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EMBO WORKSHOP REPORT

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Introduction

Ageing is not only an important biological issue, but is also an important social and emotional issue that affects an increasing number of elderly people in industrialized countries. Understanding the molecular mechanisms underlying the physiological ageing process may ultimately lead to the understanding and prevention of age-related diseases. The EMBO Workshop on Molecular and Cellular Gerontology has offered an ideal opportunity to promote the awareness that Europe should increase its capacity for research into ageing, as well as to open the field for young molecular biologists. It has brought together delegates from 14 different countries representing 21 nationalities. The sessions covered a wide range of complementary research areas: molecular and cellular aspects and theories of ageing, molecular basis of the loss of homeostatic maintenance mechanisms, model organisms including fungi, worms, flies, transgenic animal models, genetics of human longevity, clinical and molecular aspects of age-related diseases, and integrated systems like brain and immune system (IS) ageing.

This workshop report presents some of the highlights of the meeting. We apologize to all participants whose contribution has not been specifically mentioned due to space limitations. The proceedings of this meeting will be published in detail (Toussaint *et al*., 2000).

Keynote lectures

In the first keynote lecture, George Martin (Seattle, WA) summarized the present concepts and research status, and gave an outlook to new directions for research on the biology of ageing. The ageing process is probably under the influence of genes and the environment (nutrition, lifestyle and chance) at a ratio of ~30:70. This explains the stochastic nature of many age-related alterations, a theme that came up in many subsequent talks and was stressed also by Tom Kirkwood (Manchester, UK) in the second keynote lecture (Finch and Kirkwood, 1999). Random accumulation of molecular and cellular damage and loss of repair functions are at the basis of the network theory of ageing (Kirkwood and Kowald, 1997). Intrinsic developmental chance may be another major factor contributing to the divergence of the senescent phenotype. Both speakers stressed the importance of combining highly reductionist analysis of molecular processes with integrative model systems as being the major challenge for the future in biological gerontology (for further reviews see Schächter *et al*., 1993; Lithgow and Kirkwood, 1996; Martin *et al*., 1996; Osiewacz, 1997; Kirkwood, 1998). These aspects were covered in more detail during the following days of this workshop.

Model organisms: from yeast, worms and flies to humans

The first day of the workshop brought together presentations on a complete spectrum of organisms utilized in experimental gerontology. The workshop participants were subject to a comprehensive review of molecular ageing research from yeast systems, through invertebrates, to human population genetic studies. In addition to review material, a great deal of new data were presented with many speakers choosing the workshop to present new and important findings for the first time.

All of the systems studied take organismal lifespan and mortality rates as a direct way of measuring changes in ageing wrought by environmental or genetic interventions. Genetic interventions in particular have provided a clearer picture of the determinates of ageing. Michal Jazwinski (New Orleans, LA) pulled together many of the most important concepts in the current debate on the physiological causes of ageing, those of genetic stability, metabolic control and stress resistance (Jazwinski, 1999). Much attention has been paid to the activities of RAS2 in determining the lifespan of the budding yeast, *Saccharomyces cerevisiae*. RAS2 influences many processes including cell division, stress resistance and transcriptional silencing. The gene also determines lifespan, and a new potential mechanism for longevity was presented. Earlier this year, Jazwinski published an account of the influence of the retrograde response on lifespan. In yeast, the retrograde response is defined as a signalling pathway from the mitochondrion to the nucleus in response to mitochondrial impairments (Kirchman *et al*., 1999). It leads to the activation of nuclear genes (e.g. *Cit2*, encoding peroxisomal citrate synthase) and is dependent on retrograde transcription factors Rtg1p, Rtg2p and Rtgp3 (Liao and Butow, 1993). Jazwinski reported that RAS2 modulates this response, e.g. by modulating the activity of retrograde transcription factors, and this may be the mechanism whereby RAS2 influences yeast ageing rate.

Yeast is not the only fungal species being utilized in ageing research. Heinz Osiewacz (Frankfurt, Germany) presented the explanatory power of the *Podospora anserina* model (Osiewacz and Kimpel, 1999). This filamentous fungus is at the forefront of studies that demonstrate the importance of the mitochondria to lifespan determination (see below).

Shin Murakami (Boulder, CO) and Gordon Lithgow (Manchester, UK) presented the *Caenorhabditis elegans* system (Lithgow, 1998). This organism has proved highly tractable for ageing studies principally due to the identification of single gene mutations that extend mean and maximum lifespan (Age mutations). These mutations have been utilized to construct genetic pathways and the mutated genes have been cloned. As a result, we know more about genetic influences on ageing in *C.elegans* than in any other species. Both presentations on this worm related the relationship between stress resistance and ageing with a strong emphasis on signal transduction pathways that determine ageing rate. However, both presentations went beyond the simple isolation of Age mutants and demonstrated interventions in ageing incorporating transgenic manipulations and pharmaceutical agents.

Murakami presented many novel findings centred on altering the expression of a tyrosine kinase receptor encoded by a gene called *old-1* (previously *tkr-1*). Overexpression of *tkr-1* by trangenesis results in an extension of lifespan by 65% and resistance to heat and UV irradiation (Murakami and Johnson, 1998). The human fibroblast growth factor receptor (FGFR) shares homology to worm *trk-1*, and Murakami demonstrated that the kinase domain of human FGFR would functionally complement the worm gene. Murakami also utilized RNA interference for the first time in an ageing experiment with clear and carefully controlled results.

Lithgow presented a genetic screen for novel Age mutations by first screening for genetic variants with enhanced resistance to heat shock. Such a screen is much faster than conventional screening for Age mutations, and evidence was presented for novel mutations extending lifespan (Walker *et al*., 1998). Lithgow also showed that some of the known Age mutations that occur in genes encoding an insulin-signalling pathway confer overexpression of stress response genes. Both heat-shock protein (HSP) genes and metallothionine genes are overexpressed in Age mutants, leading to the hypothesis that stress genes affect lifespan. This hypothesis was tested directly, and indeed overexpression of HSP-16 results in extended lifespan. Work in our laboratory also demonstrated that lifespan could be dramatically increased by treating worms with drugs that act as catalytic antioxidants. These drugs are mimetics of superoxide dismutase (SOD) and catalase, and this result illustrates that oxidative stress plays a significant role in determining *C.elegans* lifespan.

Another major genetic experimental system, *Drosophila melanogaster*, was represented by two talks that demonstrated the full potential of the system (for reviews see Brack *et al*., 1996; Tower, 1996). John Tower (Los Angeles, CA) discussed the current state of the art in transgenic manipulation. Tower has developed a modified doxicyclin-inducible expression system for studying the effects of antioxidant and heat-shock protein genes on ageing. He has shown that overexpression of the cytoplasmic Cu/Zn SOD or the mitochondrial MnSOD significantly extends lifespan. This represents very good evidence that oxidative stress plays a direct role in the ageing of *Drosophila*.

The second presentation on *Drosophila* highlighted another strength of the model system, that of being able to maintain large outbred populations (Partridge *et al*., 1999; Sgro and Partridge, 1999). A number of previous studies have demonstrated that selection for late reproduction in such populations results in a lengthening of mean and maximum lifespan. Linda Partridge (London, UK) has demonstrated for the first time that such a lifespan extension is only seen between lines that are fertile. Partridge cross-selected long-lived lines and control lines into a sterile genetic background (ovoD) and discovered that mortality rate differences disappeared. This is consistent with evolution theory, which predicts that genes selected for early life processes such as reproduction will also detrimentally affect the organism in late life. This is some of the first direct evidence that such trade-off associated with ageing genes occurs as a result of the cost of reproduction.

Whether mechanisms of ageing are conserved from species to species is one of the most important questions on ageing research at present as it is hoped that the progress being made in model systems will be of value in addressing age-related disease. Two talks addressed the progress being made in primate ageing. In the first, George Roth (Baltimore, MD) outlined the current status of the major primate studies on calorie restriction (for reviews see Roth, 1997; Roth *et al*., 1999). Extensive molecular, biochemical and physiological analysis indicates that primates respond to calorie restriction in a manner similar to rodents.

In the second talk, Eline Slagboom (Leiden, The Netherlands) presented an overview of mapping late-acting genes that contribute to human disease. Of particular importance are efforts to identify susceptibility genes for osteoporosis, osteoarthritis, diabetes and dementia. Emphasis was put on the great value of sib pair analysis, twins and centenarians in these population genetic association studies. François Schächter took up some theoretical aspects of the genetics of human ageing and interpreted them on the basis of the concept of compensatory adaptation. Compensatory adaptation refers to the reorganization of metabolic pathways and coordinated functions in reponse to primary ageing and to external stresses (Schächter, 1998).

Stress-induced premature senescence (SIPS) and molecular scars

The session on replicative senescence and oxidative stress highlighted two major concepts. First, the concept of SIPS arose from studies on different *in vitro* systems where various cell types such as human diploid melanocytes or fibroblasts are exposed to sublethal stresses (UV, hyperoxia, *tert*-butylhydroperoxide, hydrogen peroxide, etc.) (for review see Toussaint and Remacle, 1996).

Replicative senescence is reached when diploid fibroblasts and other proliferative normal cell types reach exhaustion of the replicative potential. This *in vitro* model

is convenient for studying irreversible growth arrest. In the ageing organism, however, accumulation of senescent cells might be very slow. Nevertheless, the exposure of cells to repeated or chronic non-lethal stresses may lead to an accumulation of stress-induced senescent cells. It was suggested at the Workshop that local accumulation of these cells might be responsible for alterations of the tissue function and differentiation status of neighbouring cells. The stress-induced senescent cells could also be removed by immune cells or undergo apoptosis, thereby participating in tissue ageing through modifications of the extracellular environment, creation of a micro-inflammatory state or activation of neighbouring cells.

Using the melanocyte model, Estela Medrano (Houston, TX) proposed that the upregulation of the cyclin-dependent kinase inhibitor $p16^{INK-4}$ may be essential for maintenance of terminal growth arrest. She speculated that reactive oxygen species, generated during the process of melanin synthesis, may lead to SIPS of heavily pigmented melanocytes, in addition to UV-induced SIPS (Medrano *et al*., 1999). Failure to respond to this stress-induced senescence programme may favour immortalization, a step required in the development of melanomas. Thomas von Zglinicki (Berlin, Germany) presented data obtained from a model of long-term hyperoxia during which cells accumulate strand breaks leading to increased rate of telomere shortening. When cells are treated with G-rich telomeric oligonucleotides, they undergo a p53-dependent growth arrest, the duration of which might be dependent on the presence of telomerase (Petersen *et al*., 1998). Olivier Toussaint (Namur, Belgium) and Qin Chen (Tucson, AZ) presented new data on SIPS induced by *tert*-butylhydroperoxide and H_2O_2 (Dumont *et al*., 2000), which suggest that the retinoblastoma protein plays a fundamental role not only in the regulation of the cell cycle in SIPS but also in the control of the appearance of biomarkers of replicative senescence (morphology, gene expression) triggered by exposure of human fibroblasts to these stressors.

The second concept highlighted in the session was concerned with molecular scars, identified as a result of the comparison of the proteomes of normally senescing human fibroblasts and human fibroblasts undergoing SIPS after sublethal stresses of *tert*-butylhydroperoxide or ethanol. Highly reproducible results arose from the analysis of six replicates of high-resolution two-dimensional gels of proteins labelled 2 days after the stresses. This analysis of the long-term response to sublethal stresses shows that beside changes common to normal senescence and SIPS, various proteins undergo expression changes specific either to normal senescence or to the long-term stress response (O.Toussaint, Namur).

Age-related changes in cellular repair systems

Many essential maintenance and repair systems become defective in senescent cells and organisms (for review see Toussaint and Rattan, 1996). Jan Hoeijmakers (Rotterdam, The Netherlands) proposed a link between endogenous DNA damage, transcription and premature ageing revealed by trichothiodystrophy (TTD) transgenic mice bearing defects in the nucleotide excision repair (NER) of DNA (de Laat *et al*., 1999). The NER apparatus contains two subpathways: global genome (GG)–NER covering the entire genome, and transcription-coupled (TC)–NER for fast repair of transcription-blocking lesions. TTD mice are defective in TC–NER and reflect various signs of premature ageing that are dramatically enhanced in mice bearing complete TC– and GG–NER defects. Similar observations with mutants for *CSA* and *CSB* genes suggest that the Cockayne syndrome symptoms of premature ageing are due to accumulation of DNA damage interfering with transcription and contributing to the dramatic features of ageing of the double mutants. Similar observations were obtained with mutants of the *ERCC1* NER gene.

Progress was reported on the role of poly(ADP-ribosyl)-ation in genomic instability and longevity (Bürkle, 1999). Alexander Bürkle's laboratory (Heidelberg, Germany) isolated stably transfected hamster cells overexpressing poly(ADP-ribose) polymerase (PARP) 5-fold upon induction. In these transfectants, PARP strongly suppressed alkylation damage-induced sister-chromatid exchanges. These results provided evidence that PARP may be a factor responsible for tuning the rate of genomic instability events provoked by constant attack of the genome to a level just appropriate for the longevity potential of a given organism or species.

Efstathios Gonos (Athens, Greece) focused on the molecular genetics of human ageing and longevity. His laboratory has cloned several genes that associate with mammalian ageing *in vitro* (Derventzi *et al*., 1996). Current work is focusing on fibroblasts and lymphocytes from centenarians in order to identify the genetic and biochemical parameters that associate with human longevity (Gonos and Franceschi).

Among the cellular repair and maintenance systems, the proteasome is a key element in charge of protein turnover and degradation of altered, damaged and ubiquitylated proteins (for review see Grune *et al*., 1997). Bertrand Friguet (Paris, France) put the emphasis on the age-related decline both in proteasome peptidase activities and proteasome content in different tissues such as rat liver and human epidermis. They may contribute to the accumulation of oxidatively modified proteins that increase with ageing and in certain age-related diseases [e.g. in Alzheimer's disease (AD) brains].

Dimitris Kletsas' group (Athens, Greece) has developed an *in vitro* system (3D gel of polymerized collagen) mimicking the *in vivo* tissue conditions to study the interplay of cells, growth factors and extracellular matrix components involved in wound repair (for review see Stathakos *et al*., 1996). They show how the interplay of growth factors (paracrine and autocrine) with the extracellular environment regulates cell proliferation. Furthermore, they confirmed that the ratio of the secreted collagenases and their inhibitors (TIMPs) is altered during ageing, which is probably relevant to the quality of the wound repair process in the elderly.

Mitochondria, mitochondrial DNA and oxidative stress in ageing

Several contributions focused on the role of mitochondria and mitochondria–nuclear interactions in ageing of different biological systems ranging from simple organisms to mammals. The retrograde response pathway in yeast, which is signalling the metabolic state of mitochondria to the nucleus (M.Jazwinski) has been mentioned above.

In another fungal model, the ascomycete *Podospora anserina*, mitochondrial oxidative stress is the result of dysfunctional mitochondria that accumulate during ageing (for review see Osiewacz and Kimpel, 1999). The accumulation appears to be due to electron leakage at the inner mitochondrial membrane and the formation of reactive oxygen species (ROS). These aggressive components lead to damage of macromolecules, thereby increasing the generation of ROS. In older cultures, due to an almost quantitative reorganization of the mitochondrial DNA (mtDNA), compromised mitochondria cannot be replaced by functional ones. As a consequence, senescent strains of *P.anserina* die because of energy deficits. Analysis of long-lived nuclear mutants demonstrated clearly the role of nuclear–mitochondrial interactions in the control of lifespan in this organism. Heinz Osiewacz (Frankfurt, Germany) reported on the long-lived mutant *Grisea*, in which the uptake of copper is affected, leading to the stabilization of the mtDNA and an increase in lifespan by 60%. Growth of the mutant in a copper-supplemented medium rescues the wild-type phenotype including the characteristic age-related mtDNA reorganizations and the shorter lifespan. These results indicate that the molecular machinery leading to mtDNA rearrangements is copper dependent, probably mediated by a component containing this transition metal as a cofactor.

Calorie restriction is one of the few regimes that positively influences most aspects of ageing in all organisms tested so far. Brian Merry (Liverpool, UK) reported on the positive effect calorie restriction has on the lifespan of rodents, Rhesus monkeys and squirrel monkeys. In rats, calorie restriction alters the rate of ageing, resulting in life extension. When these calorie-restricted animals are refed normal diet, ageing is again accelerated. Calorie restriction leads to reduced production of mitochondrial ROS and thus to a reduction in mitochondrial oxidative stress. Mitochondria from calorie-restricted animals show alterations in the mitochondrial membrane composition and as a consequence a reduction in electron leakage and mitochondrial ROS production. Most strikingly, this type of adaptation is similar to that found constitutively in mitochondria of pigeons, which are characterized by a similar metabolic rate to calorie-restricted rats but have a significantly greater maximal lifespan. Up to now no common mechanism for the effect of calorie restriction can be postulated. Depending on the tissue or organ examined, calorie restriction affects oxidation of lipids, DNA or proteins to different extents.

A different approach was taken by Simon Melov (Novato) to investigate the significance of mitochondrial oxidative stress in mammals (for review see Melov *et al*., 1999a). He summarized experiments on genetically modified mice carrying a mutation in a protein of the mitochondrial protective system against oxidative stress, the mitochondrial superoxide dismutase (MnSOD). Knock-out of the MnSOD gene is lethal, leading to severe defects in the heart, brain, skeletal muscles and liver. Treatment of these mice with specific synthetic antioxidants can cure the defects at least partially, giving rise to greatly increased lifespan. The SOD/catalase mimetic drug Euk-8, for example, can cross the blood–brain barrier and rescue the spongiform encephalopathy in MnSOD knock-out mice. Comparison of antioxidant-treated wild-type and knock-out mice by differential display microarrays reveals a number of differences in gene expression.

The role of mtDNA mutations in human diseases and ageing was summarized by David Cottrell (Newcastle, UK). MtDNA mutations have been characterized in >100 pathological defects (for reviews see Linnane *et al*., 1998; Esposito *et al*., 1999; Wallace, 1999; Wallace *et al*., 1999). Both point mutations and deletions of mtDNA are known to be of relevance. The major feature of mtDNA-associated human diseases is the presence of cells with a low activity of cytochrome oxidase, which was demonstrated by a histochemical technique. Cottrell reported on recent investigations of mtDNA deletions in the central nervous system, indicating that cytochrome oxidase negative neurones accumulate with age in the hippocampus. This class of neurones appears to be present at even higher number in brains from patients with neurodegenerative disease like Alzheimer's disease (AD). PCR analysis made on mtDNA of individual cytochrome oxidase negative neurones revealed clonal expansion of single mtDNA mutations (Melov *et al*., 1999b). Similar analyses were performed on other postmitocic tissues: individual muscle fibers analysed by PCR show that mtDNA mutations are specific for each individual and each tissue (Kovalenko *et al*., 1998), confirming the hypothesis that accumulation of mitochondrial mutations with age is a stochastic process (Tony Linnane and Sergej Kovalenko, Melbourne, Australia).

Giovanna De Benedictis (Arcavacate-di-Rende, Italy) reported on extensive population genetic studies of mtDNA ageing (De Benedictis *et al*., 1999). They demonstrate that mitochondrial haplotypes differ in different sub-populations (e.g. AD patients, centenarians), suggesting that the mitochondrial genome is a susceptibility locus in complex traits like successful or unsuccessful (for review see De Benedictis, 1996).

IS ageing, brain ageing and neurodegeneration

Two sessions were dedicated to ageing of complex organ systems: the brain (Ball and West, 1998) and the IS (Franceschi *et al*., 1998; Grubeck-Loebenstein and Wick, 1999). AD being one of the most important age-related neurodegenerative diseases affecting 20 million people world-wide was the focus of the session on brain ageing. Extensive stereological studies performed by Mark West's group (Aarhus, Denmark) addressed the question of whether sporadic AD represents an accelerated form of ageing, or whether it is a true disease. These studies confirmed his earlier findings (West, 1990): a massive cell loss in specific brain regions is characteristic for AD brains but not for control ageing brains. The CA1 region of the hippocampus is a hotspot for neuronal cell loss in early AD. Correlation of minimental state and cell number in the CA1 allows prediction of the total cell number in this brain region in early AD. Matthias Staufenbiel (Basel, Switzerland) reported on the latest progress on transgenic mouse models of AD (Sturchler-Pierrat *et al*., 1997). Mice overexpressing the human *APP* gene with the AD-linked Swedish double mutation (APP 23 mouse) show many characteristic pathological features of AD such as a high load of β-amyloid plaques that increase with age, which are surrounded by cells indicative of inflammatory

processes and that are associated with other AD specific proteins (ApoE, hyper-phosphorylated tau), as well as accumulation of cholinesterase in dystrophic neurites in the vicinity of plaques. Interestingly, stereological analysis shows a significant loss of neurones in the hippocampal CA1 region. Similar to the human AD brains, the neurone number in APP 23 mice negatively correlates with plaque load and is specific for the hippocampal CA1 region (Calhoun *et al*., 1998). Crossing these mice with a transgenic line carrying the AD-linked presenilin mutation, but not with lines carrying wild-type presinilins, enhances the AD-specific pathologies. These APP transgenic mice are, therefore, excellent tools to study the contribution of Aβ to AD pathogenesis (for review see Sturchler-Pierrat and Sommer, 1999).

One of the highlights of the meeting was the talk of Fred van Leeuwen (Amsterdam, The Netherlands) who reported further progress on molecular misreading, a novel type of RNA mutations that accumulate during ageing. As a result of dinucleotide deletions in GAGA repeats the reading frame shifts to the +1 frame resulting in so-called +1 proteins. Molecular misreading was first discovered in neuronal proteins, notably proteins linked to AD: β-amyloid precursor protein, APP, and ubiquitin-B (Ubi) (van Leeuwen *et al*., 1998a,b). Immunoreactivity to these APP+1 and Ubi+1 proteins was found in the cortex of sporadic AD, but also in brains of other neurodegenerative disorders (Down's syndrome, Pick's disease, argyrophilic grain disease, partial Huntington's). As these +1 proteins are also found in elderly, non-demented controls, but not in young brains, molecular misreading may act as a general ageing factor. Since dinucleotide repeats exist in almost every gene (e.g. other genes associated with AD: presenilins, tau, ApoE4), and since +1 proteins have also been detected in proliferating cells, molecular misreading can be regarded as a general source of transcription errors that may be involved in cellular derangements in age-related pathologies, including cancer. The exact mechanism of RNA misreading is not yet clear. Van Leeuwen postulated the existence of an mRNA surveillance system involved in the degradation of nonsense RNA, the accuracy of which decreases with ageing. It will be important to show whether misreading also occurs in proteins of the transcription and translation machinery, as these provide the positive feedback loop postulated in Leslie Orgel's error catastrophe theory in 1970 (Orgel, 1970).

The IS is one of the best studied ageing models in humans. As pointed out by Claudio Franceschi (Bologna, Italy), the IS presents a good example of constant remodelling and adaptation of cells during ageing. Franceschi discussed evolutionary aspects of immunosenescence, suggesting that antigens are chronic stressors which constituted a major selective pressure in IS emergence and evolution (Franceschi and Ottaviani, 1997). Whereas the network of ancestral defence mechanisms (natural immunity, inflammation, stress response) centred around the macrophage is well preserved or upregulated during ageing (particularly prominent in centenarians), the evolutionarily more recent clonal immune cells (T and B lymphocytes) are subject to age-related changes. The Italian studies with centenarians suggest that the proinflammatory status in healthy elderly and centenarians is elevated and that IL-6 plasma levels correlate with risk of death. George Wick summarized the most prominent age-related diseases of the IS, discussing the putative value of biomarkers like changes in IL-2R expression, increase in autoimmune antibody titres and antibodies to heat-shock proteins (hsp65) that might play a role in arteriosclerosis (Wick *et al*., 1999).

Conclusions and future perspectives

During the final round table discussions the various model systems were evaluated and their advantages/disadvantages for progress in molecular gerontology were pointed out. Lively discussions aimed at developing new strategies for future co-operation between European laboratories, and political and social aspects of research into ageing were debated. Two general statements were agreed upon by most participants. First, almost all aspects presented at this workshop deal in one way or another with oxidative stress: is it the most common mechanism of ageing? In his concluding sum-up of the workshop, Suresh Rattan (Aarhus, Denmark) pointed out that although severe stress certainly accelerates ageing, low levels of (repeated) stress—a state called hormesis—become stimulatory for healthy survival (Rattan, 1998). Thus mild irradiation, calorie restriction, heat shock, or a low pro-oxidative state improve repair and defence mechanisms, by stimulating the so-called longevity assurance genes. As to the question of increasing human longevity, there was general assent that the goal of molecular gerontology should be to improve the health of the elderly population and not to increase the human lifespan. Because ageing research is really a multidisciplinary task, workshops like the present one offer an ideal platform for interaction between molecular biologists, cell biologists and clinicians.

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