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Utilizing preclinical models of genetic diversity to improve translation of phytochemical activities from rodents to humans and inform personalized nutrition

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Abstract:

Mouse models are an essential tool in different areas of research, including nutrition and phytochemical research. Traditional inbred mouse models have allowed the discovery of therapeutical targets and mechanisms of action and expanded our knowledge of health and disease. However, these models lack the genetic variability typically found in human populations, which hinders the translatability of the results found in mice to humans. The development of genetically diverse mouse models, such as the collaborative cross (CC) or the diversity outbred (DO) models, has been a useful tool to overcome this obstacle in many fields, such as cancer, immunology and toxicology. However, these tools have not yet been widely adopted in the field of phytochemical research. As demonstrated in other disciplines, use of CC and DO models has the potential to provide invaluable insights for translation of phytochemicals from rodents to humans, which are desperately needed given the challenges and numerous failed clinical trials in this field. These models may prove informative for personalized use of phytochemicals in humans, including: predicting interindividual variability in phytochemical bioavailability and efficacy, identifying genetic loci or genes governing response to phytochemicals, identifying phytochemical mechanisms of action and therapeutic targets, and understanding the impact of genetic variability on individual response to phytochemicals. Such insights would prove invaluable for personalized implementation of phytochemicals in humans. This review will focus on the current work performed with genetically diverse mouse populations, and the research opportunities and advantages that these models can offer to phytochemical research.

1. Introduction:

Plant secondary metabolites (phytochemicals) are known to possess a wider range of beneficial biological activities [1–3]. For example, phenolic compounds are well-known for their antioxidant functions [4], and plant sterols for low-density lipoprotein cholesterol (LDL-C) lowering effects [5]. However, humans present significant variability in response to phytochemicals, and this is referred to as interindividual variability. Human genetics play a significant role in this interindividual variability. It is well know that several genetic variations are able to modulate the bioavailability and metabolism of phytochemicals in humans [6–9]. These range from genes range from digestive enzymes, uptake/efflux transporters, such as ATP-binding cassette (ABC) transporters, metabolizing enzymes such as uridine diphosphate-glucuronosyltransferases (UGTs) or sulfotransferases (SULTs), among other factors. Notably, genetic variability in humans goes beyond phytochemical bioavailability and metabolism modulation. In fact, phytochemical bioactivity and efficacy can be affected by the genetics of the consumer For example, the variability in the response to plant sterols has been liked to variation in genes governing cholesterol absorption, synthesis, and turnover. In this sense, polymorphisms in 7 α-hydroxylase (CYP7A1) and ATP-binding cassette G5 (ABCG5), among other genes, impact the LDL-C lowering effects of plant sterols, which segregates populations into responders and non-responders [5,10]. Other clear examples of examples interindividual variability in the response to phytochemicals include the cardiometabolic effects of ellagitannins, anthocyanins and other phenolic compounds [11,12]. Of note, besides genetics, other factors can contribute to interindividual variability of response to phytochemicals [8]. Perhaps most important is the role of gut microbiota, understood as the bacteria strains and its percentage residing in the host's digestive tract, which also provides its own genetic variability. Also, the coadministration of other nutrients or xenobiotics along with the compound of interest, can affect the bioavailability and metabolism, tested compound between experiments. In fact, food matrix has relevant implications in phytochemical bioaccessibility, which modulates bioavailability, metabolism and bioactivity [13–16]. A comprehensive review of many of these factors is beyond the scope of this review. The focus of the present review is the role on genetics on observed activities of dietary phytochemicals.

There is great interest in exploiting phytochemicals as preventative strategies, therapeutic agents, or adjuvant treatments for chronic diseases in humans. Animal models are an essential component of research efforts to develop interventions for preventing, ameliorating or treating disease, as this requires knowledge on their basic underlaying mechanisms and preclinical data to justify human intervention trials [17]. Mice in particular are a commonly used model because of their genetic similarity with humans, as ~ 80 % of their genes have a 1:1 gene ortholog in humans [18], and the wide availability of mice with useful genetic

modifications compared to other rodents [19].

Preclinical animal models, particularly mice, are indispensable tools for research focused on advancing human health via use of phytochemicals. Fields such as nutrition, toxicology, pharmacognosy, pharmacology, and genetics rely on useful mouse models. These models are critical for identifying potential health benefits of phytochemicals, and the subsequent mechanistic, toxicological and pharmacological studies that precede human clinical trials.

There exists a general preference for the use of inbred strains, and this is due primarily to the desire for as little phenotypic variability in any given strain [20]. In this context, "phenotype" can refer to inherent characteristics/traits, or response to treatment or intervention. Although these traditional inbred mouse models have been and will continue to be useful models to understand human biology and gain knowledge on disease mechanisms, these models do have significant limitations in terms of their relevance to human populations. [17]. These limitations have likely been significant contributing factors to the repeated failure to translate robust, promising preclinical data from mice to humans in clinical settings [21]. There are plenty of examples of this in the literature [22–26], but we can find a clear one in cancer research [27,28], where the success rate in translating results from animal models to clinical trials is less than 8 % [28]. Another example is behavioral sciences, where about 90 % of the results found in mice do not translate to humans. Immunology is another area where translation has been problematic [29]. Indeed, there exist plenty of differences between mice and human immunology [30], which can affect any given response ultimately leading to a lack of translatability between species. However, different studies using genetically diverse mice populations have demonstrated the value of these models in advancing in mice translatability to humans [31–33].

There exist a wide variety of commercially-available mouse strains, each with different phenotypic characteristics. For example, C57BL/6J mice are susceptible to diet-induced obesity [34], type 2 diabetes [35] and atherosclerosis [36]; LP/J mice are susceptible to audiogenic seizures [37]; BALB/cJ mice are susceptible to *Listeria* [38] and *Leishmania* [39] infections; AKR/J mice present a high incidence of leukemia [40]; DBA/2J mice present progressive eye abnormalities similar to the ones reported in humans [41]; C3H/HeJ mice are resistant to endotoxin [42]; and A/J mice present a high incidence of lung adenomas [43]. The specific models employed depend on the field of research. Strains are typically selected based on their specific phenotypic characteristics and consistency. For example, to study diet-induced obesity, C57BL/6J mice are widely used due to the rapid, reproducible increase in weight and adipose tissue, particularly in males [44,45]. This reproducibility is valuable and convenient for researchers in order to provide consistency between studies both within and between lab groups, and to provide "tight" data,

facilitating detection of statistically significant differences with relatively small sample sizes. However, the advantages of inbred mouse models for laboratory science also correspond to significant disadvantages for translation to genetically diverse human populations. The extremely low genetic variability in which most mouse studies are performed does not typically reflect or model the genetic variability found in human populations, and this issue is particularly critical for sporadic diseases (such as obesity) that do not arise from one or a few well-defined genetic mutations. As a result, some of the effects (such as phenotypes or responses to specific interventions) reported in specific animal strains are likely overestimated. Exaggerated responses or phenotypes arise when the selected strain is phenotypically too homogenous compared to humans (which is almost always the case, intentionally), or happens to be a "hyper-responder" to the selected treatment. Such result may suggest intervention effects (such as protective effects of phytochemicals against a disease) that researchers may pursue exhaustively, despite the fact that the finding cannot reasonably be expected to be translated to a more diverse population. Conversely, some other effects might be underestimated or not observed at all. This occurs when the chosen strain does not effectively mimic the disease etiology or phenotype in humans, or is an "under-responder" (minimally responsive or unresponsive to the selected treatment). In this sense, when a single inbred mouse strain (or a few strains) is used and provides a negative result, the researchers may conclude that the selected intervention (such as a phytochemical) is inactive or ineffective. However, this null finding may simply be an artifact of strain selection, and there could exist other mouse strains that are responsive to the treatment. The converse is also true: significant or promising findings in a single mouse strain may be limited to that specific genetic context, and may not be observed in other genetic backgrounds (or in diverse populations). For example, quercetin has been shown to regulate body weight in several studies using C57BL/6J mice [46–48], but many human studies have failed to report similar results [49–51]. To address the issue of the pre-clinical findings potentially being single-strain artifacts, we recently demonstrated that the anti-obesity properties of quercetin are highly dependent on the genetic background and sex of the mice [52].

The critical question thus arises: if individual strains are prone to highly strain-specific findings, what mouse strain(s) should be selected to facilitate pre-clinical discovery as well as translation to humans? The answer to this question has the potential to drastically improve the value of preclinical research and ultimately may prove to be a game changing approach to preclinical pipelines to benefit human health. The present review will attempt to address this question. Genetically diverse populations are useful tools for mapping the genetic basis of complex traits and identifying candidate genes for personalized medicine and nutrition approaches [53]. Some examples include obesity [54,55], cancer [56], diabetes [57,58] and infectious diseases [59,60]. However, lack of genetic diversity is also a problem in human studies, as most of them mainly include individuals with European ancestry, thus failing to portray the global genetic

diversity in humans [61]. Moreover, human genetic studies tend to point to etiology and mechanisms of a disease, rather than focusing on therapeutic interventions [62].

This review will focus on the potential application of genetically diverse models in the fields related to discovering and implementing human health benefits of phytochemicals (**Figure 1**). This field significantly lags behind many other fields in terms of applying preclinical genetic diversity strategies to improve translation to humans. If we retake our previous example on quercetin anti-obesity effects on C57BL/6J mice and its lack of translation into humans, it would be interesting to study the anti-obesity effects of quercetin in a genetically diverse mouse population to identify led genes responsible for positive and negative anti-obesity effects. These genes could then be matched to human orthologues, and these candidate gene would be then used as selection criteria/grouping for an anti-obesity human study. Also, previous anti-obesity studies in humans could be re-analyzed considering these new candidate genes too. This specific approach could provide valuable insights into what sub-population groups can benefit the most from quercetin intake as for its anti-obesity effects. Relevant areas that have benefited from the use of genetic diversity mouse models, which provided valuable translational insight into human health an disease, include cancer [63], tuberculosis disease [64,65], Alzheimer's disease [66], among other areas [67–69]. For example, through the use of a genetically diverse mouse population Koyuncu *et al*. [65] discovered a new biomarker for tuberculosis, CXCK1, which was then also identified as a human serum biomarker for tuberculosis. We first review inbred, recombinant inbred and diversity outbred models, and then discuss potential applications of these models to phytochemicals research.

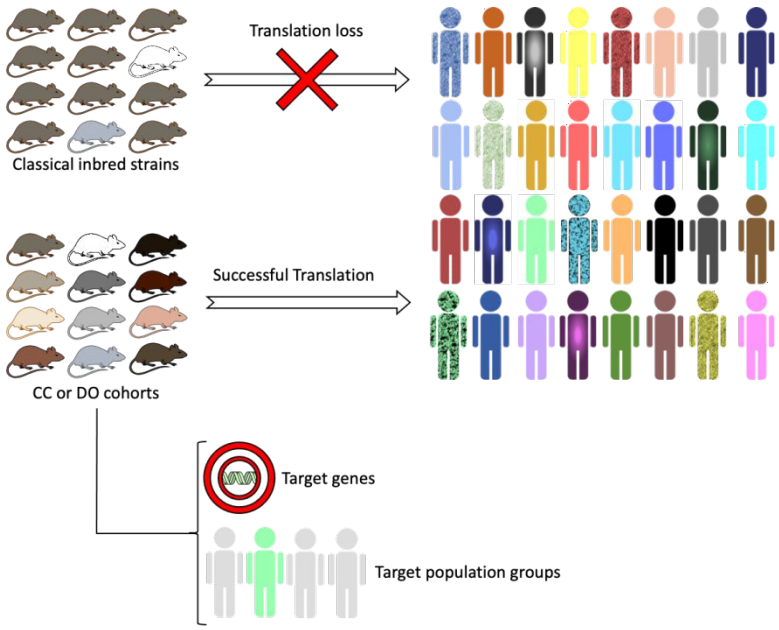

Figure 1: *Currently, most studies evaluating the effects of phytochemicals in multifactorial diseases are performed in a few specific mouse strains, usually inbred (isogenic). This strategy has likely been a hindering factor in the discovery and translation of mouse results to human populations, as preclinical results are obtained in very specific genetic backgrounds that do not reflect the genetic variability typically found in human populations. As a result, a wide range of results in mouse models are not reproduced in human clinical trials. The use of genetically diverse mouse cohorts, like the collaborative cross (CC) or diversity outbred (DO), will likely allow for better translation science. Also, the use of these mouse models will allow the discovery of genes previously unknown to be responsible for phytochemical-associated responses and phenotypic traits, new mechanisms of action, and new therapeutical targets. Moreover, the use of mouse populations like the CC or DO in the field of phytochemicals will allow development of more precise dietary recommendations (precision nutrition) and allow the recruitment of better-suited sub-population groups of interest for clinical trials.*

In addition to genetically diverse mouse populations, other strategies have been developed to enhance translational relevance of rodent studies. These include the use of "humanized" mouse models [70,71]. Humanized mice, or mouse-human chimeras, were developed to create *in vivo* models closer to humans. Some examples of humanized mouse models include immunodeficient mice engrafted with hematopoietic cells or tissues that express human genes [70], mice that express glucuronosyltransferase (UGTs) human genes [72,73], mice that express angiotensin-converting enzyme-2 (ACE2) human genes [74–77], or mice transplanted with human gut microbiota [78–80], among others [81–83]. These humanized mouse models have helped to generate insights into human health and disease, which includes immune response to, pathogenesis of and treatment for the current global SARS-CoV-2 pandemic. However, like all models, humanized mice present limitations too. In the context of this review, the most relevant one would be the fact that besides the introduced human genes or expressed material, the rest of the genetic background comes from inbred mouse lines with no genetic variability. Nevertheless, humanized mouse models can offer valuable insights in some specific cases where host the genetic diversity produces has a low impact on very specific and selected traits. This is the case of mice transplanted with human microbiota and their metabolic capacity to produce trimethylamine *N*-oxide (TMAO), a pro-atherogenic metabolite of choline produced through a microbiome-host axis. In this particular case, TMAO production mainly depends on the gut microbiota capacity to produce trimethylamine (TMA) and, by extent, their gut microbiota composition. The genetic diversity of hepatic flavin-containing monooxygenases (FMOs), enzymes catalyzing the oxidation of TMA into TMAO, plays a small role into dictating the circulating levels of TMAO, except for the rare genetic mutations that completely inhibit FMOs activity [2]. As a matter of fact, humanized mouse models have been key to establish the role of the gut microbiome and TMAO production

capacity as relevant factors for cardiovascular disease [78].

2. Inbred vs. genetically diverse animal models:

Inbred mice, which have tightly controlled genetics and thus minimal genetic variation, are useful for producing specific phenotypes with minimal variation that match research model needs. These strains are particularly useful when they exhibit a unique phenotype or mutation that correlates very well with a human disease. A representative example is the use of $Apc^{Min+/-}$ mice to study familial colon cancer (Familial Adenomatous Polyposis, FAP). The most common mutation that originates inherited colorectal cancer in humans is the mutation in the suppressor gene of APC (adenomatous polyposis coli), and $Apc^{Min+/-}$ mice replicate this by carrying an autosomal dominant loss of function of the APC gene [84,85]. In this case, the mouse model specifically mimics the etiology of the human disease of interest with high fidelity. However, it would not make sense to use these mice model to study sporadic colon cancer in most cases. Furthermore, even though $Apc^{Min+/-}$ recapitulate FAP in mice, these mice are on an inbred background and thus do not account for background genetic variability that may affect gene penetrance in humans. In this sense, the use of genetically diverse models better fits the needs required to study multifactorial diseases, such as obesity [86] or sporadic cancer [87,88], where diet, genetic, social, environmental and other uncontrollable factors may play a significant role, than inbred mice. Classical inbred mice also fail to capture genetic and response variation, which are typically found in human populations. In an effort to solve the limitations presented by a lack of genetic diversity in mouse models, two different models of genetically diverse mice emerged: Collaborative Cross (CC) and Diversity Outbred (DO) mice [89,90].

Classical inbred and modern genetically diverse mouse models each have different advantages and drawbacks. Due to the commercial availability, reproducibility and statistically "tight" data provided by inbred strains (achieved by shrinking genetic variation and maximizing phenotypic consistency) inbred strains have been widely used in research [53]. A strain of mice is regarded as "inbred" when it has been mated brother × sister for at least twenty consecutive generations, and can be traced to a single ancestral breeding pair of mice. As a result, genetic variants become fixed and inbred mice become homozygous in 98.6 % of the loci on average, providing a very specific genetic background. All individual animals within a given strain are thus regarded as essentially genetically identical, or isogenic [91]. Due to this, it is assumed that in a given inbred strain, phenotypic variability equals the environmental variability (as genetic variability is negligible) [20]. However, spontaneous mutations inevitably occur, generating genetic variability within a strain. These mutations can accumulate and become fixed, which causes inbred strains to change over time [92]. This process is known as genetic drift, and it is usually seen as a relevant drawback. Genetic drift can cause significant phenotypic variations, especially in fields like immunology

[93–96]. To slow down this process, different vendors (i.e., Jackson Laboratory or Charles River) "refresh" breeding stocks of inbred strains by the use of cryopreserved mouse embryos, which replace the foundation colony after a specified number of generations (i.e., every 5 – 10 generations). This resets the "genetic clock" by going back to a known starting point for breeding the colony and provides researchers with animals that possess a consistent, fixed genetic background over time overcoming genetic drift [93]. However, genetic drift has provided new valuable strains (i.e., *ob/ob* and *db/db* strains) and allowed the discovery of many genes relevant to human diseases [93,96–98].

The first inbred strain (dilute brown albino, DBA) was developed by Dr. Clarence Cook Little in 1909 at Harvard University. DBA mice were produced in order to eliminate the uncontrolled inherited variability in order to facilitate the study of study cancer heredity [91,99,100]. Since then, inbred mice have been an extensively used and are an invaluable tool in research. Most inbred mouse strains are derived from *Mus musculus*, and belong to one of the three most representative subspecies: *Mus musculus domesticus, Mus musculus musculus* and *Mus musculus castaneus* [101]. Classical inbred mice present standardized genomes with extremely low genetic variation, which provide reproducible phenotypes and performance [62]. The homozygosity (i.e., both alleles are identical for a given gene) found in inbred strains adds the advantage of almost complete reproducibility of phenotype measurements [102]. Overall, this allows direct comparisons across stocks, laboratories and years, which has led to its widespread use in research [62]. Additionally, this minimizes experimental noise, facilitating detection of statistical significance with smaller *n* sizes. Despite their advantages, the standardized use of a few inbred strains (i.e., WT and *ob/ob* C57BL/6J) in certain fields presents significant limitations and does not facilitate the performance of certain research applications [62]. Also, unlike outbred animals, inbred animals lack heterozygosity, which makes them more susceptible to recessive alleles, which may confound findings in these strains [53]. Additionally, the use of isogenic, homozygous animals produces artificially "tight" data, which is valuable for some aspects of experimental science but which likely leads to statistically significant differences that often cannot be reproduced in genetically variable populations, and is thus of questionable translational relevance.

Genetically diverse populations, such as outbred mice populations, are highly valuable as they allow the mapping of complex traits, understood as traits arising from a combination of genetic an environmental effects, to phenotypes of interest [53]. Inbred mice cannot be used to correlate genetic variation to traits, as there is essentially no significant variation in genetics or traits. Some clear examples include obesity and cancer [54–56,103,104]. The use of high-diversity mouse populations, such as the Collaborative Cross, Diversity Outbred stock, and their founder strains are a valuable tool to identify complex disease mechanisms [62]. In contrast to inbred strains, genetically diverse populations have significant variation in

both genetics and traits, and thus facilitate genome-wide association studies (GWAS). Studies that aim to identify genetic loci responsible for traits benefit from populations with a diverse genetic background, as finding dependence of trait outcomes requires variations in both phenotypes and genetic loci. When there is no genetic variation, observed phenotypic variation is due to experimental noise (such as inevitable variability in experimental techniques or instruments) and/or environmental factors. These types of studies can also be performed by crossing inbred populations and using Quantitative Trait Locus analysis (QTL) but QTL regions (loci) are large (~ tens of megabases) and contain hundreds of genes, and the subsequent studies are needed to refine the QTL to a testable number of candidate genes are difficult and costly [102]. For example, in the study of Yang *et al.* [105] the application of GWAS in a F2 mice population originating from C3H/HeJ and C57BL/6J, and thus with segregated alleles, allowed the identification of 292 genes involved in aortic lesion generation. More importantly, authors then validated the mechanistic role of *C3ar1* as a gene involved in aorta lesion generation in a targeted knock-out model. Also, by the use of QTL, Fench *et al*. [106] found that genetic variability in two genes coding for sulfotransferase enzymes (*Gm4794* and *Sult3a1*) was responsible for protection against benzene-induced DNA damage. Moreover, by the use of GWAS in a hybrid mouse diversity panel, Hartiala *et al.* [107] found a loci in chromosome 3 and a QTL loci for solute carrier family 30 member 7 gene associated with plasma trimethylamine N-oxide levels. Obviously, such studies are not possible in populations that do not provide significant variation in traits or genes. GWAS in genetically diverse mouse populations requires only a population of a few hundred animals to reliably identify > 50 % of loci associated to a particular genotype, which contrasts with the tens of thousands of humans required to identify loci that typically explain a small fraction of the phenotypic variance [102]. The use of genetically diverse rodent populations helps to overcome challenges usually found in human populations, such as sample size, collection of prospective data and lack of associations not generalized in human subpopulations [62]. Moreover, findings from genetically diverse mouse populations are more likely to generalize across strains, and even species [62]. Noteworthily, there exist numerous orthologous genes, pathways and molecular networks in both species (mouse and human) that provide efficient and inexpensive research opportunities in mechanistic and translation sciences relevant to humans [62]. For example, Church *et al.* [33] discovered 46 mouse genes with human orthologues that could potentially explain the variation in the toxicity of the green tea compound epigallocatechin gallate (EGCG) by using a cohort of genetically diverse mice. Wang *et al.* [108] identified 140 human gene orthologues that could potentially explain the variability in gastric tumour susceptibility. Identification of novel loci and/or genes responsible for a phenotypic response (potentially including response to phytochemicals) can lead to the discovery of new mechanisms of action and therapeutical targets based on the functions of the identified genes. For example, the study of Yang *et al.* [105] on atherosclerosis identified different genes responsible for aorta plaque lesion that presented a corresponding human

orthologue demonstrated to be associated with coronary artery disease in humans. Other examples of the potential of newly discovered genes/QTLs include the identification of several QTLs, genes and single nucleotide polymorphisms (SNPs) as factors responsible for hypertension in different mouse models with different genetic backgrounds [109–111] and human studies [112], which opened the door for mechanistic and therapeutical targeted studies focused on those genes and their activities. By QTL analysis in a CC mice cohort, Bienebaum *et al.* [113] found that the genetic mechanisms underlying susceptibility to high-fat diet induced obesity between male and female mice are different, which suggests that different targets should be considered for therapeutical purposes between sexes.

With the understanding that strain genetics and phenotype are inextricably linked, the Mouse Phenome Database (MPD; https://phenome.jax.org) was conceived as a resource to compile and share phenotype data from 40 common inbred mouse strains, known as the "phenome panel". Phenotype data are shared, and mouse models can be rationally selected by researchers for specific studies based on these historical phenotype data. The development of Collaborative Cross and Diversity Outbred mice (discussed below) further widened the use of the MPD [114]. Currently, the MPD provides useful analysis and visualization tools for the data it contains. These data are provided by researchers who conduct phenotypic analyses in mice, which include biochemical and behavioural phenotypes of commonly used mouse strains [115,116]. The availability of these data not only allows researchers to reproduce experiments but also to re-analyse data with up-to-date bioinformatic resources and unravel gene-trait relationships with different sets of data [114]. For example, Gielen *et al.* [103] performed a meta-analysis that uncovered an association between telomer length with body mass index using data from the MPD.

It is worth mentioning that commonly used cell lines have limitations with regards to genetic variability and translatability, similar to highly inbred mouse strains. Primary cultures typically stem from a single individual; thus, several donors should be used to capture the variability in genetically diverse populations. Moreover, in some fields of research such as cancer biology, the accepted standard for *in vitro* studies is that several cell lines must be used to validate findings [117,118]. This is also a problem with available commercial human cell lines. Many of the available cell lines have been produced from cancer cells, from a single subject such as Caco-2, derived from a colorectal carcinoma sample from a 72-year-old white male, or C2BBe1, derived from a colorectal carcinoma sample from a 44-yeard-old white woman [119]. Furthermore, the gene expression profiles of these cells might differ from the one reported from human biopsies [119]. In addition to *in vivo* studies, primary cells, stem cells and organoids derived from genetically diverse mice allow the possibility of *in vitro* screening, validation and cell function studies with

greater genetic diversity [120]. For example, fibroblasts from DO mice have been used to reveal the heritability of circadian phenotypes [121].

2.1. Collaborative cross mice:

The Collaborative Cross (CC) panel is an effort to increase genetic variability carried out in the mid 2000's [89]. In response to limited genetic diversity in available stock mouse strains, the CC was developed to generate a panel, of recombinant strains, which then allows the mapping of traits and its use for system biology studies [102]. The CC lines are referred to as "recombinant inbred" (RI) lines because they are derived by recombination of 8 different founder strains (**Figure 2**), followed by inbreeding to produce a genetically homogenous strain (note that not all RI strains are CC mice; CC mice are specific RI strains from the 8 founders). Thus, the panel of strains is highly diversified, with low diversity within each strain due to inbreeding. These eight different inbred strains, known as "the founders", were selected to develop the CC. These included laboratory and wild-derived *Mus musculus* subspecies (*M. m. musculus*, *M. m. domesticus* and *M. m. castaneous*) and *Mus spretus*, namely: A/J, C57BL/6J, 129Sv/ImJ, NOD/LtJ, NZO/H1J, CAST/EiJ, PWK/PhJ and WSB/EiJ [122]. Of note, the 5 classical inbred founder strains (A/J, C57BL/6J, 129Sv/ImJ, NOD/LtJ and NZO/H1J) lack variants in many genes, and the inclusion of the 3 more recently wild-derived strains (CAST/EiJ, PWK/PhJ and WSB/EiJ) intentionally introduced a significant amount of genetic diversity in the CC that would otherwise be lacking [102]. The inclusion of wild-derived strains has shown value in different research areas, specially behavioral sciences [123]. The advantage of CC lines over founder strains is that CC lines provide new gene-to-gene interactions of the eight original genomes, which may result in new combinations that can perform better than founder strains alone [124]. However, the inclusion of the recent wild-derived strains, specifically the PWK/PhJ, also introduced some problems, such as male infertility and a high extinction rate of CC lines [125].

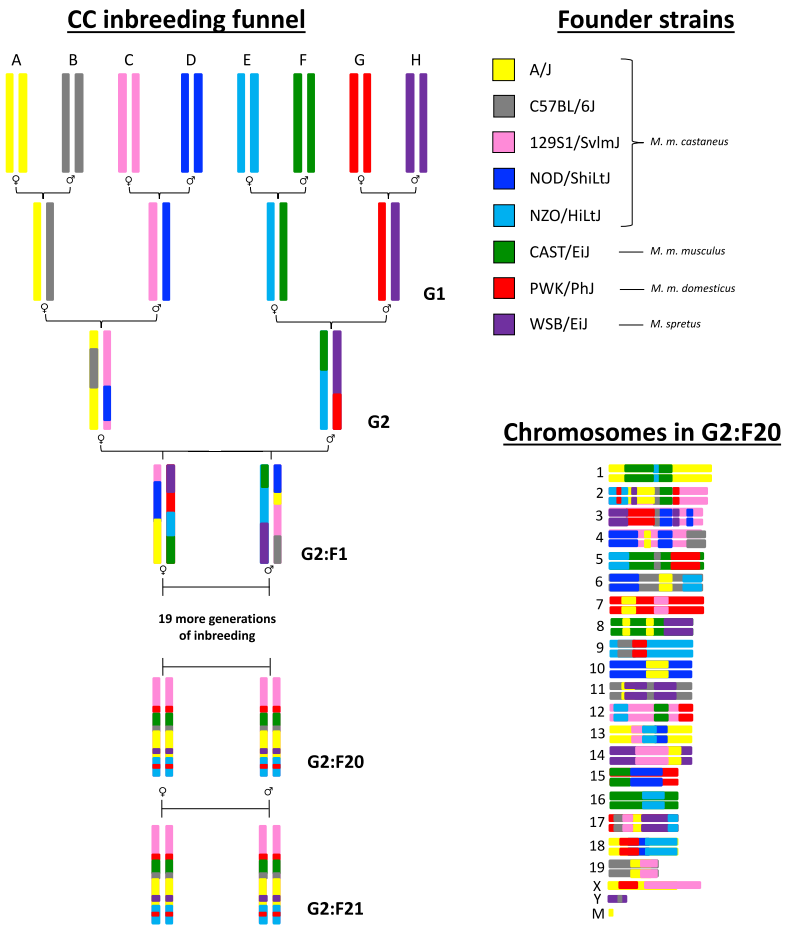

Figure 2: *Generation of a hypothetical collaborative cross (CC) line. To generate a CC line, the eight homozygous founder strains are mated in pairs, generating G1. Then two pairs of male and female mice generated from different original mates are mated, generating G2. A pair of G2 individuals is then mated, generating the G2:F1, which is the very first generation with genetic contributions of all 8 founder strains. By sibling matting for at least 20 generations (inbreeding), a CC (recombinant inbred, RI) line is created. CC lines are homozygotes at all loci. Further matings between siblings will produce identical individuals.*

The development of the CC included a mating system for the founder strains to reduce random genomic interactions between strains and optimize the contribution from each founder strain which, in turn, reduced population structure effects that limit genetic mapping resolution [62,126]. CC lines are generated by 4 mates from each of the 8 founder strains, which generate a G1 progeny. Then, two mates of the G1 progeny follow, generating the G2:F1 progeny. The G2:F1 is the very first generation to contain genetic contribution of all 8 founder strains. By sibling mating this generation by inbreeding for at least 20 generations (>G2:F20), an inbred CC line is created (**Figure 2**). Depending on the initial matings, hundreds of different CC lines can be created, and all of them are unique, highly inbred genetic "mosaics" of the original 8

founder strain genomes [127]. The subsequent cross of progeny in CC lines tend to exhibit transgressive variations or novel traits that were not present in the original founder strains [124], which is another advantage of CC lines over the founder strains. However, when developing the CC to maximize genetic diversity, founder strains were also chosen to give emphasis on disease susceptibility, especially for cancer and diabetes [62]. Indeed, the CC has been used for various aims within the field of diabetes [57,58,128] and cancer [56,108,129]. Although the CC is still comprised of inbred (RI) mice strains, there is extreme variation between the strains and thus it captures ~89 % (some estimates are higher) of the genetic diversity found in widely available mice. Other available RI schemes and their strains capture a lower percentage of genetic variation (i.e., 16 % in BXH, 15 % AXB/AXB, 14 % CXB, and BXD 16 %). The CC also captures a higher genetic diversity than outbred strains panels (i.e., Northport HS 36 %). Moreover, the distribution of the genetic variation in the CC is uniformly maintained with approximately even (12.5% of each founder) contribution to each genome, while other diversity panels (i.e., Laboratory Strain Diversity Panel) have variable genetic variation and a high degree of relatedness presenting drastic changes in the level of variation [126]. The CC presents a higher minor allele frequency than other panels (i.e., Northport HS and Laboratory Strain Diversity Panel) [126], and this facilitates discovery of rare susceptible individuals with smaller sample sizes compared to other panels. This can be used to generate unique phenotypes useful for specific experiments, and also identify the alleles responsible. For example, Wang *et al.* [108] discovered a CC strain extremely prone to gastric tumorigenesis with only 293 mice. It has been estimated that the CC panel presents up to 53.7 million possible genetic variants, 40 million of which are already present in the founder strains [130]. Finally, the introduction of the recent wild-derived strains during the generation of the CC increased the likelihood of the presence of genetic variants that perturb a trait of interest [102]. By increasing the genetic diversity and variations in mouse models, the range of different observed phenotypes that are possible also increases [124], which is key to maximize the identification of possible genetic factors/traits affecting a phenotype. Of note, genetic background is not the only source that provides variability in CC lines, or any other mouse model for that matter. For example, azoxymethane acute toxicity has been reported to be modulated not only by the CC lines genetic background, but also by their gut microbiota composition [131]. Since its inception, the CC has been used to study host-pathogen interactions to identify loci associated with susceptibility to pathogens [132], susceptibility to diet-induced obesity [133], glucose tolerance and prevention of type-2 diabetes [57,134], risk factor genes for drug toxicity [131,135], among others [136,137]. For example, in the specific case of glucose tolerance, the use of CC lines has helped to undercover the role of different genes involved in glucose tolerance, such as Mboat4 (membrane-bound *O*-acyltransferase domain containing 4), a gene that mediates the octanoylation of ghrelin, and Leprotl1 (leptin receptor overlapping transcript-like 1), which controls hepatic growth hormone resistance [57], among others [128].

Due to its high level of genetic diversity between strains (due to recombination) and with simultaneous strain population reproducibility (as each strain is generated from defined mating and then inbred), CC populations can be used to characterize trait correlations, and phenotypic studies in specific strains of interest can then be reasonably compared across experiments and between laboratories [62]. For example, the meta-analysis performed by Gielen *et al.* [103] using 87 different studies involving CC cohorts revealed that telomer length was negatively associated with body mass index. Also, trait correlation studies can be extended using recombinant inbred cross strains (RIX), which are the offspring of systematically intercrossed pairs of inbred strains and are heterozygotes at most loci [62], which resembles the human population of heterogeneous individuals heterozygous at many different loci. For example, RIX strains have been used to study locomotor activity and reinforcing effects of cocaine [138], and to identify basal immune predictors of severe clinical outcomes upon SARS-CoV infection [139], among other examples [140,141]. Founder strains are typically selected to produce subsequent RI strains based on extremes of the selected phenotype(s), and subsequent RI strains can be further crossed based on desired genetic or phenotypic outcomes.

While classical inbred mouse disease models typically feature a single (i.e, *ob/ob*, $LDLR^{-/-}$, $Apoe^{-/-}$ and $Apc^{Min+/-}$) or a few (i.e., 3xTg-AD mice with $PS1_{M146V}$, APP_{Swe} and tau_{P301L} transgenes) defined gene perturbation(s) on a single genetic background (C57BL/6J) [85,142–144], CC strains better reflect population heterogeneity as each typically presents multiple variants of multiple pathways associated to different diseases [62]. Thus, CC populations can also be used to discover new complex disease model strains, such as extreme strains (express a phenotype more strongly than founder strains) and multivariate outlier strains (presenting traits within the normal range but do not present trait correlation). CC can also be used to identify strains with cumulative high- and/or low-risk genetic variants with contributions from multiple genes [62]. For example, Levy *et al.* [145] identified Rhbdf2 as a novel gene in skeletal homeostasis by using 34 different CC strains. Their results were then confirmed in extreme-phenotype and knockout mice studies. Wang *et al.* [108] discovered the CC-derived strain CC036 as a spontaneous laboratory mouse model for the study of human gastric tumorigenesis. Also, Gelinas *et al.* [146] identified strains that were resistant or sensitive to bleomycin, an antineoplastic drug. Extreme strains can also be used to corroborate the role of a gene in a trait, as evidenced by Levy *et al.* [145].

Finally, CC populations are a useful tool for studying hereditability. For example, Atamni *et al.* [57] studied the heritability of glucose tolerance on a cohort of 501 mice from 58 different CC lines, and Shustreman *et al.* [137] studied the heritability of bone volume and bone bacterial infection susceptibility in 272 male and female mice from 23 CC lines. Hereditability indicates the importance of the genetic background on the performance of evaluated traits. A high hereditability value is indicative of a trait that is

strongly influenced by the genetic background of the host [124]. Founder strains can also be used as a robust, informative and generalized model to establish heritability of a trait in a reproducible fashion [62,147]. In summary, CC mice are reproducible, homozygous recombinant inbred strains with extreme variation between strains but similar homogeneity within strains compared to traditional inbred lines.

2.2. Diversity outbred mice:

Unlike inbred mice, which are maintained by sibling mating [127], outbred animals are those generated by randomly breeding mice [90,148]. There exist different commercial vendors (i.e., Charles River, Harlan or Jackson Laboratories) and different outbred strains (i.e., Black Swiss, Crl:CF1, Hsd:ND4 or Northport) that are commercially available [53,126]. In outbred models, the phenotypic variation is assumed to be combination of both environmental variability (like in inbred strains) plus their own inherent genetic variability [20]. Thus, due to their genetic variability, outbred models are thought to display a higher phenotypic variability than inbred models [149]. However, classical outbred animal models, such as CD-1 and NMRI, have been found to present phenotypic variability similar to classical inbred mouse strains (i.e., BALB/c and C57BL/6J) [20,149].

The Diversity Outbred (DO) model is a CC-derived mouse model created in an effort to obtain more genetically diverse mouse cohorts. This model was developed as a way of modeling genetic diversity while avoiding the experimental problems arising from environmental variability, population structure and rare allele presence typically found in human populations [90]. DO was designed to offer high-resolution genetic mapping, and, although each mouse is genetically unique like other outbred models, each allele and SNP of a given mouse in a DO cohort is present in at least one CC strain that originated the DO cohort [53]. Put simply, DO populations are outbred cohorts derived from random breeding of the 8 original founder strains or subsequent CC (RI) strains that accumulate recombinant events at each outbreeding generation [150]. DO mice are maintained outbred by random mating with an estimated contribution of each CC founder strain of 11.3 – 13.8% , which, in turn, allows precise genetic mapping [114,148,150]. In fact, this high mapping resolution is derived due to their heterozygosity in most loci and high minimum minor allele presence of 12.5 % [62]. Of note, a specific DO population is comprised of genetically unique individuals (each individuals is a genetically unique "strain" with n=1), and only a single individual is representative for a DO genotype [90,114] (**Figure 3**). This uniqueness is reflected in a high level of heterozygosity, which allows precise estimation of QTL and investigation of allelic effects [151]. The heterogeneity obtained by DO outbreeding process allows one to reach generalizable conclusions while avoiding monolithic phenotypic responses that occur in a limited genetic background and minimizing the likelihood of missing a potential genetic effect due to testing in a sensitive genetic background model [90,127]. Indeed, results

generated in DO cohorts are likely to be robust and generalizable to diverse populations [62]. In this sense, Chitre *et al.* [104] replicated their previous results [55] when identifying loci associated with adiposity trails. DO models can also replicate interindividual variability in response to treatments commonly found in human populations, which allows the identification of subpopulations resistant or susceptible to a certain treatment [33], which could be extremely useful for personalized nutrition applications of phytochemicals. In this sense, DO mice showed high interindividual variation in response to cisplatin-triggered renal injury [152] and EGCG-associated hepatotoxicity [33], which replicated the interindividual variation found in humans. Nevertheless, the genetic polymorphisms in mice might not be the same as the ones reported in humans. However, DO cohorts can still be used to estimate variability in response due to genetic diversity, which would be broadly useful in human contexts, and identify genes that provide mechanistic insights into a phenotypic response [150], which would be useful if human orthologues exist. For example, while LDLR$^{-/-}$ mice have been widely used to study familial hypercholesterolemia [153], DO mice have been used to genetic influence on study serum cholesterol traits and identified 5 candidate SNPs in the upstream region of the gene Foxo1 [148]. However, it is worth noting that in some cases, the phenotypic variation of specific DO cohorts can be equal to, or even lower, than the phenotypic response observed in inbred strains and classical outbred models [20], depending on the outcome of interest and the specific genetics of the cohort.

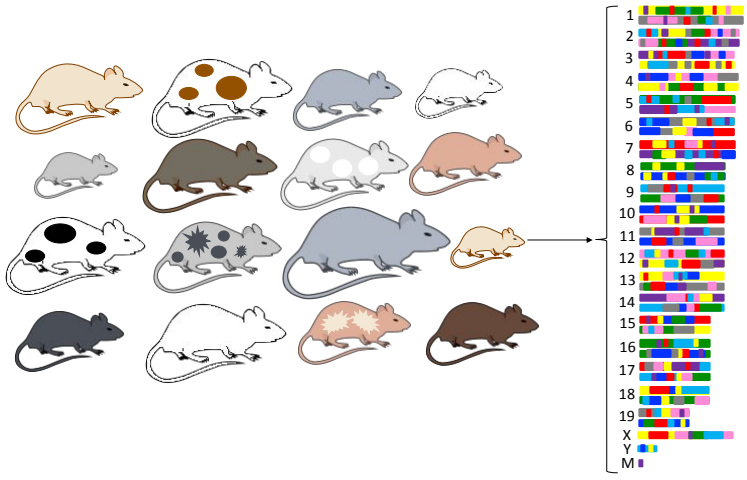

Figure 3: *Representation of a DO cohort. In DO cohorts, each mouse is a single and unique representant of its strain (n=1). At a genetic level, mice in DO cohorts are heterozygotes at most loci. Phenotypically, each mouse is diverse too, presenting different fur colors, sizes and behavior traits, amongst other aspects.*

As stated above, due to random mating, neither a specific cohort nor the individual animals within each cohort can be replicated, even if the cohort originates from the same parents (or parents from the same inbred strain). Nevertheless, the genetic loci associated with a phenotypic response can be subsequently

reproduced in targeted CC cohorts [90], with $n>1$. Hypotheses generated in DO cohorts can be tested and validated in CC populations selected and generated based on loci identified by DO studies [62]. Thus, the DO model can be viewed primarily as a hypothesis-generation model, while the CC model can then be used to test those hypotheses [90] with specific RI strains selected based on the loci of interest. DO models can also generate hypotheses that can be tested in humans and/or human samples. For example, the F1 of a DO cohort allowed the identification of 3 genes (*Rwdd4*, *Cenpu* and *Casp3*) as metastasis modulators in prostate cancer. Further analysis in human samples from > 5,300 prostate cancer patients presented revealed correlations between the presence of genetic variants on the identified genes and their expression levels, cancer aggressiveness and patient survival [63].

The fact that each individual in a DO cohort is the only representant of its own unique genotype maximizes variability, but logically this has some drawbacks. This is not optimal when pursuing gene-by-treatment studies, while CC allows the use of unlimited isogenic biological replicates [127]. On top of that, the complete genome sequences of CC panel lines are known and available for free. This implies that quantitative trait loci (QTL) mapping can be performed after phenotype data collection by investigators without requiring any new genotyping [127]. This is obviously not the case of DO populations, in which each cohort is new and thus genotyping is required after phenotype data collection due to the uniqueness of each animal [127]. Some examples where genotyping was required after phenotypic data collection using DO populations include the studies of Church *et al.* [33] and Gatti *et al.* [151]. However, QTL studies can still be performed [62]. For example, Logan *et al.* [123] identified different QTL associated with anxiety and activity traits in a cohort of 280 male and female DO mice. Svenson *et al.* [148] identified different QTL associated with coat color and cholesterol levels in a cohort of 150 DO mice. The main advantage of DO populations is that they allow precise genetic mapping due to their wide amount of recombinations, genetic diversity and randomization, as well as an unlimited amount of genetic backgrounds to include in a cohort (as each mouse is its own and unique "strain", and therefore variability is proportional to the chosen sample size) [62]. Moreover, DO mice allow the evaluation of the effects of causal genetic variants within a wide variety of genetic backgrounds [114]. A clear example is the study on the hepatotoxic effect of green tea component EGCG [33]. Nevertheless, DO cohorts can be useful for applications that do not require genetic mapping, and this includes toxicology screens [90].

The main disadvantage with CC and DO models is the large amount of animals required to conduct most studies [150], but this is a common trait in heterogenous population studies [33,154]. CC studies typically use a large number of strains with a small (n=2) sample size per strain (unless a single specific CC/RI strain has been identified for a subsequent treatment arm study, in which case the n size per group would be calculated as in any standard rodent experiment), whereas DO studies employ large cohorts of

individuals (*n*=1 per strain, as individual = strain). For example, the DO cohort of animals used by Church *et al.* [33] was of 272 mice, and the CC cohort used by Nashef *et al.* [155] was of 78 mice. However, these large populations are still reasonable, especially for hypothesis-generating screening studies with highly focused outcomes [90].

3. Use of Founder Strains, CC and DO models in phytochemicals and bioactives research.

As discussed above, mouse models of genetic diversity are useful for associating specific phenotypes or traits with genes and their variants. While some research fields present obvious phenotype/trait characteristics for study (i.e., glucose levels in diabetes, body weight or adiposity in obesity, tumor burden or invasiveness in cancer), in the field of phytochemicals, those often remain to be clearly defined. Some traits worth investigating include the pharmacokinetic behavior and bioavailability of phytochemicals (epithelial absorption, transport and efflux, as well as phase-II metabolism and excretion of specific compounds); receptor and promoter activation by specific compounds; effect on a given disease; or study of pathway modulation. Of note, gut microbiota is also a huge source of variability in humans and animal models such as mice [156]. As a matter of fact, gut microbiota diversity plays an important role in phytochemical bioavailability and metabolism, which can later affect their bioactivity [157,158]. For example, individuals whose gut microbiota is capable of metabolizing isoflavones such as daidzein into equol report more relevant cardioprotective effects than non-producers [159]. However, the influence of variability of the gut composition and function on human and animal response to phytochemicals is beyond the scope of this review.

In the field of phytochemicals research, we have historically assessed phytochemical-animal (mouse or human) interactions, such as pharmacokinetic behaviors or pharmacological effects, in models with little or no genetic variability. Thus, we tend to think of these observed biological activities as inherent or intrinsic properties of phytochemicals (with the often-implicit assumption that these findings have broad applicability). Based on this incomplete conceptual framework, we tend to view the potential translatability of reproducible animal data to humans with optimism. However, attempts to translate promising preclinical phytochemical data to clinical practice have more often than not proven futile [160,161]. It is our opinion that a paradigm shift in our thinking is required, where each interaction of a specific phytochemical with an animal (rodent or human) can be considered as a phenotype or trait that is likely to depend highly on genetic factors. Thus, studies in the context of mice with limited or essentially no genetic diversity are inherently limited in that the applicability of these findings to other genetic contexts, as exists in humans with significantly greater genetic variability, is unknown at best and unlikely at worst. Specific mutations in mice that recapitulate the etiology and progression of a human disease with high fidelity (see the example

of APC^{min} mice in human FAP research above) may still provide useful results for application to humans [84,85]. However, the study of multifactorial processes with as yet poorly understood genetic influence, such as phytochemical bioavailability or phytochemical modulation of obesity, in mouse populations with artificially low genetic variability is akin to phenomenology: the study of unique experiences or outcomes that are almost completely dependent on specific contexts as opposed to the "nature of being" (in other words, context-dependent behaviors as opposed to inherent properties) [162,163]. This is not to say that previous studies in single or a few animal strains are not valuable, and we have performed numerous such studies ourselves [164–168]. Rather, the difficulties often encountered in translating preclinical mouse findings from phytochemicals to humans suggest that a broader understanding of the potential benefits of phytochemicals that encompasses the significant influence of genetic background needs to be taken if we are to maximize the use of preclinical data to drive meaningful improvements in human health.

Other fields such as toxicology [33,106,131,169,170], cancer [108,129], and immunology [132,136,139,171,172] have made significant advancements by using genetically diverse preclinical models to elucidate genetic drivers of phenotypes, as well as to understand gene-treatment interactions (the latter approach is highly relevant to phytochemical interventions). This is the goal of "precision nutrition" or "personalized nutrition" [173,174], but thus far the opportunities presented by genetically diverse mouse models remain largely unexploited by phytochemical researchers. This presents an intriguing and potentially fruitful opportunity to make major advances in the use of phytochemicals to improve human health.

For example, to evaluate the response to phytochemicals in the context of hypertension, traits to investigate in genetic diversity models could include systolic blood pressure reduction in response to phytochemical intervention, as some phytochemicals have shown this property in rodent [175,176] and human studies [177,178]. Thus far, little work has been reported in the area of phytochemicals and diverse mouse populations [33,52,124,154,179], and traditional phytochemical research has not viewed these phytochemical activities as a context-dependent "phenotypes" or "traits", but rather as inherent properties of phytochemicals. Also, most phytochemical research, like in other areas, is based (either explicitly or implicitly) on the assumption that these phytochemical activities do not vary much and that common inbred mouse models (i.e., C57BL/6J) will translate into humans (or are at least the best or only models available). For example, investigations of the potential anti-obesity effects of quercetin have been largely performed in male inbred C57BL/6J mice [46,180–186]. In this sense, there remain several challenges (or unexploited opportunities, depending on the perspective) to overcome in phytochemical research translation from mice to humans [161,187]. The use of genetically diverse models could help overcome some of these challenges, especially the overuse or exclusive use, of specific inbred lines in certain fields of research. So far, only the

effects of catechol-O-methyl transferase (COMT) gene polymorphisms have been widely studied as a factor that modulates polyphenol bioavailability, metabolism and bioactivity [188–194]. However, further studies are required to identify other genetic loci that affect the bioavailability, metabolism and bioactivity of phytochemicals. This may also serve to identify previously unknown genes that are associated with phytochemical behavior, potentially leading to discovery of unknown mechanisms of action and novel therapeutic targets. The value of such discoveries for translation of phytochemical benefits from mice to humans cannot be overstated.

There are multiple excellent reviews that delineate the utility of CC and DO models to study genetic factors that contribute the most to a phenotype as well as a treatment or intervention outcome (i.e., toxic or beneficial effects) [53,59,62,114,127,170,195,196]. Most studies involving CC and DO models aim to elucidate which genetic factors contribute significantly to a chosen phenotype [56,123,128,152,197–202], and a substantial part of them are framed within the field of immunology [32,60,136,172,203–208]. The research field of pharmaceuticals and toxicology has also used and benefited from CC and DO models [138,151,169,170,201,202,209,210], with clear parallels to phytochemical research. Although most dietary phytochemicals (the vast majority of phenolics, carotenoids, alkaloids, etc.) are non-nutrient xenobiotics, and thus follow drug-like xenobiotic detoxification metabolism [211,212], only a handful of studies involving food bioactives are known to have been performed in CC and DO models [33,52,124,154,179]. This evidence suggests that genetically diverse mouse populations are valuable tools that are generally overlooked in the field of phytochemicals. Given the wide variation in phenotypes, traits and treatment outcomes dependent on genetic background that have been identified in other fields [57,137,155,207,208,213,214], it is highly likely that bioavailability of and sensitivity to phytochemicals is also highly dependent on genetic background. Thus, identifying novel phytochemical-gene interactions is likely to significantly advance the felid of precision nutrition. This section will summarize the main research findings in the field of food phytochemicals that used founder strains, CC and DO models, and the potential of these models to further advance in food bioactive research.

3.1. Obesity and body weight.

Obesity is a multifactorial disease in which both environmental and genetic factors play an important role. However, it has been estimated that about 70 % of population variance in obesity is due to genetic factors [215]. Genetically diverse populations have been used to study the genetic architecture of obesity, mapping and identifying genes associated with adiposity traits. For example, Chitre *et al.* [104] identified 32 different loci associated with adiposity traits as well as several candidate genes for functional studies in a cohort of heterogeneous stock (HS) rats. However, genetically diverse populations can also be used to

find strategies to prevent and/or manage obesity. Dietary phytochemicals have been widely researched for their potential to treat and/or prevent obesity and obesity-related pathologies. Most of these studies are carried out in inbred C57BL/6J male mice fed a an obesogenic diet [216–219], or other well-defined inbred mouse strains (*ob/ob*, etc.). As discussed above, this is primarily due to the exaggerated phenotype, convenience and consistency: the results are rapid, consistent and predictable. However, while the phenotype is convenient for researchers, the results from this model do not account for various etiologies of obesity, genetic variability, or sex differences. Furthermore, the C57BL/6J male mouse only represents a snapshot of the genetic factors that influence critical phytochemical parameters such as bioavailability, pharmacokinetics and metabolism, interactions with cell signaling mechanisms (receptors, transcription factors, etc.), epigenetic effects, etc. It is generally unknown where the C57BL/6J male mouse (or any other inbred mouse model) lies on the possible spectrum of these phytochemical activities related to other models, or most importantly, to humans. Thus, the translatability of these results in the context of phytochemical efficacy to genetically diverse humans remain questionable. With the added caveat that the genetic determinants of efficacy for various phytochemical classes and subclasses and possibly individual phytochemicals) likely differ, there is much work (and much opportunity) to be done to elucidate gene-phytochemical interactions and translate these to precision human nutrition.

In order to demonstrate the effect of genetic background on phytochemical efficacy in the context of genetic background and sex, we recently employed male and female CC founder strains to evaluate how diet-induced obesity and hyperglycemia are modulated in various mouse strains fed a high-fat diet supplemented with quercetin [52], a phenolic compound found in large quantities in onions, hot peppers and capers with cardioprotective, anti-obesity and anti-inflammatory bioactivities [220]. We employed mice from 6 of the 8 CC founders: A/J, C57BL/6J, 129Sv/ImJ, CAST/EiJ, PWK/PhJ and WSB/EiJ micer. The NOD/LtJ and NZO/H1J strains were excluded, as these are spontaneously diabetic [221–223] and obese [224,225], respectively, and this study focused on diet-induced obesity. In this study, we showed that response to high-fat diet administration and quercetin administration presents multiple strain-specific traits [52]. For example, while the body weight of C57BL/6J, 129Sv/ImJ and A/J increased during the experiment, CAST/EiJ and WSB/EiJ did not increase in body weight. This further confirms evidence that body weight gain depends on strain [226–228]. Indeed, most mice studies on obesity are performed on male C57BL/6J due to its rapid and consistent body weight gain [229–231]. Also, we showed sex-specific response to high-fat diet administration [52]. For example, high-fat diet produced an increase of body weight in male PWK/PhJ mice, but not in female PWK/PhJ mice. This shows the importance of including both sexes in studies in which the last goal is to provide data to develop clinical studies in humans. Most significantly for this review, the protective effects of quercetin were highly dependent on mouse strain and

sex. For example, in C57BL/6J mice, quercetin supplementation seemed to protect against high-fat diet induced body gain in females, but not in males. Amongst all strains, only in PWK/PhJ male mice did quercetin significantly reduce body weight and glucose fasting levels during high-fat diet administration. Indeed, quercetin generally increased blood fasting glucose and insulin levels. In CAST/EiJ mice, males and females presented an inverse response to quercetin in fasting blood glucose levels, and quercetin did not worsen female CAST/EiJ fasting insulin levels. Interestingly, no changes in fasting insulin levels were reported for female 129Sv/ImJ. Overall, this study generated proof-of-concept data on the complex interactions between genetic background, sex, diet-induced obesity and quercetin intake. This suggests that there are significant genetic factors, which remain largely unknown, that govern quercetin efficacy. Strategic use of genetically diverse mice could uncover these factors as well as predict variation in response across a diverse human population. Such studies would likely increase transability to humans, at least in the context of quercetin and obesity.

The effect of phenolic compounds on body weight gain and obesity development has also been recently studied in CC lines [124]. In the study of Amer-Sarsour *et al.* [124], the body weight management properties of a non-dialyzable material from a polyphenol-rich cranberry extract were evaluated in 13 male and female CC lines (average *n* of 6). Briefly, mice were fed a high-fat diet for 18 weeks, and during the last 6 weeks they were intraperitoneally injected with 50 mg/Kg of the cranberry treatment three times per week. During the high-fat diet (cranberry-free) period, both males (10.41 – 68.65 %) and females (9.78 – 64.74 %) increased their body weights. There was high variability between CC lines, but males reported a higher body weight increase than females (on average, 9.61 g vs. 7.81 g). The effect of the cranberry extract depended significantly on the genetic background and sex of the mice. Five of the 13 lines reported a significant decrease in body weight in males (- 5.68 – - 15.69 %) due to cranberry, while one line reported a significant increase in body weight (+ 8.31) and 7 did not report a significant change in body weight. In females, the cranberry extract produced a significant decrease in body weight in 5/13 lines too (- 3.90 – - 10.75 %), and no significant effect in the 8 remaining CC lines. Noteworthily, effects in the same line between different sexes were generally not conserved. Indeed, 9 of the lines presented a sex-specific response. Only 3 lines reported a significant body weight reduction in both sexes, and only 1 a lack of effects in both sexes. In line with our study [52], Amer-Sarsour *et al.* [124] further demonstrated that the effects of phenolic compounds in body weight management depend on both the genetic background and sex of the mice model. Moreover, the use of CC lines in the study of Amer-Sarsour *et al.* [124] allowed the calculation of estimated heritability, which was found to be high both in males and females, indicating that the traits under study were under a strong influence of the host genetic background.

3.2. Redox status

Norris *et al*. [179] evaluated the effect of a common dietary anthocyanin on glutathione (GSH) homeostasis in 5 female inbred CC founder stains (A/J, C57BL/6J, 129Sv/ImJ, NOD/LtJ and CAST/EiJ). After six weeks of dietary supplementation with cyanidin-3-*O*-β-glucoside (100 mg/Kg in chow diet), Norris *et al*. [179] observed that, regardless of control or cyanidin-3-*O*-β-glucoside intervention, NOD/LtJ mice consumed significantly higher amounts of food that the other strains, and that CAST/EiJ reported a lower body weight. More importantly, changes in GSH homeostasis depended on strain genetic background. Overall, female NOD/LtJ mice were not responsive to cyanidin-3-*O*-β-glucoside administration, and C57BL/6J female mice only reported a modest (12.5 %) increase in pancreatic GSH/GSSG ratio. However, A/J, 129Sv/ImJ and CAST/EiJ reported strain-specific changes in GSH homeostasis due to cyanidin-3-*O*-β-glucoside supplementation. Both A/J and 129Sv/ImJ reported a decrease (40 and 43 %, respectively) in hepatic GSH/GSSG ratio, which was triggered by different mechanisms. In A/J mice, cyanidin-3-*O*-β-glucoside decreased GSH levels, while cyanidin-3-*O*-β-glucoside increased GSSG levels in 129Sv/ImJ, and this was accompanied with an increased expression of glutathione peroxidase-1 gene expression. Unlike A/J and 129Sv/ImJ, cyanidin-3-*O*-β-glucoside produced a two-fold increase in hepatic GSH/GSSG ratio in CAST/EiJ. Overall, CAST/EiJ were the most responsive strain to cyanidin-3-*O*-β-glucoside, reporting a four-fold increase of GSH/GSSG ratio in the heart, an increase of GSH in the kidneys, and a decrease on GSSG in the pancreas. The different effects of cyanidin-3-*O*-β-glucoside may be due to sensitivity to hepatotoxic effect of phenolic compounds, which has been demonstrated to depend on genetic background by Church *et al*. [33]. This research by Norris *et al*. [179] demonstrates that the modulation of GSH homeostasis by cyanidin-3-*O*-β-glucoside depends on genetic background. The variability in response to cyanidin-3-*O*-β-glucoside suggests that one explanation for inconsistent results in clinical and epidemiological studies may be the genetic variability in human populations.

3.3. Hepatotoxicity

The use of DO models can be useful to identify genetic variants related to toxicological effects of xenobiotics. These types of studies have been going on since late 2000's with drugs and toxins [106,232,233], but are notably lacking in the field of phytochemicals. One of the few examples is the study performed by Church *et al*. [33] in the context of EGCG-mediated hepatotoxicity. EGCG is a flavonoid abundant in green tea. Although tea consumption has been associated with health effects (i.e., weight management), and EGCG seems to be an important component in these health effects [234], tea-associated hepatotoxicity occurs in human [235] and animals [236], especially at high doses. In their study of > 250

male DO mice, Church *et al*. [33] evaluated the hepatotoxic effects of 50 mg/Kg/day intraperitoneal-administered ECGC over 3 days. The authors reported a phenotypic variation of ALT levels, hepatic necrosis and hepatic DNA strain break in response to ECGC. For example, the fold change in ALT between pre- and post-treatment administration ranged from 0.15 to 495.5; hepatic necrosis ranged from 0 – 86.8 %, and was only severe (> 10 %) in 16 % of the population; and DNA strand breaks ranged from 0.01 – 26.7 %, and only 26 % of the population had > 1%. The ALT results gathered by Church *et al*. [33] allowed the association of ALT levels with a segment (142.56– 151.8 Mb) of chromosome 4, and a total of 25 SNPs. Out of these 25 SNPs, 88 % of them were located within the 142.6– 151.8 Mb region. The authors also reported that alleles inherited from the NOD/ShiLtJ strain conferred a protective effect against hepatic injury, while alleles from the seven remaining founder strains conferred a higher degree of hepatic injury risk. Interestingly, within the region 142.6 – 151.8 Mb from chromosome 4, there existed 49 genes with sequence variants exclusive to the NOD/ShiLtJ strain, and 46 of those present human ortholog genes. This information was then used to explore gene variants in humans (n=15) with reported hepatotoxicity associated with green tea extract consumption. Within those patients, 3 SNPs belonging to 3 different genes were associated with EGCG hepatotoxicity, namely mitofusin 2, periodic circadian clock 3 and vacuolar protein sorting 13 d. Overall, the work performed by Church *et al*. [33] exemplifies that DO studies have potential to be used as population-based safety assessment studies in the field of xenobiotics, including phytochemicals. Moreover, their results also demonstrated that DO studies can be used to identify lead genes and its variants associated with toxic effects. Indeed, their results have opened the door to a possible mechanistic study of mitofusin 2, periodic circadian clock 3 and vacuolar protein sorting 13 d on EGCG-associated hepatotoxicity. Finally, these data suggest that phytochemical metabolism, efficacy, and toxicity could be strongly modulated by host genetic background. Such work could easily be extended to other phytochemicals.

3.4. Lifespan

Strong *et al.* [154] evaluated the effect of resveratrol, green tea extract, curcumin, oxaloacetic acid and medium-chain triglyceride oil supplementation on the lifespan and body weight of male and female genetically heterogenous mice fed a standard diet. In their study, genetically heterogeneous mice, originated from four-way crossed BALB/cByJ, C57BL/6J, C3H/HeJ and DBA/2J original inbred strains, were used. This approach generates genetically unique individuals, which avoids effects occurring due to the use of a single inbred strain, while allowing studies to closely reproduce this genetically diverse population. In terms of body weight, female mice were more responsive to supplementation than male mice when compared to sex-matched control mice. Females reported decreases in body weight by middle-chain triglyceride oil, curcumin and resveratrol, and increases in body weight by green tea extract. Male

mice only reported a body weight increase due to green tea extract administration. Green tea extract and the main component of curcumin, tetrahydrocurcumin, have been shown to increase life span in C57BL/6 male mice [237], but this effect was not reported in the study of Strong *et al.* [154]. In terms of lifespan, only green tea extract diminished midlife (660 – 800 days) mortality, and only in female mice. This lack of reproducibility in lifespan effects from green tea extract and curcumin may reflect the fact that, when using a single inbred strain, results may be dependent on a single genotype, and thus outlier findings may be reported that cannot be translated to genetically diverse populations. This also affects translational science, since strategies developed in a single or a few mice strains aiming to targe humans do not agglutinate the genetic diversity found in humans. It warrants mention that implementing four-way crosses from BALB/cByJ, C57BL/6J, C3H/HeJ and DBA/2J mice to generate diverse populations may be a good starting point for phytochemicals research. Studies using this approach require less genotyping, and the "founder" studies are less expensive, than those originating from the CC/DO founders.

4. Study and application of genetic diversity for phytochemical use in humans

It is known that individual requirements for, and response to, nutrients and bioactive compounds differ significantly. For example, humans show inter-individual variability in cardiometabolic biomarkers in response to supplementation with hydroxycinnamic acid-containing foods [238]. The objective of precision nutrition is to stratify people into different sub-groups based on different predictive biomarkers (i.e., genetic variations, epigenetics, microbiome, lifestyle, diet intake, environmental exposure, etc.) and then use those stratifications to better estimate dietary requirements and come up with better, more precise recommendations and/or interventions [173,174]. Genetic variation plays an important role in nutritional requirements [173,174,239–242] and the effects of food bioactives [243–245]. For example, different genetic polymorphisms alter choline recommended intake [242]. The use of genetically diverse mouse populations could therefore identify these genetic factors and provide invaluable information to inform subsequent human studies the field of precise nutrition employing phytochemicals. For example, Yam *et al.* [213] identified CC strains prone to develop obesity in a cohort with 22 CC strains, highlighting the importance of genetics when making dietary recommendations. The studies performed by Griffin *et al*. [52], Amer-Sarsour *et al*. [124], Norris *et al*. [179], Church *et al*. [33] and Strong *et al.* [154] have demonstrated the potential of founder strains (CC founders and other founders), CC (and other recombinant inbred crosses) and DO cohorts in the field of phytochemicals and health. Their results demonstrate that the bioactive and toxic effects of dietary phytochemicals depend heavily on the genetic background of mice [33,52,124,154,179], which is in line with other studies on drug toxicity [135,146,201]. The logical extension of these findings is that human genetics are likely to influence the effects of phytochemicals. This

opens the door for using these genetic diversity models to inform and improve precision nutrition applications of phytochemicals in humans.

Currently, in most fields of research, variability is seen as something undesirable. Hence, the use of a few well-characterized and consistent mouse strains in certain research fields [44,45]. However, as discussed above, genetic homogeneity in preclinical research presents significant limitations for translation to populations with significant genetic variation. The advantage of consistency in single inbred strains becomes a disadvantage when searching for genetic determinants of traits and response to interventions. Therefore, variability can be used intentionally as a tool to introduce and then elucidate genetic determinates and mechanisms of action, as reported by the studies of Hartiala *et al.* [107] and Church *et al*. [33], among others [123,148,179].

The use of genetic diversity models could further advance the knowledge in many areas of phytochemical research, such as bioavailability and metabolism, bioactivity and toxicity. As a matter of fact, different genetic variants have been identified as factors affecting the bioavailability, metabolism and bioactivity of phytochemicals [246,247]. Of note, although the phenotypic contribution of a single SNP might be low, the cumulative contribution of several of them can explain a large extent of the variability found in humans [248]. For example, it is well known that COMT polymorphism affects the urinary excretion profile (a biomarker of bioavailability and metabolism) of tea polyphenols and their metabolites [188]. The COMT enzyme gene presents a SNP at rs4680, where a guanine transition to adenine results in a 66 – 75 % reduction in COMT activity [249,250]. Homozygote individuals for the low-activity SNP exhibited lower urinary levels of tea polyphenols and their metabolites than heterozygotes and high-activity homozygotes [188], and authors hypothesize that tea polyphenols in low-activity homozygotes remain in their bodies for longer which potentially results in a more potent bioactive effects. Although unlikely due to COMT polymorphism, poorer absorption of phenolic compounds could also be a mechanism of action. Indeed, this COMT polymorphism has also been associated with modulation in polyphenol bioactivity. Dostal *et al.* [189] showed that low-activity homozygote women reported higher bioactivity (reduction in plasma adiponectin and increase of plasma insulin) after a 12-month supplementation with a green tea extract. This effect is tentatively attributed to the fact that tea polyphenols remain unmetabolized for longer times in individuals with the low activity COMT isoform, allowing the polyphenols to retain their bioactivity longer and resulting in a greater bioactive effect [189]. COMT polymorphism has also been shown to modulate the protective effects of plant extracts against DNA damage in lymphocytes [190], the protective effects of tea consumption against breast cancer [191], and the regulation of energy expenditure and fat oxidation promoted by tea catechins consumption [192], among other effects [193,194]. There are other examples of genetic variation in genes involved in the bioavailability and metabolism of

phytochemicals that affect the bioactivity of these compounds. For example, Deming *et al.* [251] found that the homozygote variant (G/G) of the polymorphism rs2070959 in the UGT1A1 gene was associated with a reduced risk of endometrial cancer.

Beyond genes regulating the bioavailability and metabolism of phytochemicals, other genetic variations can affect the bioactivity of phytochemicals [252–256]. For example, George *et al*. [252] showed that genetic variation in the endothelial nitric oxide synthase (eNOS) gene produced a different endothelium-dependent vasodilation response due to administration of a fruit and vegetable drink rich in phytochemicals; while homozygote GG subjects reported a vasodilation effect, no effect was reported for GT heterozygote subjects. Lin *et al*. [253] found that among tea drinkers, tea consumption had a higher reduction in the risk of lung cancer development in (CA19)/(CA19) and (CA19)/X individuals for insulin growth factor 1 than X/X carriers. Schwarz *et al*. [257] reported that the potential of different phytochemicals (i.e., hypericin and hyperforin) to inhibit NADPH-dependent oxidation of estradiol depended on the genetic variants of CYP1A1. However, there are other gene variations that may affect the bioavailability and metabolism of phytochemicals, but they may or may not have a relevant impact in certain diseases. For example, urinary excretion of flavonols and flavan-3-ols is higher in women null for the glutathione-*S*-transferase M1 (GSTM1) than those positive for GSTM1 [258]. Although urinary content in flavan-3-ols and flavonols was associated with a reduced risk of breast cancer in null GTMS1 [258], genetic variants of GSTM1, as well as GSMT2, did not affect the protective effects of tea consumption against esophageal and stomach cancers [259]. Moreover, the effects of phytochemicals can change depending on the genetic background of the host. For example, women positive for both GSTM1 and GSTT1 breast cancer risk increased with urinary levels of flavonols, while urinary flavonol and flava-3-ol levels were associated with a reduced risk of breast cancer in individuals null for GSTM1 and GSTT1 [258].

It is important to note that the studies described above examined the impact of polymorphisms in genes that were previously known to impact phytochemical bioavailability or were thought to be targets of phytochemicals. Further studies should be performed on the impact of polymorphisms in genes that are already mechanistically linked to phytochemical activities (phase-I, II and III enzymes, carotenoid oxygenases, PPARs, RXR, FXR, Nrf2, etc.). However, some aspects of the regulation of phytochemical (i.e., polyphenols and carotenoids) bioavailability and metabolism are still unknown [260]. This is of special relevance, as current evidence suggest that phytochemical metabolites play an important role in phytochemical bioactivity [1,261]. Both bioactivity and toxicity of phytochemicals are related to their bioavailability and metabolism. Additionally, many phytochemical mechanisms of action and effective therapeutic targets likely remain unknown. Such gene-phytochemical interactions must be identified before they can be tested in mice (targeted CC lines) and humans (screening and recruitment for clinical trials).

Implementation of mouse models of genetic diversity combined with GWAS/QTL could result in a quantum forward leap in phytochemical-based precision nutrition, by facilitating identification of loci and eventually genes heretofore unknown to be linked to phytochemical activities. The intriguing findings of COMT polymorphisms and polyphenol bioavailability and bioactivity could potentially be repeated with genes previously unknown to affect phytochemical behavior in humans. The identification of such loci and genes and their potential human orthologues will not only facilitate stratification of subjects for focused studies (and eventually clinical practice) based on anticipated efficacy, but may also suggest previously unknown mechanisms of action and therapeutic targets that can be exploited. The variability in diversity mouse panels could also be used to identify and predict responder vs. non-responder individuals [33,108], and phenotypic responses can be linked to genotypic traits. Research on host-related genetic determinants for phytochemical bioactivity/toxicity could be performed in diversity mice models, which would further expand our knowledge on this field. Of note, these types of approaches can suggest specific targets and mechanism of action. Some examples include the research conducted by Church *et al*. [33] and Norris *et al*. [179]. Such innovation is desperately needed, as mechanistic research and translation to humans have both come up against serious obstacles in the past decade [24,160,161,262,263]. Overall, understanding the genetic factors modulating bioavailability, metabolism, toxicity and bioactivity of phytochemicals will lead to optimized individual personal recommendations.

Epidemiological or clinical studies in whole populations sometimes fail to report any specific associations [191,254,264]. For example, in the study of Yuan et al. [254], the authors found no association between frequency of tea consumption and breast cancer risk when evaluating the whole population. However, when women were separated between angiotensin II converting enzyme (ACE) low-activity and high-activity genotypes, frequency of tea consumption was associated with a decrease in breast cancer risk only in the high-activity genotype. Rizzi *et al.* [264] did not find a significant effect in the lipid profile of high and low polyphenol intake individuals. However, when groups were stratified by different PON1 genotypes, 4 different PON1 genotypes were reported to increase HDL levels under high polyphenol intake. The use of diversity mouse models could identify target genes that could then be used to profile and stratify human populations based on genotype, leading to useful predictions of likely response.

5. Research opportunities in the field of phytochemicals

As reviewed previously, little work has been done in the field of phytochemicals with new genetically diverse populations (i.e., CC and DO cohorts) [33,52,124,154,179]. This field of research could benefit from a paradigm shift in thinking, to consider phenotypic and genetic variability as a tool to answer questions and generate hypothesis instead of an obstacle. For example, variability in phytochemical

bioavailability and bioactivity could be considered as traits/phenotypes of interest rather than seeing that as an inherent characteristic of the compound under study. This approach has already been applied in the closely-related fields of pharmacology and toxicology [127]. However, even in the pharmacological field of study, studies addressing genetic determinants of key aspects of compound bioavailability and activity (i.e., pharmacokinetic studies) are extremely rare [265]. Although there have been attempts to study phytochemical bioavailability and bioactivity in classical genetically diverse cohorts (i.e., use of Swiss outbred mice to study EGCG bioavailability [266] or use of CD-1 outbred mice to study olive polyphenol bioactivity [267–269], among others [270–274]), the majority of studies do not take advantage of the full potential of genetically diverse models. Instead, these classic models are used much like any other inbred strain. As reviewed in this manuscript, mouse cohorts like CC and DO offer the potential to discover new mouse strains, mechanisms of action, genes regulating traits/phenotypes, target genes for therapeutical uses, QTL and GWAS studies. All these promising applications remain to be exploited at a significant level in the field of phytochemicals. For example, a cluster of application of phytochemicals is the treatment of obesity and its associated pathologies [50,52,275–280]. Genetically diverse mouse models could be used as standard preclinical models to study multifactorial diseases such as obesity. CC and DO cohorts could be used to identify strains/individuals with a specific (or opposite) phenotypic trait, as previously exemplified in this review. QTL and GWAS could then be applied to identify the responsible loci and genes for those phenotypic traits [63,104,106,148]. Studies with specific targeted CC lines or knock-out mice could the confirm the results found in CC and/or DO cohorts [105,145]. As previously discussed, the MPD offers a wide array of data and information on mouse phenotype. However, the information on phytochemicals' effect in the MPD is scarce. Further efforts must be made to gather and upload information on phytochemicals' metabolism, toxicity and alterations in major biomarkers in relevant disease models. In due course, these data should be uploaded in the MPD for public availability (or, a complementary database focused on phytochemicals could be developed). Overall, these approaches can provide new genes/loci of interest, reveal new mechanisms of action as well as targets to exploit in phytochemical studies. Another potential application of genetically diverse mouse models in the field of phytochemicals it their application for translational sciences. In this sense, DO cohorts have been shown to reproduce phenotypic variability typically found in humans [33,172]. DO cohorts, from founder or CC strains with opposing phenotypes or gene polymorphisms, could be used to find what genetic traits are required for an individual to be responsive or non-responsive to a given treatment, which would ultimately contribute to advances in the field of precision nutrition. For example, tea polyphenols have been shown to provide protective effects against metabolic syndrome, obesity and hypertension, and this has been confirmed with different meta analyses [275,276,281,282]. However, although the general trend is to find these positive results, some studies do not report these beneficial effects. For example, some studies report lack of anti-hypertensive effects of

green tea [278,279,283]. The effect of tea on some parameters (i.e., modulation of blood glucose and triglycerides) are very variable and mixed, reporting both positive (protective) and negative results [275]. Thus, the beneficial effects of tea on those are not validated, potentially due to genetic factors. In this particular example, the use of DO and DO-derived (i.e., F1 or F2) cohorts could be useful to identify combinations of genetic variables responsible for tea responsive or non-responsive phenotypes. It warrants mention that use of large number of mice in DO/CC studies will necessitate implementation of a few very targeted, efficient, and high-throughput phenotyping methods. This information could then be validated in humans, and applied to stratify population groups to: 1) select individuals for interventional studies; and 2) recommend specific dietary guidelines to specific population subgroups. In clinical trials, subjects could be recruited and stratified *a priori* (ideally, to recruit specific polymorphisms into the study) or *post hoc* (less ideally) when performing a clinical trial. The potential outcomes would be: 1) more successful human intervention trials, with clearer effects of tea; and 2) direct application to human population to improve health. In a nutshell, what these types of approaches provide is strong data for translation sciences.

6. Conclusion.

Phytochemicals face significant obstacles for successful translation from preclinical mouse models to successful human clinical trials. One possible explanation for the reported challenges is that many promising preclinical findings may actually be artifacts of the specific genetic background or polymorphisms in specific genes in a few inbred strains, which are not broadly translatable to genetically diverse humans. Mouse models of genetic diversity have proven useful in areas of research closely related to phytochemicals (toxicology, pharmacology, obesity, cancer, immunology, etc.). Such models have identified genetic drivers of phenotypes and xenobiotic responses, identified mechanisms of action for xenobiotics, and suggested novel therapeutic targets. However, these mouse models have not yet been widely adopted by researchers studying phytochemicals. Two fundamental shifts in phytochemicals research could be implemented to improve the translational relevance of preclinical mouse data: 1) viewing phytochemical bioactivities as traits that are dependent on host genetics, and 2) implementing mouse models of genetic diversity to identify gene-phytochemical interactions, as well as novel phytochemical mechanisms of action and therapeutic target. Utilization of genetic diversity models for preclinical phytochemicals research is thus a logical, and much needed, experimental strategy in preclinical research pipelines to improve translation of phytochemical research into humans and enhance precision nutrition. Logical initial approaches include recruitment of geneticists to phytochemical research collaborations, and implementing founder strain studies to determine whether potential gene-phytochemical interactions exist (for specific phytochemicals and outcomes). Targeted DO, CC and knockout mouse approaches can then be used as described above, which will inform design of clinical trials with improved likelihood of success.

The potential insights gleaned from such approaches are likely to be game changers for implementing phytochemicals to improve human health.

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