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Methodology

Scientific guidelines for preclinical research on potentised preparations manufactured according to current pharmacopoeias—the PrePoP guidelines

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ABSTRACT

Objective: Pharmacopoeias regulate the manufacture of potentised pharmaceutical preparations used in different branches of complementary and integrative medicine. The physicochemical properties and biological activity of these preparations are often investigated in preclinical research, yet no guidelines for experimental research currently exist in this area. The present PrePoP guidelines aim to provide recommendations to promote high-quality, statistically sound, and reproducible preclinical research on potentised preparations.
Methods: Input was gathered from researchers nominated by the relevant scientific societies using a simplified Delphi consensus approach covering the most relevant aspects of basic research methodology in the field including appropriate controls, sample preparation and handling, and statistics. After three rounds of feedback, a consensus was finally reached on the most important aspects and considerations for conducting high-quality research on potentised preparations.
Results: We present a series of recommendations on a range of topics including experimental controls, system stability, blinding and randomisation, environmental influences, and procedures for the preparation of potentised samples and controls, and we address some specific challenges of this research field.
Conclusion: This expert consensus process resulted in a robust set of methodological guidelines for

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research on potentised preparations and provides a valuable framework that will inform and improve the quality of subsequent research in this emerging field.

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1. Introduction

There are various pharmacopoeias that regulate the production of pharmaceutical preparations [1–4]. International agencies such as the European Medicines Agency [5], the U.S. Food and Drug Administration [6], and the World Health Organisation promote scientific guidelines for different aspects of research concerning drug quality, treatment effects, and drug interactions of pharmaceutical preparations [7]. The EQUATOR Network (https://www.equator-network.org/) provides an overview of these and other related guidelines.

These regulations also include the production of potentised preparations (as "Praeparationes homoeopathicae" in the *European Pharmacopoeia*) [1–4], which are used in different branches of complementary and integrative medicine, such as phytotherapy, homeopathy, and anthroposophic medicine. Potentised preparations are manufactured by a series of dilution (typically 1:10 and 1:100 ratios) and succussion (vigorous shaking) steps, starting from a substance of interest, e.g., the mother tincture of a plant. The manufacture of potentised preparations and their specific effects are the subject of ongoing research in this rapidly developing field [8–10].

Recent reviews of this field have underlined the need for increased quality in the research [8–10]. To complement the guidelines available for clinical research in homeopathy and align with similar international efforts in other fields, the aim of the present PrePoP guidelines is to establish recommendations for adequately performing experiments in the specific area of basic and preclinical research (e.g., physicochemical experimentation and cell-, plant-, and animal-based experimentations) into potentised preparations [11–17]. While reporting guidelines already exist [18,19], insufficient attention has been paid to the specific research procedures employed in this field. The present publication aims to address this deficiency. In particular, the following topics are covered: choice of appropriate controls, experimental design and associated statistical considerations, experimental system stability, production of potentised preparations, and publication and reporting.

2. Methods

The expert consensus process used for this publication was a simplified version of the Delphi process [20,21] whereby the lead authors (Tournier AL and Baumgartner S) generated an initial draft of the guidelines which were circulated to the co-authors for comments, which were then incorporated into a new draft. This updated draft was then presented to the group again. Comments were gathered and incorporated in an iterative process until a final consensus was reached.

Based on earlier publications addressing methodological issues [22,23] and the reporting guidelines by Stock-Schröer et al. [18,19], the lead authors Tournier AL and Baumgartner S went through a series of iterations to create the first draft covering the different aspects which they considered relevant to the question at hand. This already included the feedback gathered from several experts at a workshop held at the Homeopathy Research Institute Research Conference in London in 2019.

We then contacted the three well-established scientific societies in the field: the Groupe International de Recherche sur l'Infinitésimal [24], the Wissenschaftlichen Gesellschaft für Homöopathie [25], and the Société Savante d'Homéopathie [26]. The presidents of these societies were asked to nominate researchers with relevant expertise in the field to join our expert consensus process. All nominated experts were then invited to comment on the first and subsequent drafts. The expert consensus process was demonstrated in the Supplementary file 1.

3. Methodological considerations when conceiving experiments

Experiments in basic research typically involve the comparison of a potentised preparation of interest with appropriate controls. However, underlying assumptions are often not clearly stated, leading to confusion and inconsistencies in the choice of controls. To address this issue, it is recommended that the working hypothesis be clearly articulated. Formalising the system of assumptions and expectations behind a research project not only helps improve the experimental design but also clarifies the rationale behind the experimental design choices for the subsequent readers.

In order to scientifically address questions such as if potentised preparations have specific physical properties different from controls or if they induce specific chemical and/or biological effects different from controls, we need to define appropriate controls and establish experimental setups to avoid—as much as possible—false positive and false negative results. The following considerations serve to address these issues.

3.1. Choice of controls and delivery system

Negative controls are reference samples to which potentised preparations are compared in order to establish the presence or absence of an effect. Within the context of a specific experimental system, negative controls are samples for which we expect to have no specific effect, i.e., they are biologically and/or chemically inactive (*cf.* a placebo in clinical settings). Negative controls tend to exhibit small random effects, comparable to those observed in the absence of treatment. Negative controls are often simply referred as "controls" in most publications, so we will refer to them as "controls" in the following sections.

On the other hand, positive controls are reference samples that are expected to produce a known effect, according to established knowledge (e.g., substances in pharmacological concentrations). Potentised preparations are generally not used as positive controls. Positive controls can be used to verify the proper functioning of the experimental system and to compare the effect size of potentised samples to conventional treatments.

Negative and positive controls are distinct from systematic negative control (SNC) and systematic positive control (SPC)

experiments which are used to investigate the stability of the experimental system. SNC and SPC experiments use an experimental setup which usually compares potentised samples with controls but replaces both sample sets with identical negative and positive controls respectively.

Fig. 1 demonstrates the many possible combinations of methodologies available when preparing potentised preparations. Each such combination, together with the specific research question, will dictate the choice of the appropriate controls. As is quickly apparent, an exhaustive list of all possible combinations involved is not possible and we will therefore only discuss the general principles involved.

3.1.1. Choice of appropriate controls

Determining the most appropriate controls for different experimental setups and research questions investigating potentised preparations deserves careful consideration. We will now present four of the main research questions to exemplify possible adequate controls, which were determined by the modified Delphi process. The discussion is by no means exhaustive but hopefully illustrates the different issues one faces when selecting controls. Table 1 presents the most common controls and three examples to put the different controls into context.

3.1.1.1. Role of succussion. Succussion is a key step in the manufacturing process of potentised preparations, and the succussion of a liquid in ambient air leads to a number of effects such as formation of air bubbles of different sizes with different lifetimes, increased dissolution of air components in the medium (N_2, O_2, and CO_2), and increased dissolution of material from the potentisation vessel wall (e.g., Si, B, Na, and K) [27]. Some of the micro-bubbles formed during succussion implode upon themselves through a mechanism called cavitation. This process is known to tear material from the walls through the high pressures and speeds generated locally [28]. These processes may lead to further consequences such as increased oxidative processes due to increased O_2 dissolution, changes in pH due to CO_2 dissolution and acid formation, changes in nuclear magnetic relaxation caused by O_2 as relaxation agent, increased silica-hydrogel formation due to increased Si dissolution, radical formation due to cavitation, and potentially other biological effects (e.g., affecting growth rate in systems dependent on oxygen metabolism) [29,30]. In experiments investigating the role of succussion, it is recommended that non-succussed controls be considered, such as the medium used for dilution (e.g., 95% ethanol) and the simply diluted starting substance. Another closely related research question is whether succussion is required to prepare effective potentised medicines. In such cases, a further succussed control should be included to ensure that any differences found are not due solely to succussion.

3.1.1.2. Specific effects. When investigating the hypothesis that the potentisation of a given material generates preparations with *specific* effects (i.e., related to the potentised substance), we would like to distinguish such effects from the unspecific succussion effects. From this point of view, only succussed or potentised controls can be considered valid controls. Furthermore, the succussion and potentisation process used for preparing the controls should be identical to that used for the preparation of the verum.

Although the potentised dilution medium would appear to be the ideal control for investigating the specific effects of potentised medicines, further considerations indicate the matter is not so straightforward. Indeed, homeopathic provings of waters from specific sources indicate that, under certain conditions, "just water" might not be completely neutral. Such provings, also called as homeopathic pathogenetic trials, are used to test the effects of a substance on healthy individuals and document the symptoms it produces to determine its potential therapeutic uses [31]. Provings of waters from different origins can be found in the online database of provings [32] and this topic is discussed in detail in a book titled "*Aqua—water remedies in homeopathy*" [33]. Therefore, the question arises whether potentised medium might have unknown specific effects that would affect the experiment in unpredictable ways—it would thus appear that succussed medium might not be the ideal control when looking into specific effects of potentised preparations. Similar considerations show that lactose and ethanol have their own associated homeopathic symptom pictures [34,35] and also may not be "neutral" when being potentised [36,37].

Therefore, in cases where this is applicable, we suggest that potentised preparations of substances known to have no measurable effect on the experimental outcome parameters of a given test system can be used as negative controls. Example of such controls: potencies of gibberellic acid did not affect arsenic-stressed duckweed in contrast to potentised *Arsenicum album* [38], and potencies of abscisic acid did not affect gibberellic acid-deficient dwarf peas in contrast to potentised gibberellic acid [39].

When using biological systems, especially complex systems (e.g., plants and animals) [40,41], homeopathic theory tells us that we should differentiate between two types of assays: experiments on healthy/unimpaired organisms and experiments on stressed/challenged organisms [42]. The effects of a well-chosen potentised preparation on a diseased system are expected to be more pronounced compared to those on a healthy system, as this will result in a healing reaction that can be readily observed [42]. Based on the Simile principle, the vast majority of homeopathic

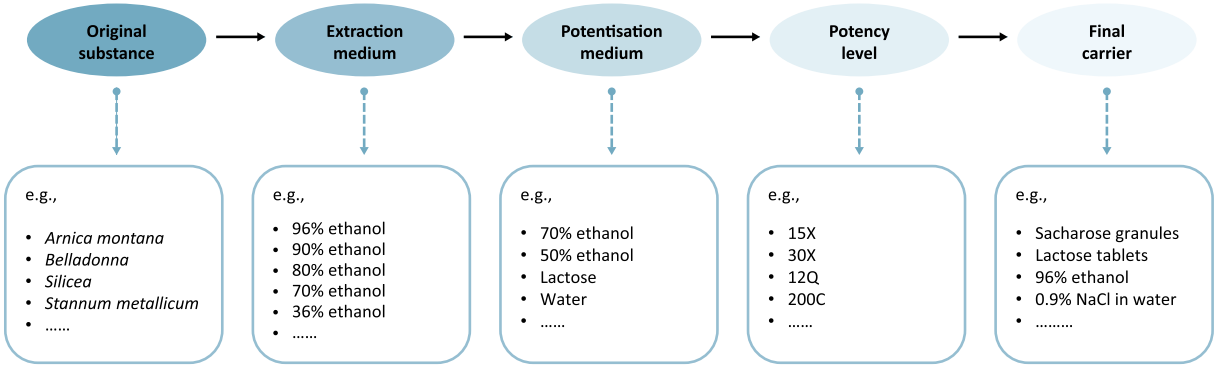

Fig. 1. Overview of the essential steps during the manufacturing process of homeopathic preparations. The ethanol concentrations are presented in % v/v, following the *European Pharmacopeia* [2]. The choice of the controls depends on the specific production process of the preparation on the exact research question (see also Table 1 for specific examples). "X" stands for 1:10 ratio, "C" stands for 1:100 ratio, and "Q" stands for 1:50,000 ratio.

Table 1
A number of possible controls exist, and the final choice will depend on the specific research question being investigated. Three examples were provided to illustrate different possibilities.

Example	Manufacturing process	Potential control					
		Potentised extraction medium	Potentisation medium			Different preparations with the same potency level	Plain carrier
			Plain	Successed	Potentised		
Arnica montana	Extracted in 43% ethanol, potentised in 95% ethanol to 30C with 95% ethanol as final carrier	43% ethanol potentised in 95% ethanol to 30C [a]	95% ethanol	Successed 95% ethanol[a]	95% ethanol potentised in 95% ethanol to 30C	*Atropa Belladonna* 30C [a]	95% ethanol [b]
Calendula officinalis	Extracted in 43% ethanol, potentised in 95% ethanol to 15C with SG as final carrier	43% ethanol potentised in 95% ethanol to 15C on SG [a]	95% ethanol on SG	Successed 95% ethanol on SG [a]	95% ethanol potentised in 95% ethanol on SG to 15C	*Arnica montana* 15C on SG [a]	SG [b]
Stannum metallicum	Extracted in lactose, potentised in water to 30X with water as final carrier	Lactose potentised in water to 30X [a]	Water	Successed Water [a]	Water potentised in water to 30X	*Zincum metallicum* 30X [a]	Water [b]

The ethanol concentrations are presented in % v/v, following the *European Pharmacopeia* [2]. "X" stands for 1:10 ratio and "C" stands for 1:100 ratio. SG: saccharose granules.
[a] indicates the recommended controls to investigate specific effects of homeopathic medicines.
[b] indicates the appropriate controls for real-world effectiveness research.

medicines are expected to have no measurable effect when measuring the secondary reaction. The Simile principle, also known as "like cures like" (*similia similibus curentur*) principle, posits that a substance causing specific symptoms in a healthy individual can be used to treat similar symptoms in a patient [42]. Hence, only those medicines that are a good match for the presenting symptoms are expected to significantly affect the system [38,43,44]. In such cases, potentised preparations of substances which have no effect on the outcome parameters can be used as negative controls. Different research questions require the use of unimpaired/healthy organisms, where it is anticipated that no healing reaction will occur, and we can expect the emergence of proving or milder symptoms when the biological system is pushed out of its natural equilibrium [45]. For such an experiment, establishing good controls is more challenging as potentially many potentised medicines are expected to have effects on the system.

Potentised preparations of a given substance might act in different ways than material doses, and we should therefore remain open to a wide range of possibly relevant outcome measures [30,46]. In the development phase of a basic research system, it may be beneficial to screen a range of potential outcome measures. Furthermore, it is possible that potentised preparations not only act on the average of an outcome measure but also act on other aspects of the data distribution such as increasing or decreasing the variability associated with a given outcome measure [47,48].

3.1.1.3. Potency level dependency. When researching specific effects of potentised preparations, not only the starting substance but also the potency level can be of interest. Thus, possible differences between potencies can be a topic of research. For this type of question, an appropriate control may be the same starting substance but at a different potency level. Similarly, a whole series of potency levels can be useful and is quite common for this type of question.

3.1.1.4. Real-world effectiveness. With this type of research, one is interested in the overall effect of a treatment, including context factors, e.g., in clinical application, consultation time and attention. In clinical research for such research questions, adequate controls are not placebo but usual care or no treatment. Correspondingly, in basic research, some questions are turned towards application in agriculture and livestock breeding [49,50,51]. In such cases, where one is not interested in the specificity of the effects or the underlying physicochemical mode of action, then "no treatment" and plain unsuccessed controls are also appropriate controls. This research approach focuses on the effectiveness of potentised preparations in the real world, which is different from the investigations addressing specific remedy efficacy.

3.1.2. Positive controls

If possible and appropriate, we recommend the use of a positive control, i.e., a substance in a molecular concentration that is known to create a particular effect, to: (i) document the system's reactivity, (ii) compare the effect size with that of a given potentised preparation, (iii) compare and contrast the mode of action of potentised preparations with that of conventional compounds.

An example is the use of histamine 2C (0.1 mmol/L) in comparison to potentised histamine (15C–19C) in the human basophil degranulation model [52,53]. Another example is the use of diazepam (1 mg/kg) and buspirone (5 mg/kg) in comparison to potentised *Gelsemium sempervirens* (4C, 5C, 7C, 9C, and 30C) in a mouse anxiety model [54]. Substances in pharmacological concentrations may have different mechanisms of action from potentised preparations [55]. Depending on the experimental setting, mother tinctures diluted or potentised to appropriate pharmacological concentrations could also be used as positive controls.

3.1.3. Administration and dosage

Depending on the experimental system, the choice of appropriate controls is not always straightforward due to the use of specific growth mediums (e.g., cell culture medium for in vitro assays and pure water or specific growth media for plant-based bioassays). Experimenters must also decide whether all potentisation steps or only the last potentisation steps are performed in the corresponding medium, or whether the potentised substance is diluted in the growth medium in a final step. In any case, the controls have to be treated in the same way as the potentised substance (see 3.4).

Dosage of homeopathic preparation is not straightforward in preclinical research, since the optimal dose is unknown for many preclinical systems. Thus, dose–response investigations are recommended if feasible. In this context, a similar dose-related question concerns the effect of repeat administration of homeopathic preparations. In clinical settings, a phenomenon called "homeopathic aggravation" is known to occur when the preparation is given either too often or in a too high potency, or when the selected preparation is not appropriate [56]. Thus, studying such effects in laboratory settings would enable a better understanding of the effects of repeat administrations.

3.2. Experimental design and statistical considerations

3.2.1. Blinding

The possibility of an unwanted influence of the researcher on the experimental outcome cannot be discarded when performing measurements that rely on human intervention (e.g., measurement of the root length of plants involving manual handling). Blinding is common scientific practice and highly recommended in such situations to avoid bias.

Effectively blinding the experimenter to the nature of the specimen is often difficult owing to the specific colour, smell, or consistency of the starting substance. However, this issue does not arise in homeopathy research as the samples are typically diluted beyond the point where any specific colour, smell, or consistency is discernible by even a careful observer.

A standard blinding procedure includes assigning codes to each sample such that the experimenter cannot tell which group a particular sample belongs to (see 3.2.3 for specific procedures for generating codes). Ideally, this blinding is performed by a person otherwise not involved in the experiment, such as an external statistician without conflict of interest regarding the experiment. Ideally, in confirmatory experiments with predefined hypotheses, the data analyst should also be blinded, with the unblinding taking place at the very end of the analysis thus avoiding any possible bias. In exploratory experiments, a blinded external statistician may be inefficient since only the experimenter himself knows all the details of the experimental procedure and setup that need to be considered for an in-depth statistical analysis. In any case, the best scientific practice dictates that all data should be collected under the blinding and the exact blinding procedure should be well documented so that it is possible to assess whether the data was adequately blinded.

3.2.2. Repetitions and replications

Experiments can be influenced on different levels by a variety of external factors. A common way to determine and statistically reduce the effect of such influences is repeating experimental units and experiments within a given experimental series. Multiple repetitions of the primary experimental unit are commonly used: for example, multiple individual plants, multiple wells in microtiter plates used for cell-based in vitro assays, or multiple individual samples in physical measurements. In the cases where these samples are independent, they can also be used as the statistical unit of the statistical evaluation (see 3.2.5).

Depending on the experimental setup, repetitions in *space* can be introduced. These are common in field experiments for plants, where different plots are used. Repetitions are usually also performed over *time*. This applies to repeating individual experiments one after the other to assess the variability of the experimental outcome over the course of time and to identify possible influencing factors modulating experimental outcome, e.g., the influence of electromagnetic fields (EMFs) and phases of the moon (see 3.3.6). Furthermore, the production of the verum and control samples can be repeated over space and time, e.g., in different laboratories and/or at different time points (see 3.2.4). The experiments can be independently replicated by different groups at other laboratories (e.g., a multi-centre study) and statistically evaluated as a whole for a more rigorous assessment.

All these repetitions and replications make it possible to assess the variability introduced by the different factors linked to the experimental procedure. They can and should be integrated in the statistical evaluation (see 3.2.5).

3.2.3. Randomisation

In order to minimise confounding external influences, measurements of both verum and control samples would be ideally simultaneous and at the same location. This is not feasible in practice; however, it is possible and advisable to minimise spatial and temporal differences to improve the stability of the experimental system. Common external influences include light, temperature, and humidity. The importance of these factors for biological systems as well as common measurement equipment is evident, yet sometimes ignored. For example, greenhouses always have light and temperature gradients, laboratory growth chambers may have a warmer side or a more humid corner, and temperature drifts may occur during sample preparation. Such heterogeneities or gradients may influence the experimental assays or the samples themselves. Measuring instruments also have time and temperature drifts that need to be taken into account. Other external influences may be the presence of local EMFs or differing soil quality in field and greenhouse experiments.

To minimise the impact of these effects, it is highly recommended that the samples be suitably randomised. One approach is to distribute the repeats within each experiment randomly over the space and/or in time. Another possibility is to use SNC experiments to establish a fixed blocked randomisation scheme optimised for a given experimental setup (see also 3.3.1 and 3.3.2). In this approach, the repeats of each condition are distributed over the experimental field in such a way that any systematic variability in the environment is distributed equally among groups. As an example, consider a scenario: 100 beakers containing duckweed (*Lemna gibba* L.) to be allocated to 20 experimental conditions with 5 repeats each [45]. These five repeats are positioned in the growth chamber in such a way that the average growth rate is as similar as possible for all 20 conditions. The optimal arrangement can be empirically determined using SNC experiments. In subsequent experiments, the spatial arrangement, i.e., the positions of the five repeat beakers in the growth chamber, is then kept the same, and the 20 experimental conditions are allocated randomly.

Adequate randomisation can be achieved by using methods such as repeated coin-tossing or throwing dice [57,58]. More usually, randomisation is achieved by referring to a list of random assignments generated by a computer. The use of computer-generated codes or real stochastic processes (e.g., throwing dice) is preferred over subjectively human-generated codes (e.g., assigning random numbers) [59]. More sophisticated randomisation procedures, such as blocked randomisation, can be used to randomise samples into groups of fixed sizes. The randomisation procedure should be well documented in any publication.

3.2.4. Multiple production lots

In order to avoid exaggerating random differences generated during the production process, it is strongly advised to use samples prepared multiple times from starting materials (e.g., mother tincture) rather than to repeatedly using the same sample or production lot. Multiple testing of the same production lot can amplify differences associated with the production process, e.g., variations in levels of contaminants, which could erroneously lead to highly significant effects (see Box 1). In order to determine the variability introduced by the production process itself, it is recommended that a minimum of three repetitions of the full production of verum and control and the ensuing set of measurements be conducted, with the latter being sufficiently statistically powered.

Box 1. The necessity of multiple independent production lots.

New potentisation vessels (DURAN® glass, hydrolytic class 1) were carefully washed according to trace analytical standards and pre-treated with acid to minimise ion release from the vessel walls. The analysis of trace compounds in water stored over several days in these vessels revealed up to a 100-fold differences in the levels of certain elements (e.g., Na, Mg, Si, B, and Fe). These differences were attributed to the highly variable levels of leaching between vessels, even for those made of high-quality glass, according to unpublished data from Baumgartner et al.

Thus, major physicochemical differences could have arisen if these vessels had been used for production of potentised preparations, solely due to differences between vessels. Therefore, randomisation by means of multiple independent production lots is mandatory to avoid false positives, i.e., differences which are actually only due to small variations in the vessel walls.

Some research questions investigate the use of potentised preparations in the "real world" using medicines provided by established manufacturers [60]. In such cases, comparing manufacturers and obtaining different lots from the same manufacturer makes sense to investigate inter- and intra-manufacturer variability.

3.2.5. Statistical methods

Statistical methods are used to cope with the unavoidable natural variability, the experimental "noise" that occurs in any experimental setting, be it biological or physicochemical. Statistics can be used to purely describe the data set obtained in measurements ("descriptive statistics") and then to further test the probability of certain hypotheses ("inferential statistics"). Descriptive statistics such as mean, standard deviation and standard error should be considered a must. When comparing potentised preparations with controls, we recommend that statistical tests are performed to assess the probability that any observed differences are due to chance alone. Choosing an appropriate statistical method is not easy. One wants to avoid false positives and false negatives. False positives, also called type I errors, are incorrect conclusions, that interpret random fluctuations (stochastic noise) in the experimental system as real treatment effects. False negatives, also called type II errors, are also wrong conclusions, but they state that no effects of the treatments were observed, when in fact there were such effects.

Different statistical tests have different properties. For example, more conservative tests tend to result in a false negative (type II) rather than a false positive (type I) error. The choice of a statistical test is also sometimes influenced by a researcher's concern about committing a particular type of error. In our opinion, the best way to resolve such issues is to use systematic control experiments (see 3.3.1) to test the appropriateness of different possible statistical models, and to identify the most appropriate statistical model in a given situation. The sample size needed to reach power calculation will depend on the type of statistical test, the magnitude of the treatment effect size, and the inherent variability in the response to treatment.

To illustrate a typical type I error, consider a scenario where the weights of a treated plant and a control are both measured 1000 times. If we are to base our statistical evaluation on these 2×1000 data points, we are very likely to obtain a highly significant difference. However, such a result can hardly help draw meaningful conclusions regarding the efficacy of the treatment, as it does not take the natural variability of the plants into account.

The basic statistical units to be used for the statistical evaluation also need to be correctly defined, as they must be truly independent for the resulting statistics to be meaningful. The nature of the basic unit will depend on the scale of the system under investigation. A single well in a microplate can be the appropriate unit, as well as an individual or group of organisms, depending on the intended level of generalisability.

Determining the correct statistical unit for a given experiment can sometimes be tricky. For example, in an aquaculture experiment investigating the behaviour of fish in differently treated tanks, if the fish within each tank interact with each other, then taking individual fish as the basic statistical unit will statistically overamplify random differences. Consequently, in such cases, the average per tank should be used as the basic statistical unit for statistical evaluation. Conversely, if there are no interactions among the fish, it is possible to reduce the statistical unit to a single fish, thereby increasing statistical power. Systematic control experiments are a good way to empirically determine if the chosen statistical unit is appropriate or not.

A common phenomenon in basic research is the occurrence of time-varying effects linked to unidentified external influences, which can increase the variance of the observed parameter to the point where the results are not statistically significant anymore. We therefore recommend using statistical methods such as analysis of variance (ANOVA) and analysis of covariance (ANCOVA), which are able to detect correlations between the measurements and time-related parameters (e.g., experiment or sample number). Larger sample sizes may be required to resolve interactions over time and among the different variables, for example, when multiple hypothesis testing is applied which requires corrections such as the Bonferroni correction.

Apart from providing probabilities (*P*-value) and confidence intervals, providing measures of the effect size such as eta squared (η^2) is useful in assessing the relevance of the results.

In any case, we recommend seeking the advice of a statistician at the planning phase of an investigation in order to determine the appropriate statistical tests to be used. To avoid mistakes, the use of statistical software is recommended.

3.3. System stability

In basic research on potentised preparations, stable experimental systems are needed. External influences can be of a physical nature such as spatial or temporal variability in temperature, or it can be experimenter-dependent (e.g., observer bias). Usual ways to avoid this problem are randomisation, blinding, and the use of statistical analysis. In the following paragraphs, these approaches are specifically discussed in the context of basic research on potentised preparations.

As elaborated below in more detail, systematic control experiments, in most cases SNC experiments, are a great tool in basic research. They can be used to assess the natural variability of an experimental system, to identify systematic errors such as spatial gradients or temporal drifts, and to check the suitability of the statistical model used to evaluate the experimental data.

3.3.1. SNC experiments

The stability and natural variability of a given experimental system must be meticulously assessed. An excellent way to achieve this is the use of SNC experiments. Such experiments use an experimental setup which would usually compare potentised samples against some controls but replace both sample sets with identical negative controls. Thus, SNC experiments only involve identical control samples, e.g., identical water samples (see Box 2). The value of such SNC experiments lies in the different ways in which the results can be utilised. For example, in cases where the experimen-

tal setup involves a spatial allocation of samples, any potential gradients can be assessed by analysing spatial correlations found in the SNC experiment. Also, the data can be analysed as a function of time to detect any temporal patterns linked with the sequence of measurements. Since the investigated samples are identical, any systematic differences found must be due to external spatial or temporal influences. In addition, SNC experiments can be used to check the suitability of potential randomisation schemes to be used in the actual experiments.

Box 2. Example of a systematic negative control experiment.

Suppose we would like to investigate the stability and natural variability of the experimental setup in a plant growth experiment. Plants are grown in a growth chamber for over 2 weeks. During this time, the height and weight of plants are regularly measured. A total of 200 plants are randomly allocated to 10 groups with 20 independent plants in individual pots per group distributed over the 2.5 m^2 area of the growth chamber. In this case, conducting a systematic negative control experiment means subjecting the 200 plants to an identical control treatment (e.g., water), which should yield statistically identical results for all dependent parameters (e.g., height and weight) for all 10 groups. This approach allows for the detection and correction of artefacts (e.g., temperature gradient), which could otherwise potentially lead to false-positive results.

Such SNC experiments are not expected to yield any statistically significant results, given that the negative control samples should be indistinguishable from one another. If there are significant effects, this is likely caused by spatial gradients or temporal drifts that lead to systematic errors or a large natural variability which may not be well-suited to the initially selected statistical model. In the first case, the experimental setup should be revised; in the second case, another more appropriate statistical model should be applied. Thus, SNC experiments can be of great help in optimising experimental procedures since they are able to identify and correct systematic variability in the test environment (see 3.2.3).

Performing such SNC experiments provides more confidence in the stability of the experimental setup and in the robustness of any statistical results generated. The results of such systematic control experiments could be provided in publications to document the stability and sensitivity of the experimental design [38,45,61,62].

Small treatment effects are typically observed in basic research on potentised substances, so it often happens that one is working at the limit of detection of the experimental setup or instrument. It is thus important to get a good handle on the intrinsic variability of the experimental setup, i.e., how much does the experimental signal vary naturally. This will determine the actual detection limit one can expect from this setup.

As well as investigating drift and gradients linked to the experimental setup, SNC experiments can also be used to investigate the variability associated with the manufacturing process. In such an SNC experiment, the samples are individually prepared using the same standard procedure, which in the case of centesimal dilutions involves that samples are each prepared in identically manufactured glass vials. These vials actually vary slightly from one another in terms of composition thereby introducing a potential source of variability which can be assessed with SNC experiments.

In principle, systematic control experiments can use either positive or negative controls. We recommend using SNC experiments if the potentised samples are expected to induce rather small effects which will be primarily compared to negative controls—as is often the case in this type of research. SPC experiments are recommended if the samples are expected to induce comparably large effects.

3.3.2. SPC experiments

SPC experiments are nearly identical to SNC experiments, but positive controls are used instead of negative controls. This makes the most sense for experiments involving low dilution levels (e.g., 2X and 3X) where larger effects are expected [63]. Also, gradients or temporal drifts that may appear negligible in the context of the SNC may be significant to the experimental treatment. Therefore, SPC experiments are recommended when the treatment effect is expected to have a similar magnitude as the positive controls and to differ considerably from negative controls.

3.3.3. Field effects

Several researchers have reported field-like effects whereby a sample or experimental system can be affected by the presence of nearby potentised sample (within 10 cm) [64,65]. It was observed that placing a control sample for approximately 18 h close to a verum sample may transfer verum properties to the control sample, making it no longer valid as a control [66,67]. Therefore, while designing the randomisation, handling, and storing procedures, special attention should be paid to such possible cross-contamination between the verum and control samples. It is also recommended that potentised samples and controls not be stored in close proximity, or that a barrier be used to separate different preparations, as has been done in previous studies, e.g., mu-metal [66] and aluminium [61,68]. Measures to prevent such cross-contamination should be taken and the spatial arrangement of samples such as distance between samples during the experiments should be documented.

3.3.4. Possible experimenter effects

It has been discussed that the mental state of the experimenter might interfere with experiments in research on potentised preparations [69]. Though this question has been a topic of many informal discussions, as far as we know, there is only one publication investigated one aspect (in situ randomisation/unblinding *vs* external randomisation/unblinding) of such a possible experimenter–experiment interaction in basic research on potentised substances [70]. It was hypothesised that external randomisation would destroy the quantum superposition state responsible for the observed effects, whereas an in situ randomisation would preserve it [70]. Given the preliminary nature of the current findings, we recommend that relevant aspects of this potential human influence be documented, thereby enabling analysis in the future [71].

3.3.5. Other possible effects

Unknown external factors may have impact on the effects of potentised preparations. For example, effects of them could change from positive to negative in the repeated experiments when environmental conditions varied [72–74]. Whether these effects are truly present or not can be assessed by the application of adequate statistical models such as multifactorial ANOVA.

In plant experiments involving seeds, distinct seed lots may have different susceptibilities to potentised preparations [73,75]. Since the critical factors have not yet been identified, seed origin, history, and cultivation conditions should be documented in as much detail as possible.

Other factors that could interfere with the effects of potentised preparations and should, therefore, be documented include (i) use of certain metals during the manufacturing process or experimental procedure, (ii) use of electrical equipment such as vortexers which emit EMFs, and (iii) exposure to sunlight or high-temperature, among others.

Preliminary observations indicate that the sensitivity of an experimental system to potentised preparations may depend on the inherent variability of the system, as measured by the variance of the outcome parameter, according to unpublished data from Ücker et al.. This would indicate that systems with higher inherent variability are potentially more sensitive to potentised preparations. Depending on the system, a higher or lower variance may affect the size of the effect observed. Thus, we recommend monitoring the variance of the system in repeated experiments.

3.3.6. Monitoring environmental conditions

As it is nearly impossible to control all external influences on an experiment, a good practice is to record them in order to detect their effect through statistical analysis after the experiments. Atmospheric temperature, pressure, and humidity can be recorded as well as EMFs.

It is less widely known but equally important that chronobiological effects, which manifest on timescales from seconds to years, may lead to changes in the susceptibility of bioassays to potentised preparations [76,77]. In two experiments, duckweed (*Lemna gibba* L.) was observed to respond to potentised preparations only when in a certain physiological state, which appeared to be correlated with the seasons [78,79]. For this reason, recording the precise time and date of each experiment can enable the detection of such effects through post hoc statistical analysis. Also, the observation of the moon phase might be another important variable to be documented and evaluated [80,81].

Apart from human-made EMFs, the earth's background EMF is also particularly important. The repetition of experiments in different geographic locations, especially northern *vs* southern hemisphere-based experiments should be considered to investigate the impact of the earth's EMF on experiments. In some cases, a good and inexpensive way of controlling EMFs can be achieved using a Faraday cage.

Another case of relevant environmental conditions was observed by Endler PC and his colleagues [82] that the metamorphosis of the frog *Rana temporaria* could be influenced by potentised thyroxine 30X, but only in animals from highland ecotopes, while animals from lowland ecotopes reacted only after additional stimulation [83].

3.4. Producing potentised preparations and controls

3.4.1. Standard procedures

In order to have the greatest real-world relevance, potentised preparations should be manufactured in accordance with the guidelines provided by the pharmacopeia; these guidelines are followed by manufacturers all over the world [1–4]. However, these regulations do not cover all details of the production process and leave areas open to interpretation and variation, reinforcing the importance of publications in which these standard procedures are discussed. Meanwhile, specific research setups or research questions might require deviations from standard procedures—dilutions in water instead of alcohol for plant experiments or in cell culture medium for in vitro assays are typical examples.

It is therefore imperative that the production procedures be well and comprehensively documented to facilitate exact reproduction of these procedures. First, a good documentation about the origin and manufacturing of the starting substance or mother tincture for potentisation is needed. Further relevant details concerning potentisation should also be documented, such as the material and volume of the potentisation vessel, headspace volume (i.e., volume of air remaining once the vial is closed), type and intensity of movement used for the succussion process, number of succussion strokes or duration of agitation [84–86], description of intended fluid movement, room conditions, whether machines were used in the process, and whether there were specific instructions for the human operator. It is recommended that the time and location of manufacture be recorded in order to facilitate the investigation of potential environmental factors. These may include weather, season, and moon phase, which can be looked up in national weather and astronomy databases, and their potential effects investigated (see 3.3.6).

At this point, in our understanding of the effects of potentised preparations, the exact role and impact of many factors involved in the manufacturing process remain unknown, so it is advised to stick to established processes. Potentised preparations and corresponding controls can be produced in the laboratory by the researcher or in a dedicated institution such as a pharmacy or a pharmaceutical company, either on demand or off the shelf. These different possibilities have their own advantages and disadvantages.

In-house production allows greater flexibility, such as production on the day of experiment as needed for nonsterile aqueous preparations in plant experiments, production under sterile conditions for sterile samples, or variations in production procedures for comparative investigations. All production details are under full control of the researchers and are straightforward to document.

Pharmaceutical companies follow Good Manufacturing Practice procedures. Their production processes are thoroughly controlled and documented, and the amount of work for the researcher is clearly reduced. Companies and pharmacies may have access to specific machines that are not easily accessible otherwise (e.g., machines for potentisation and globule impregnation).

When producing potentised medicines, materials used for potentisation can be either discarded or cleaned and reused. Both options have their own advantages and disadvantages. Inexpensive materials, such as pipette tips, are usually used only once and then discarded. However, for more expensive materials such as potentisation vessels, which are made of high-quality glass, cleaning and reusing is a feasible option for economic and ecological reasons. However, due to the unclear mode of action of potentised medicines, it is not known which procedures remove the "active ingredient" from potentisation vessels most effectively. Since incomplete cleaning could lead to cross-contamination between samples and correspondingly to erroneous results, we recommend implementing cleaning procedures that have been used in previous investigations, where specific effects of potentised preparations have been observed in order to reduce the danger of cross-contamination [38,61]. The cleaning procedures used should be documented.

When making potentised preparations for an experiment, considerations should be given preparing multiple aliquots of each treatment to enable future replications of the experiment and/or further investigations of the preparations.

Analytical methods can be used to control the dilution process during potentisation, and to demonstrate that no material cross-contamination occurred during production. Simple inorganic compounds (e.g., As_2O_3, NaCl, and Si) might be easier to analyse compared to organic extracts (e.g., *Arnica montana* extract), where analytical methods are not always available and are often less sensitive compared to inorganic analytical methods. On the other hand, testing certain ubiquitous inorganic compounds (e.g., Ca, Na, Si, and B) introduces additional challenges as one can expect to find them in trace amounts in even the purest potentisation medium and thus they will be found even at high potency levels [27,87].

3.4.2. Medium used for potentisation

Standard potentisation media include ethanol–water mixtures for liquid potentisation and lactose for solid-state potentisation (trituration). For specific experiments, however, different types of media are used. For some bioassays, ethanol must be avoided due to its toxic effects on the test organisms (e.g., plants or isolated cells). Furthermore, in some bioassays, specific growth media are needed. Also, for some physicochemical investigations, pure water samples may be of specific interest. In these cases, special consideration has to be given to potential microbial contamination. Microbes are able to grow and multiply even in ultra-pure water and may therefore lead to erroneous results if not properly controlled [88]. Thus, purely aqueous samples should either be prepared under sterile conditions or be used within a maximum of 48 h. Conditions required for such preparations include using only sterile material under a laminar flow hood and using sterile potentisation medium which can be achieved through autoclaving or sterile filtration. Multiple production lots (see 3.2.4) can also be used to randomise chance contaminations.

It is currently unclear whether autoclaving or sterile filtration influences the efficacy of potentised preparations. Therefore, these procedures should be avoided as much as possible when manufacturing potentised preparations. Sterile end products can still be achieved through the use of autoclaved materials and sterilized ultra-pure water as starting materials and through production in a sterile environment (e.g., laminar flow bench). Long-term experiments can be performed by storing potentised preparations made in 20%–70% ethanol a few potency levels below the potency level under investigation. The final potencies can then be prepared in water under sterile conditions up to 24 h before use. Thus, the same batch of potentised preparations can be used to produce several test preparations with low risk of contamination.

It is almost impossible to have pure water free of any trace element [27,87,89]. Some researchers actually suggest that the presence of defined solutes promotes and might even be necessary for the creation of the putative structures responsible for the specific effects. These may include, for example, silicates and sodium, which one would expect to find in "normal" everyday water and molecules such as ethanol [90–92]. Therefore, consideration should be given to whether ultra-pure water is the most appropriate medium for the production of potentised preparations or whether a medium with addition of some defined material such as NaCl or ethanol dissolved at a known concentration might not yield better results [90,91]. It is also worth mentioning that some scientists think that distilled and double-distilled water are more neutral than ultra-pure water, which is prepared with a reverse osmosis purification system, due to the heating involved in the distillation process. However, no evidence supporting this belief has been published, to our knowledge. In any case, the potentisation media used should be documented in detail.

3.4.3. Possible batch effects

In order to minimise variations in the production of samples and controls, it is often recommended to use the same batch of potentisation medium to produce the different lines of potentised preparations and controls. However, some researchers have speculated that using the same batch of solvent might lead to effects where verum and control samples remain "linked" somehow and thus tend to react more similarly than expected. This would tend to reduce the overall sensitivity of the experiment leading to false negative effects [93]. These possible batch effects are currently little explored, yet worth keeping in mind when conceiving experiments.

3.5. Publication and reporting

3.5.1. Current reporting guidelines

In a Delphi process initiated by Stock-Schröer et al., publication guidelines for fundamental research on potentised preparations had been developed [94] and published [19]. From our point of view, these reporting guidelines are still valid, and no amendment is necessary.

3.5.2. Publication of research protocols

In clinical research, it is now considered a best practice to publish research protocols in advance. One of the main reasons for this practice is to prevent selective reporting of results. It is especially important for confirmative clinical research with predefined primary outcomes.

In basic research, we recommend publishing research protocols in advance in case of confirmative and/or reproduction experiments where the main research question is to test the existence of a certain effect. In many other areas of preclinical research such as method development or mode of action research, research methods and questions are often adapted during the research process based on intermediate results which would often render research protocols obsolete shortly after start of the investigation. A publication of research protocols therefore makes no sense in most cases. This is reflected by the fact the research protocols are regularly not published in any area of basic and preclinical research.

3.5.3. Reporting on the experimental refinement process

Another aspect to consider is that in fundamental research arriving at a stable and sensitive system is more often a long and arduous process of optimisation until experimental parameters are found that maximise the effect size, i.e., maximising the ratio of the experimental effect compared to the variability of the system. This process can often take a considerable amount of time and resources that remain hidden from the reader. The reporting of the optimisation process that led to the development of a particular experimental protocol can be very beneficial, as it can assist researchers in identifying potential issues and challenging aspects of the protocol, thereby facilitating the refinement of their own protocols. Furthermore, it is worthwhile mentioning any theories that were being considered during this refinement process as it will help other researchers understand the rationale behind the decisions made.

3.6. Summary

In this publication we have covered many aspects of the design of experiments in basic research on potentised preparations, many of which are seldom mentioned in final publications. The main aspects are summarised in Table 2, which is also intended to serve as a checklist.

The guidelines and considerations presented will enable future researchers in this field to quickly familiarise themselves with some of the intricacies and peculiarities involved in basic research into potentised preparations. We also hope that we have shown that the design of such experiments requires careful consideration, as the aspects to be taken into account go beyond those required in conventional research practice. This is due to the fact the mode of action of these potentised preparations is still largely unknown and is likely to be quite different to that of conventional biochemicals.

Table 2
The checklist of essential items in designing experiments in fundamental and basic research in homeopathy*.

Item	Nr.	Descriptor
Control and research question		
Negative control	1	Is/are the negative control(s) appropriate for the given research question?
Positive control	2	Are positive controls possible/required for the given research question?
Succussed/potentised control	3	Does the research question require succussed/potentised controls?
Expected action	4	What is the expected action of the potentised preparation according to the experimental design?
Experimental design and statistics		
Blinding	5	Can the experiment be meaningfully blinded? Are proper procedures in place?
Repetition and replication	6	Are there sufficient repeats to obtain good statistical power? Are independent replication experiments planned?
Randomization	7	Is the randomization scheme adequate for the research question?
Multiple production lots	8	Does the research question require multiple production lots?
Statistical analysis	9	Is sufficient knowledge of statistics available? Are the statistical procedures adequate?
System stability		
Systematic control experiment	10	Are systematic negative/positive control experiments implemented?
Possible field effect	11	Are there precautions in place to prevent a possible cross-contamination of control solutions and potentised preparations?
Monitoring environmental condition	12	Are environmental conditions in the lab well monitored to enable post hoc analysis of any potential influences?
Production of control and sample		
Standard procedure	13	Are the standard production procedures adequate?
Documentation	14	Are procedures in place to enable adequate documentation of the experiment throughout?
Publication and reporting		
Following publication guideline	15	Is there awareness of the available publication guidelines?
Experimental design process	16	Did the experimental design process go through different phases that deserve to be documented?

* A similar table by Stock-Schröer et al. [19] provides a checklist of items to be included at the time of writing up the experiment for publication.

4. Conclusions

Only through thorough research of the highest quality, which can be achieved by following the present PrePoP guidelines, will it be possible to resolve the various open questions in this field, such as the mode of action from physicochemical and biological perspectives, the stability of homeopathic preparations over time and against external influences, the role of the potentisation process, and questions related to production optimisation (e.g., optimal intensity of succussion, type of succussion, number of succussions). We are confident that these guidelines presented here will promote the design and execution of high-quality, rigorous research, thereby laying down the solid experimental foundations required for advancing this field.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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