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Modelling ageing and age-related disease

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An increased lifespan comes with an associated increase in disease incidence, and is the major risk factor for age-related diseases. To face this societal challenge search for new treatments has intensified requiring good preclinical models, whose complexity and accuracy is increasing. However, the influence of ageing is often overlooked. Furthermore, phenotypic assessment of ageing models is in need of standardisation to enable the accurate evaluation of pre-clinical intervention studies in line with clinical translation.

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Introduction

What has become clear over the last decade or so is that the concept that ageing is simply an accumulation of damage and physiological systems 'wearing out' are simplistic. Ageing processes are major contributors to the development of age-related disease [1–4] and can be modified, by interventions and genetically, across a range of organisms. Ageing can also impact on efficacy of disease treatments through changes in drug pharmacokinetic (PK) and pharmacodynamics (PD), which exerts its effects on therapeutic regimens. Whilst there is no unified 'theory of ageing' we are learning more and more about the physiological processes and the consequences of ageing and it is clear that this is a factor we should incorporate into modelling of disease and in preclinical studies. Within the context of such studies using aged models introduces several challenges including; increased frailty in older animals, limiting the number and types of assessment possible, increased difficulty in interpreting signs of ill health or humane endpoints, variability due to the differential rates of ageing in individual organisms, and the influence of environmental factors. In addition, models may need to be very complex to reproduce aspects of the multiple pathologies associated with frailty and multimorbidity. For this reason the standardisation of phenotypic assessment of animals and standardisation of study design is more critical than ever.

The importance of ``ageing'' in modelling age-related diseases

Mice are used to study ageing itself. For a review of how mice have been used in some studies of the ageing process please refer to Vanhooren and Libert [5]. Many disease studies do routinely incorporate ageing into their design, but many disease areas also use genetically modified organisms that have an early onset and/or acute phenotype when the disease burden is primarily a chronic or age-related condition. Examples of such models are the *Apoe* knockout mice [6,7] for the study of atherosclerosis and the *ob*/*ob* diabetic mouse [8]. While both have advanced our understanding of disease, these are extreme examples of disease with a rapid onset and do not necessarily recapitulate all aspects of human

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disease. Indeed as we gain a deeper understanding of the processes underlying age-related disease it is clear that we cannot model the complete range of phenotypes observed in patients in a single model, and it would be naïve to expect this to be so. We must therefore ensure there is a clear comprehension of the limitations and drawbacks of individual models as well as their similarities to symptoms observed in patients. Indeed the term model is often applied too freely and it should be acceptable to state that these are genetically modified organisms that aid our understanding of disease rather than labelling everything with moniker of model. This is exemplified by the recently published comment on mouse lines used to study amyotrophic lateral sclerosis (ALS) [9]. Although not an age-related disease it highlights the need for a complete understanding of disease in models and patients. As emphasised in this article, a commonly used 'model' of ALS, TDP-43 mutant mice, were found to die of a bowel obstruction rather than the progressive muscle atrophy seen in patients, thus severely limiting its usefulness in pre-clinical studies [10]. This work drives home the importance of understanding why data does not translate from putative disease models. It is not sufficient to say that mouse lines are not suitable; we must investigate if and why they are not suitable and understand any limitations, which may indeed ultimately aid our understanding of disease pathogenesis.

The rapid nature of the disease reproduced in some of these models also means there is also a limited window of opportunity for testing therapeutic interventions. In addition, because of the lack of an ageing physiology in such models, the influence of age on other aspects of therapy such as PK and PD are missing [11]. Drug toxicity is also a major problem in drug development and a significant proportion of this toxicity can be attributed to mitochondrial toxicity [12,13]. With ageing there is an accumulation of mitochondrial mutations and a concomitant decline in mitochondrial function [14,15], which could therefore sensitise the aged to toxic side-effects of drugs. Without proper pre-clinical testing in age–appropriate disease models this toxicity may go unnoticed. Even then mouse lines *per se* may have limitations as there may be differences between mitochondrial ageing between mice and humans [16]. Effectively, therapies are being tested in a worst case scenario in such models where there is a rapid and aggressive disease in young organisms rather than the chronic progression seen in older patients. As our understanding of disease in models and patients progresses, so we must refine our models accordingly. The cost of using ageing models in preclinical testing may be more than that currently encountered but these are insignificant when compared to the cost of failed clinical trials.

Using mice to study ageing, age-related diseases

As we age the risk of disease increases so surely the best murine model of age-related disease is an old mouse? Whilst some common age-related pathology can be recapitulated in mice simply by ageing mice those such as cataracts, sarcopenia, cancer, and tissue dysfunction is what is mostly observed in ageing mice. Therefore there is still a need for age-appropriate models of individual disease to recapitulate more complex aspect of age-related diseases. This is because age and age-related disease are not overlapping but rather can be seen as two parts of a multistep process [17] whereby ageing is the first step, leading to loss of tissue reserve and homeostasis and increased chances of developing one or more age-related diseases. Accumulation of multiple diseases and age-related loss of functions can result in frailty. Mechanisms of ageing such as chronic inflammation, senescence are interlinked and thought to exacerbate many age-related diseases such as atherosclerosis, neurodegeneration, and osteoarthritis [18]. Chronic inflammation can arise from obesity, with adipose tissue being an active inflammatory tissue [19], and as a result of cellular senescence whereby senescent cells release pro-inflammatory cytokines; the senescence associated secretory profile (SASP). In a model of chronic inflammation Jurk *et al.* demonstrated that persistent inflammation can in turn accelerate ageing via telomere dysfunction and cellular senescence [20]. However, not all patients get the same diseases in the same order. This is because there are other factors such as genetic components associated with specific pathways which contribute to susceptibility to disease. Consequently to push forward with personalised medicine we must understand the specific pathways contributing to disease. Specific models of disease will help our understanding of the specific pathways involved in individual pathologies, how they interact with the ageing process, and will also assist in the identification of disease biomarkers that predict later disease.

Using mice as models to improve age-related health outcomes

A key aim of ageing research is to translate this into beneficial interventions to reduce the burden of age related disease. An increase in longevity has already been shown to result in an improved healthspan [21]. However, longevity is not necessarily the best readout when considering disease parameters; an organism may live longer but still suffer from chronic diseases or disease may simply be delayed. Health span, defined as time free of diseases, is now considered a better measurement of the outcome of interventions and the main goal of ageing research is to contract the period of morbidity before death rather than just simply increase lifespan[22]. A recent study on the effect of rapamycin, a dietary restriction mimetic, demonstrated similar health benefits to dietary restriction but without a concomitant increase in longevity [23]. Whilst beneficial outcomes have been demonstrated from modulating ageing processes, this does not necessarily mean there is a generalised health benefit affecting all age-related diseases. Resveratrol treatment of mice on a high

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calorie diet resulted in increased life span but this was primarily due to a reduction in the incidence of pathologies associated with cardiopulmonary distress [23]. This is also underscored by a recent study by Neff *et al.* [24] who carried out an extensive phenotypic analysis of rapamycin treated mice to determine the influence of this treatment, known to promote longevity even when treatment begins late in life [25], on a range of disease phenotypes. Their conclusion was that although there was a significant increase in longevity and healthspan, this was primarily due to the reduction in one disease, cancer. Other beneficial effects of rapamycin occurred in young mice and therefore may not be directly related to ageing processes. Whilst the interpretations of these data [26] have been debated this study shows how critical thorough, and indeed longitudinal, phenotyping is to fully elucidate an age-related phenomenon.

As with all research reproducibility is critical to advancing research findings into the clinic, and one of the major contributing factors to the interpretation of data is standardisation of protocols. This is highlighted two major studies on caloric restriction (CR). These parallel studies on the effect of restricting CR have not resulted in a definitive conclusion as to the beneficial results of CR with the question remaining whether we are seeing the benefits of a healthier diet or a true beneficial effect of CR [27]. The data from the two macaque studies is none the less useful and informative and may indicate how different CR strategies can benefit different patient groups, but these studies are an example of how differences in protocols can result in profound differences in results, and that meta data such as diet, genetic background, and housing conditions should all be included in study design. There is further discussion on whether the benefits of caloric restriction in mice are universal [28]. The crux of the problem is similar to that encountered in the primate studies; is caloric restriction truly influencing the progress of ageing or is it simply treating the consequences of obesity [29]? Laboratory mice are quite sedentary and have a rich diet *ad libitum* so tend to become obese, especially as they age [30] and the increases in longevity and health span could simply represent a 'healthier lifestyle' rather than a true influence on the ageing process. Key to this is the correct reporting of detailed metadata including such things as body composition, genotype, diet, and age of mice, all of which have been shown to influence the outcome of caloric restriction studies. Inbred strains varying considerably in life span and disease susceptibility and as such some programmes utilise mixed backgrounds [31] and reviewed in [5]) to better represent the mixed genetic background present in patients. The use of complex strains does severely restrict the use of genetic modifications as these modifications may have to be introduced onto several strains or necessitate additional breeding steps. The NIA funded intervention testing programme makes every effort to standardise procedures across sites but despite this they noted site specific differences in their mouse studies [31].

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) have begun to address this by developing the ARRIVE guidelines to standardise reporting of animal research [32]. With the added systemic effects of ageing and the amplifying nature of extended studies it is even more critical that protocols and metadata are recorded in detail and made available to the community. Beyond standardisation of the phenotyping and housing conditions of the test mice it is also likely that we will have to consider other metadata such as health status an age of the mice used to breed test cohorts as there are clear transgenerational effects of diet and body composition on the health outcomes of offspring, particularly affecting metabolism (reviewed in [33]). The recently instigated MouseAGE cost action (http://www.cost.eu/COST_Actions/bmbs/BM1402) has the stated aim of developing such standardised protocols and methodologies for use in ageing studies.

Models of multimorbidity

Whilst mechanistic studies and delivery of interventions have been focused on individual diseases, over 60% of people aged over 65s have more than one disease at the same time and more than 20% have 5 or more [34]. Multimorbidity is the major challenge of the ageing population leading to reduced functional status, higher mortality, a poorer quality of life, and is the major expenditure of national healthcare budgets [35–37]. We know that the presence of one disease significantly increases the risk of developing another disease [38]. However, in studies to-date attempts to identify clusters of diseases occurring together with a higher frequency, other than by chance have yielded inconsistent results [39–41]. This is most likely due to the lack of standardisation, particularly the classification of the type and the number of diseases considered in the definition of multimorbidity. In addition, many studies have not been sufficiently powered to be conclusive. Therefore to better understand the pathophysiology of multimorbidity we need better clinical and preclinical studies.

Preclinical *in vivo* studies are very useful to develop a mechanistic understanding of the pathogenesis of associated diseases and to test interventions targeting multimorbidity as a whole. They rely on well characterised animal models, which should be able to develop progressive and time dependent diseases in later stages of life. This is very difficult to achieve at present. An example is described by the efforts of the Animal Models of Diabetic Complications Consortium (AMDCC). Diabetes is often associated with increased risk of cardiovascular disease [42,43]. The consortium aimed to develop new animal models that closely mimicked the human complications of diabetes, including cardiovascular disease. Epidemiological data has indicated that diabetes increases

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mortality due to atherosclerosis and/or cardiomyopathy arising independently of hypertension and coronary artery disease [43]. Diabetic cardiomyopathy (DCM) is defined primarily by diastolic dysfunction and hypertrophy in the left ventricle, leading to heart failure [44,45]. Indeed *ob/ob* and *db/db diabetic* mice develop cardiac hypertrophy, with contractile disturbances and sometimes increased chamber stiffness [46,47]. However, the development of CVD following atherosclerosis is more frequent in diabetic patients than CMD but mice are in general resistant to the development of atherosclerosis, mainly because of differences in lipoprotein metabolism [48,49]. Even after being fed with very high fat diets for 12 months, animals only develop early signs of atherosclerosis. Genetic modifications such as those in the ApoE$^{-/-}$ mouse or the low density lipoprotein (LDL) receptor–deficient mouse have been required to obtain atherosclerotic lesions [49–51]. However, crossing of *ob/ob* or *db/db* mice with ApoE$^{-/-}$ or on LDLR$^{-/-}$ mice alone or in combination [52,53] resulted in large increases in plasma lipid levels, which is not a feature of the human disease and makes it very difficult to dissect the individual contributions of hyperglycemia and hyperlipidemia in relation to the formation of atherosclerotic plaques. In addition one hallmark of atherosclerosis, present also in patients with type 2 diabetes is endothelial dysfunction, whose development precedes atherosclerosis and is characterised by accumulation of DNA damage and senescence [54,55]. However, this feature seems to be seen only in the aortas of aged *Apoe*$^{-/-}$ mice [56]. No such impairment is seen in C57BL/6J mice at any age, suggesting that a combination of genetic defect and age may be required to reproduce the full vascular phenotype. Interestingly, when ApoE$^{-/-}$ mice were inter-crossed with 4th generation (G4) TERC$^{-/-}$ mice to accelerate ageing and challenged with a high cholesterol high fat diet, the mice developed less atherosclerotic lesions compared with G4 TERC$^{+/+}$ ApoE2$^{-/-}$ mice [57]. In addition the atherosclerotic plaques were morphologically in a less advanced stages of development [57], suggesting that there are specific mechanisms of ageing underlining senescence and endothelial dysfunction and absence of telomerase activity is not involved in this process and seems to have a protective effect for atherosclerotic disease.

This example shows how complex the generation of models of associated pathologies can be. It needs an in depth knowledge of the pathogenesis in humans and generation of multiple crosses of genetically modified mice. These crosses needs to be thoroughly characterised to fully appreciate which aspect of the associated diseases they reproduce and what are their limitations. The notion that progeric mice may serve as model of multimorbidity just for the very fact that they present with accelerated ageing features is therefore simplistic. Each model requires a longitudinal phenotypic and rigorous pathologic analysis to determine the temporal and accelerated emergence of comorbidities with age later in life, together with mechanistic causal links which explain the development of the comorbidities.

Assessing frailty

In the last 50 years human life expectancy has been increasing every year mainly due to improved management of chronic, age-associated diseases (WHO, global health and ageing, http://www.who.int/ageing/publications/global_health/en/). However, life health expectancy, i.e. the number of years in one's life free of disease, has only increased by half compared to the increase in life expectancy, with the result that people live longer but suffer from an increased burden of disease and ultimately become frail [58]. The term ''frailty'' can be described as progressive accumulation of deficits across multiple systems with decreased resilience and resistance to stressors, causing vulnerability to adverse outcomes. Half of over 80 years old in the US is considered frail with poor quality of life and high costs for social and healthcare [58]. Therefore interventions which address frailty are urgently required. However, the translational path for the testing of interventions for frailty is not well defined to-date. For the approval of any new drug to be tested in patients the disease to be treated needs to be clearly defined and the definition has to be agreed at the international level, appropriate animal models needs to be available with a set of suitable measurements, which can detect improvements meaningful to patients.

At present there is not a commonly agreed definition of frailty, which is accepted by the regulators such as European medicinal Association (EMA) or the Food and Drug Administration (FDA). In patients a Frailty Index has been successfully applied to measure frailty and predict outcomes [59] and similar studies in mice have also identified useful parameters in gauging the overall health of an individual animal [60,61]. Parks *et al.* demonstrated that easily measured parameters resulted in a Frailty Index that correlated closely with more detailed measurements of cardiac dysfunction, a key disease of ageing [61]. Frailty was measured as how many standard deviations the individual mice varied from a reference range established using an adult, rather than aged, population and the cumulative score was used to determine the overall frailty of the mice. Interestingly although in this study they initially employed 31 parameters in their study as few as 8 parameters were sufficient to establish a Frailty Index. This Index was further developed to utilise parameters that can be measured easily in the majority of laboratories and requires little specialist equipment. Another index used parameters measured in patients to more directly correlate frailty in mice and humans [60]. However, it is unclear whether these indices are sufficiently sensitive to measure improvement following interventions, and whether these improvements translate in improved quality of life for the patients. One way forward is

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to focus on patients with a specific disease that is strongly associated with frailty, such as cancer cachexia and use this as an initial case to establish models and measurements suitable for drug testing which then can be translated in phase I clinical trial in patients. Cancer cachexia has several of the features of frailty with loss of weight and muscle mass [62], loss of resilience. Models of cancer cachexia may be recapitulated by using xenograft models in ageing mice rather than in young animals better reflecting the feature of the disease in humans. There are xenograft models, which are slow growing and can be treated with multiple rounds of chemotherapy over a long period of time [63,64]. They are used to study the development of resistance to chemotherapeutic agents. They would represent an excellent model to assess the onset of frailty using one of the frailty indexes available and to test whether they are sufficiently sensitive to detect improvement in performance following interventions, particularly in the case with geroprotectors such as rapamycin or metformin.

New approaches to develop models of age-related disease

Given the wide ranging impact ageing has on almost every physiological system, and the implications this has for disease, it is essential we build in an ageing component to the analysis of disease into our models. There is no doubt at all of the contributions that model organisms have made to our understanding of disease but to provide a better understanding of disease and ageing, and also to improve the translation of therapies from preclinical studies to bedside, ageing and its effects will have to be factored into many studies [65]. Phenotype-driven screens are discovery platforms that associate novel genes with phenotypes and identify novel functions of known genes. They have been successful across a range of disease areas and indeed, with the inception of the International Mouse Phenotyping Consortium (IMPC) (http://www.mousephenotype.org/), have now attained a global scale. The advantages of such approaches are standardised protocols, defined environmental conditions, a wide range of phenotyping data, and stable genetic backgrounds. At MRC Harwell we have undertaken the first large-scale phenotype driven screen to incorporate ageing as a challenge to identify genes and pathways that result in age-related disease. The programme is described in detail elsewhere [66] but briefly this is an ENU (*N*-ethyl-*N*-nitrosourea) based mutagenesis programme [67] where pedigrees of mice are aged to 18 months and phenotyped across a range of platforms. Even though this programme is in its early stages a number of age-related phenotypes have been identified across a range of disease areas including hearing, vision, osteoarthritis, neurodegeneration, and renal disease. These models are being characterised in detail and will be described elsewhere but to date over 100 phenotypes have been identified with a quarter of these not detected until six months or later. Several of the earlier mutations also have late onset additional phenotypes or progressed as a chronic condition. From a total of 16 cloned mutations identified to date that result in an age-related phenotype 15 revealed a novel gene function or associated a gene with a phenotype for the first time. These mutant lines provide preliminary support for the concept of an ageing challenge to reveal novel gene function gene function. An age-challenged pipeline has now been added to the latest phase of the IMPC.

Conclusions

With age-related disease we are facing a complex problem and simple models will not suffice. It is likely that multiple, complex models will be required in future studies, and that a convergence in study design is required; including ageing when studying disease and a greater range of phenotyping to assess health/disease/frailty/multimorbidity. Furthermore a standardised approach to protocols employed in the study of ageing and disease is required which may include a range of mouse lines and metadata on the health status and age of dams and sires of the offspring employed in these studies.

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