuities, the broader ones, ignored without reason [101], redefining the persistent ambiguities, for instance where "inhomogeneous compartments" are described [102], albeit consciously or unconsciously eluded in medical practice (Cf: [100, 103]).

The celebration of the history of the best British pharmacometric tradition has eventually included the seminal modelling contribution of the MWC, a pillar of molecular biology and pharmacology, formulated especially by Wyman [27] and later generalized to not merely quaternary but also tertiary protein conformations; nonetheless, it is admitted that theory and experimental analytical technology have achieved their limit of applicability, still irreducibly elusive, both in the higher scale of concentrations of macromolecular reagents, where for instance constitutive receptor activation may occur in the absence of the ligands mentioned above (paragraphs 3 and 4), and in the supra-macromolecular structures of 2D cooperative lattices and higher-order intra- and extra-multi-supra-cellular structures [104, 42]. It has also become necessary, for instance where analyses are feasible and models are compatible, to evaluate the amplification reaction chains of all messengers, temporal and spatial components

of the evolution of structural complexities, to overcome the current stagnation in the production of significant pharmacotherapeutic results, also geno-phenotypic, potentially patient-tailored, as is invariably repeated, though clearly without neglecting the new hot-spots of interacting cascades of overlapping signals [102, 105]. First of all, generalizing the national and global collaboration to be realized and offered, to establish as is now easier to do, by continuing to pursue their redefinitions, lists, reclassifications, after necessary validation of the available models and the identification of significance thresholds. In fact, keeping in mind Gaddum's recommendation that "they [pharmacologists] must be widely read and have good memories. They must have the energy to collect data from many sources and arrange them in any orderly way so that general principles appear through the fog of irrelevant facts" [106], current approaches tend to reconcile the current models of the mode of collection of observational and experimental data, necessarily validated, bottom-up, with mathematical, abstract, top-down ones, both detailed to represent their complex process more accurately in simulations [107], following the main road.
The Turin epidemiologist Paolo Vineis made reference to John Locke's *An Essay Concerning Human Understanding* expressing in the epigraph and in the title of his *Nel crepuscolo della probabilita'* (1999) (quoted in [108]) the opinion that the predictive ability of biomedicine rests especially in the levels of populations, rather than of individual entities, which for Susan Sontag (1978, 1988) remains in the service of a simplistic vision of reality. A probabilistic scenario which nonetheless does not appear to be superseded by the recent insights into molecular analysis [104, 91, 42], genomics/genetics [109] and even less neurology and cognitive disciplines [110, 97].

(°) It should be noted that there is a considerable difference between our university organization and that of Great Britain, as also demonstrated by the "confessions of the Master", Dr Joseph Larner, a student of the Coris' and Chairman of public and private US universities for 30 years, five of whose students are Nobel laureates for medicine-pharmacology. In his "How To" at his Festschift [111] he stresses that especially where public funds are concerned tenure should be granted only after a researcher or professor has achieved some significant goal and may thus be freer to pursue a chosen topic, possibly advised by a senior scholar. He concedes of course that a researcher may fail throughout his/her life to achieve significant or excellent results, which are rare if not exceptional. But should this happen, it would be the loss of the Institution if a talented scholar were to leave because all positions have been filled. Unfortunately this continues to occur in our own system, and it is sad to see a proposal by a senior scholar being marginalized by a collective vote made anonymous by current regulations, as when, shamefully, a PhD holder, the recipient of multiple biannual research grants and an unpaid voluntary assistant for several years and a specialized medical doctor is fortuitously supplanted by the latest graduate student, who eventually becomes a confirmed researcher after a single year, despite the fact that the scientific production of the PhD holder is seminal and topical at a distance of 15 years [112].

Note: As customary, copies of the papers and books cited are available to interested readers who request them for consultation and preparative study, an essential prerequisite for dictation in class and for discussions from the floor, and/or by assignment at the appointed times. To avoid including an excessive number of references, some have not been cited and can be found in previous papers by the authors.

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