Old Herborn University Seminar Monograph

32. AGEING AND THE MICROBIOME

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INTRODUCTORY LECTURE: SOME INSIGHTS ABOUT AGEING FROM A SHARK AND AN OYSTER

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The subject of the 32nd Old Herborn Seminar is on the impact of ageing on the microbiome. In this brief introductory lecture I would like to explore the subject of ageing. What underlies the ageing process? In general, ageing is a process that occurs as the regenerative potential of the organism diminishes. As a consequence, the functions of vital organs gradually fail. Why does this happen? Is there an ageing "clock" in the human body? Are we composed of cells that are genetically programmed to count the hours until their senescence is reached? Or is the rate of ageing controlled by a "master clock"? Can we slow down the ageing clock?

Perhaps if we examine some long-lived animals we might discern some hints about the ageing mechanism. One of the oldest known animals is a clam collected off the coast of Iceland (*Scourse* et al., 2006). Based on the "rings" of the shell of this animal, it was estimated to be about 500 years old, and called the "Ming Clam" to highlight that it began its life during the Chinese Ming dynasty. Other clams caught in that area were similar in age, suggesting that the Ming was not an unusual outlier. Clearly these individuals survived predation. As extraordinary they survived infection and malignancy, despite (or because of?) the absence of an adaptive immune system. This animal teaches us that a robust adaptive immune system is not necessarily required for longevity.

The Greenland shark was recently discovered to be the oldest living vertebrate, with the maximum age estimated to be 600 yrs. (*Nielsen* et al., 2016). The ages of the animals were determined by radiocarbon dating of the lens of the eye, which is formed during embryogenesis. Prior studies had shown that the Greenland shark reached sexual maturity at about 200 years. The ageing clock of this animal appears to run about 10-fold more slowly than the human's. This animal teaches us that tissue renewal and organ regeneration, including reproductive capacity, can be sustained in a vertebrate for hundreds of years.

In 1961 Hayflick and Moorehead proposed that an animal's longevity had its basis at the cellular level (*Hayflick* and *Moorehead*, 1961). They demonstrated that the fibroblasts from short lived animals would undergo fewer divisions in cell culture before reaching senescence compared with the cells of longer-lived animals. It was later suggested that the number of cellular divisions a cell was capable of sustaining was dependent upon the integrity of the chromosomal telomeres, and the expression of telomerase. With each successive division, the telomeres of the stem cells shortened, until chromosomal replication was compromised.

Subsequent studies failed to validate the relationship between the longevity of a species and the number of divisions to senescence. A rather telling study asked whether the fibroblasts

grown from a biopsy taken from a 90+ year old human would undergo senescence after fewer divisions *in vitro* than those from younger individuals (*Maier* et al., 2007). The results demonstrated that the fibroblasts from the nonagenarians behaved very much like young cells with respect to the maximal number of replications. These observations suggest that the replicative capacity of a stem cell is influenced by factors contributed by the *in vivo* setting. What anti-senescence factors are present *in vivo*, but absent *in vitro*? Might a gradual decrease in "anti-senescence "hormones" be responsible for ageing?

Caloric restriction has been shown to increase longevity in flies, worms, fish and mice (*Balasubramanian* et al., 2017), so better understanding of the physiological consequences of caloric restriction could provide some insight into the processes influencing longevity: IGF-1, insulin, and growth hormone concentrations fall in serum (*Bartke* and *Westbrook*, 2012; *Cady* and *Sadagurski*, 2017); plasma ketone bodies increase, providing the brain with a highly efficient source of energy, reducing its dependence on glucose; and most curious, reduced inflammation within the hypothalamus (*Cady* and *Sadagurski*, 2017).

A recent study suggests that progressive hypothalamic inflammation might be a key factor in systemic ageing (*Zhang* et al., 2013). In this study, a lentivirus expressing GFP driven by an NF-κB response element was injected to either the hypothalamus or the cortex of mice. As the animals aged, the intensity of GFP+ cells increased in the hypothalamus, but not in the cortex, demonstrating that the hypothalamus experiences an inflammatory milieu in contrast to the cortex. The hypothalamus has a central role in homeostatic regulation of metabolism, vital functions (temperature, blood pressure, etc), and growth, so inflammation could impact negatively on function. Indeed, in this study numerous hypothalamic peptides were examined to determine which, if any, changed in the older animals, and GnRH, which decreased, was identified as a candidate. Surprisingly, treatment of the older animals with parenteral GnRH stimulated neurogenesis in the brain. Most surprising GnRH treatment increased muscle strength, skin thickness, and cognitive functions, in a sense, restoring a more youthful phenotype.

So what controls ageing? Does inflammation within the hypothalamus drive generalized ageing of our bodies? Is it all that simple?

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PHYSIOLOGY OF HUMAN AGEING

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SUMMARY

The study of human ageing physiology is limited by confounders such as concomitant disease, as well as the role of environmental factors and interactions with the physical world. The objective of this chapter is to review human physiological ageing through primarily a systems-based approach, with consideration for the geriatric syndromes (multifactorial pathophysiology), resiliency, and common ageing mechanisms that may underlie declines in multiple physiologic systems. Each system has its own trajectory for age-related dysfunction with accompanying system-specific declines. There are a growing number of examples of factors that regulate the resilient responses to stressors and mitigate potential adverse physiological consequences. Common mechanisms that contribute to human age-related physiology include chronic, low-grade, non-microbial inflammation, cellular senescence, accumulation of damaged macromolecules (DNA, proteins, carbohydrates, lipids), and stem and progenitor cell dysfunction. Ageing physiology in humans is heterogeneous and almost always interdependent with other systems and extrinsic influences.

INTRODUCTION

The study of human ageing physiology is confounded by chronic diseases not easily distinguished from primary ageing processes and for which ageing is the major risk factor. Nevertheless, and to the extent possible, ageing physiology will be reviewed as a systems-based approach, highlighting the major or key ageing features seen in healthy individuals. For many system-based ageing changes, the aetiologies are multifactorial or syndromic, and the geriatric syndromes such as pressure ulcers, incontinence, falls, dementia, and osteoporosis are good examples of the clinical consequences due predominately to ageing physiology in skin, the genitourinary system, muscle, central nervous system, and bone, respectively, but also secondary to contributions from physiologic ageing in other systems and extrinsic factors (e.g., polypharmacy, amount of daily activity, etc.).

Human ageing physiology is heterogeneous among individuals, lending itself to the descriptors of "normal" and "successful" ageing and supporting the concept of resiliency in the case of the latter. Resilience to physiologic ageing has been demonstrated across several systems, although its description in humans, outside of psychological and behavioural studies, is relatively limited (*Hadley* et al., 2017). Common mechanisms contribute to ageing physiology in animal models, and control of these mechanisms likely mediate resistance

effects. In humans, early evidence suggests that the same common ageing mechanisms are at play (*Kirkland*, 2016).

LIMITATIONS TO STUDYING HUMAN AGEING PHYSIOLOGY

Declines with age in physiological systems are commonly due to the effects of disease superimposed on fundamental ageing processes. A major challenge therefore is to distinguish the effects of ageing from disease. In humans, this can only be approximated by studying healthy individuals, tissue donors, those who experience non-disease related sudden death, or probably less helpful, autopsied individuals where the circumstances of death can be obtained. With this caveat, the physiology of system-specific human ageing is often considered to reflect primary process(es).

Superimposed on primary ageing processes, are environmental factors and interactions with the physical world that may regulate the degree and pace of these processes. For example, barrier facilities for animal models limit understanding of ageing processes influenced by the microbiome and immunological responses to environmental pathogens, which are applicable to animals in the wild and to humans. Behavioural factors, which rely on interactions with individuals, are also relevant since purposeful living, social networks and support systems influence health outcomes and mortality risk (*Boyle* et al., 2010a,b; *Holt-Lunstad* et al., 2010; *Park* et al., 2014).

Death is a poor endpoint to study ageing. Mortality is tightly linked to disease state and accidental death where ageing physiology is obscured by disease pathology and events unrelated to ageing, respectively. Furthermore, in the wild most animals will never achieve longevity close to their maximum life span because of death due to accidental reasons and to predation.

Finally, a specific physiological system may be dependent on multi-system interactions, and so dysfunction in one system may be related to dysfunctional processes in another or multiple systems. This relationship is true in disease states such as cardiopulmonary, cardiorenal, and hepatorenal syndromes, where dysfunction due to disease in one system contributes to the progression of disease in another system. The geriatric syndromes represent the equivalent dysfunctional outcomes due to ageing in one system being influenced by ageing in other systems and/or multiple age-related aetiologies that may be intrinsic or extrinsic to the individual. Therefore, examination of age-related changes in a single physiological system in isolation is confounded by the contributions of ageing in interacting systems.

DISRUPTION OF PHYSIOLOGIC RHYTHMS WITH AGEING

With ageing, disruption of circadian patterns cause phase advances (1-2 hours earlier) such as in the 24-hour body temperature trough and sleep onset. Suprachiasmatic nucleus neuronal loss in the hypothalamus results in attenuated pulsatile secretions and decreases in gonadotropins, growth hormone (GH), thyrotropin, melatonin, and adrenocorticotropic hormone (ACTH) (*Veldhuis*, 1997). There is also a delayed reset to a new photoperiod with ageing (*Hofman* and *Swaab*, 2006).

AGE-RELATED CHANGES IN THE GASTROINTESTINAL SYSTEM

Oropharynx

With ageing, there is a decrease in acinar cells and saliva production that is accompanied by thinning of the oral mucosa (*Smith* et al., 2013). These changes contribute to dry mouth complaints, recession of gums over time, and the increased risk of root carries. Loss of teeth promotes less effective mastication. Abnormal transfer of the food bolus to the pharynx (due to decreased oesophageal muscle compliance) is a major cause of inadequate nutritional intake in edentate individuals and places them at increased aspiration risk (*Fulp* et al., 1990; *Frederick* et al., 1996).

Oesophagus

There are four pathophysiological processes that occur in the oesophagus during ageing, including hypertrophy, nerve loss, decreased contraction/tone, and decreased sensation. Skeletal muscle hypertrophy of the upper one-third of the oesophagus carries no functional consequences (*Hall* et al., 2005). There is loss of myenteric ganglion cells which cause a decline in the amplitude of oesophageal contractions during peristalsis, but this does not substantially cause impairment of food movement (*Hall* et al., 2005). However, major deficits in secondary oesophageal contractions and a decrease in lower oesophageal sphincter tone causes increased gastric acid exposure (*Kekki* et al., 1982; *Feldman* et al., 1996; *Hurwitz* et al., 1997; *Haruma* et al., 2000,). Impaired sensation of distension at the distal oesophagus contributes to loss of symptomatology from injury (*Lasch* et al., 1997).

Stomach

Acidification of gastric contents with ageing is intact in the basal unstimulated state; however, there is an increased prevalence of *H. pylori* infection (*Marshall*, 1994; *Haruma* et al., 2000). Higher rates of gastritis, decreased production of prostaglandins, bicarbonate, and non-parietal fluid in the setting of decreased gastric emptying and microcirculation lead to impaired mucosal defences (*Guslandi* et al., 1999; *Tarnawski* et al., 2014). There is also a greater susceptibility to injury, with impaired healing and reduced efficacy of ulcer-healing drugs (*Tarnawski* et al., 2014).

Small intestine

Moderate villus atrophy and decreased absorption of micronutrients (e.g., calcium, folic acid, vitamin B12) with ageing are accompanied by an increase in bacterial overgrowth (*Parlesak* et al., 2003; *Salles*, 2007). Loss of innervation, especially of sensory and myenteric nerves, result in a higher incidence of painless ulcers (*Hilton* et al., 2001).

Large intestine

Mucosal atrophy, atrophy of the muscularis externus and altered coordination of contraction contribute to decreased colonic motility and the much higher rates of constipation with ageing (*Bitar* and *Patil*, 2004). Loss of myenteric plexus and intrinsic sensory neurons and concomitant lower anal sphincter tone lead to faecal incontinence (*Wade* and *Hornby*, 2005). Hypertrophy of muscularis mucosa with atrophy of the muscularis externus predispose to diverticuli (*Comparato* et al., 2007; *Commane* et al., 2009). Barrier function of colonic epithelium is compromised with ageing (*Tran* and *Greenwood-Van Meerveld*, 2013).

Hepatobiliary system

Liver mass and perfusion decline with ageing (*Schmucker*, 2005). There is

impairment in synthetic function, with a slight decrease in albumen levels, lower LDL and LDL receptor levels, lower cytochrome P450 content and lower vitamin K-dependent clotting factors (*Sotaniemi* et al., 1997; *Fu* and *Nair*, 1998; *Schmucker*, 2005). The regenerative response to injury decreases and the bile lithogenic index increases with ageing (*Valdivieso* et al., 1978; *Schmucker*, 2005).

Exocrine pancreas

There are minor atrophic fibrotic changes in the ageing pancreas with an increased number of cysts and side branching of ducts (*Bulow* et al., 2014). Although there is a decline in stimulated pancreatic flow, this does not impact exocrine function.

CARDIOVASCULAR AGEING

Heart weight increases as a function of ageing, with enlargement of the left atrium and enlargement and hypertrophy of the left ventricle (*Kitzman* et al., 1988). A decrease in cardiomyocyte number is accompanied by an increase in cardiomyocyte size (*Olivetti* et al., 1991). The aortic valve and mitral annulus thickens with development of calcific deposits. Mitral annular calcification predisposes to conduction abnormalities. Increased atrial and ventricular premature beats become more common with age (*Fleg* and *Kennedy*, 1992).

Due mostly through compensatory mechanisms, ejection fraction, stroke volume, and cardiac output do not change substantially with ageing (*Ferrari* et al., 2003). There is an increase in early diastolic filling, and an increase in end-diastolic filling due to a compensatory greater contribution from atrial systole (marked by a normal S4 in older individuals). There is a relative loss of chronotropic and inotropic responsiveness to β-adrenergic stimuli/catecholamines as well as inotropic response to digitalis glycosides. Peak cardiac output to maximal exercise is decreased.

In the vasculature, arterial wall thickness (intima-media) increases with advancing age, as does pulse wave velocity and total peripheral resistance (*Mitchell*, 2008). Endothelial nitric oxide release and β-adrenergic-mediated vasodilation decreases (*Mitchell*, 2008).

AGEING OF THE RESPIRATORY SYSTEM

Three hallmark features describe ageing in the respiratory system: change in lung volumes, increased alveolar-arterial oxygen gradient, and ventilation-perfusion mismatch (*Chan* and *Welsh*, 1998). Due to decreased elastic recoil, increased chest wall rigidity, and a loss of force-generating capacity of respiratory muscles, forced vital capacity, forced expiratory volume at 1 second, and vital capacity decrease and functional residual capacity increases (*Janssens*, 2005). Diminished lung diffusion capacity results in an increase in the alveolar-arterial oxygen gradient, measured as the diffusion capacity of carbon monoxide which drops by 2-3 ml/min/mmHg per decade (*Stam* et al., 1994). Secondary to decreased elastic recoil, the intrapleural pressure becomes less negative causing areas of the lung base to close. With this closure, there is a redistribution of inspired air to underperfused apical areas. The resultant increase in physiological dead space and underventilation of

dependent lung areas is responsible for ventilation-perfusion mismatches typical of the ageing lung (*Stam* et al., 1994).

AGEING OF THE IMMUNE SYSTEM

Ageing of the immune system is reflected by diminished immune responsiveness, so-called "immunosenescence", altered immune system physiology, and impaired immune regulation. Due to immunosenescence (*Agarwal* and *Busse*, 2010), there is a decreased response to new antigens/elevated susceptibility to infection and cancer, decreased vaccine efficiency (e.g., influenza), reactivation of latent infections (e.g., herpes simplex virus, tuberculosis), and perhaps compromised immune surveillance.

Important changes in immune system physiology with ageing include decreased production and maturation of B- and T-cells (e.g., marrow decline, thymic involution), inversion in proportional representation of memory vs naïve cells (T memory cells increase and T naïve cells decrease with age), decreased isotype switching and affinity maturation of B cells, accumulation of CD28-negative T-cells, and impaired formation of the T-cell – Antigen Presenting Cell "immune synapse" (*Panda* et al., 2009).

Altered immunoregulation can be seen as an increase in autoimmune syndromes (systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis, others), oligoclonal expansion of T- and B-cells (decrease in diversity with age), monoclonal gammopathies, and increased inflammation (*Ramos-Casals* et al., 2004; *Arai* et al., 2015; *Ray* and *Yung*, 2018).

CHANGES IN THE AGEING KIDNEY

Kidney weight and volume remain unchanged with ageing in individuals who experienced sudden death and in healthy individuals, respectively (*Glassock* and *Rule*, 2012). Nephrosclerosis increases progressively with age (*Glassock* and *Rule*, 2012). A sclerosis score is the total number of histological abnormalities (global glomerular sclerosis, any tubular atrophy, interstitial fibrosis > 5%, any arteriosclerosis). Among living kidney donors with less than 10% glomerulosclerosis, glomerular density declines with age but the size of glomeruli and tubules increase; in those with greater than 10% glomerulosclerosis, glomerular density increases with age with a rise in the number of small sclerotic glomeruli and tubular atrophy. The number of functional glomeruli decreases with age in both live kidney donors and in individuals at autopsy. There is compensation by functional nephrons in older healthy individuals. Glomerular filtration rate (GFR) is mostly reported to decline with age, but is variable and may be unchanged in as much as one-third in healthy cohorts (*Lindeman* et al., 1985; *Esposito* et al., 2007). Estimated GFR tends to vary based on the formula employed, and can be underestimated in healthy older adults when age is used as a surrogate for muscle mass. Changes in renal function with age may in part be driven by a vascular process whereby vasoconstrictive responses to angiotensin are intact but the vasodilatory responses to acetylcholine or to an acute sodium load are impaired (*Hollenberg* et al., 1974).

Table 1: Changes in the haematopoietic system with age

Parameter	Change with age	Response to challenges with age
Red cell life span	=	
Iron turnover	=	
Blood volume	=	
Bone marrow mass/cellularity	↓	
Bone marrow fat	↑	
Donor suitability for haematpoietic cell transplantation	↓	
Tolerance of chemotherapy	↓	↓
Compenstory response to:		
Phlebotomy		↓
Hypoxia		↓
Colony size of stimulated erythroid progenitor cells		↓
Production of BM stimulatory hormones (e.g. SCF, GM-CSF, IL-3)*	↓	↓
Total WBC	=	
Clonal expansion of specific white blood cells	↑	
Platelets (total number)	=	
Platelet responsiveness	↑	
Bleeding time	↓	↓
Procoagulant state (↑fibrinogen, ↑Factors V, VII, VIII, IX, ↑kininogen, ↑prekalikrein	↑	↑
Fibrin degradation products (D-dimers)	↑	
Plasminogen activator inhibitor-1 (major inhibitor of fibrinolysis)	↑	
Deep vein thrombosis	↑	↑

↑: Increase; ↓: Decrease; =: Unchanged.
*SCF: Stem cell factor; GM-CGF: Granulocyte-Macrophage-Colony-stimulating factor; IL-3: Interleukin-3.

AGEING IN THE HAEMATOPOIETIC SYSTEM

Changes in the haematopoietic system with age are shown in Table 1. Major changes outside of the immune system (see above) include decreased bone marrow, marrow cellularity, and compensatory responses to phlebotomy and hypoxia as well as increased components related to the procoagulant state

Table 2: Endocrine changes with ageing

Hormone	Change with ageing
Growth hormone	N in males; ↓ in females
Insulin-like growth factor I	↓
ACTH	N
Cortisol	N
DHEA	↓
Renin	N
Aldosterone	↓
TSH	N or ↑
T_4	N
T_3	↓
PTH	↑
Calcitonin	↓
1,25 $(OH)_2$ D	↓
LH	↑ or N in males; ↑ in females
FSH	↑ or N in males; ↑ in females
Testosterone	↓ or N in males; ↑ in females
Free testosterone	↓
Atrial natriuretic factor	↑
Insulin	↑
Glucagon	↓

↑: Increase; ↓: Decrease; N: Normal.
DHEA: dehydroepiandrosterone; TSH: thyroid stimulating hormone; T: thyroxine;
PTH: parathyroid hormone; LH: luteinizing hormone; FSH: follicle stimulating hormone.

(*Lipschitz* et al., 1984; *Pinto* et al., 2003; *Franchini*, 2006). In a young adult, haematopoietic marrow (red) resides in skull, vertebra, flat bones, and proximal femoral and humeral metaphyses. Fatty marrow (yellow) predominates in remainder of skeleton, and gradually replaces red marrow with age (*Custer* and *Ahlfeldt*, 1932; *Kricun*, 1985).

ENDOCRINE CHANGES WITH AGEING

Table 2 summarizes the endocrine changes with ageing (*Lamberts* et al., 1997). In healthy individuals, levels of some hormones are not altered with age (e.g., thyroid hormone). In older individuals without disease, the homeostatic setpoint for a given hormone may be the same as in youth, at expense of alteration in another (e.g., "normal" testosterone with elevated LH), different from youth (e.g., DHEA, IGF-1 levels decline with age) or the same as in youth, except with a stressor (e.g., cortisol response).

There is an increase in frequency of endocrine disease with ageing (e.g., hypothyroidism, post-menopausal osteoporosis) and often older individuals have different presentations of endocrine disease (e.g., hypothyroidism, hyperthyroidism) (*Gambert* and *Escher*, 1988). Different presentations

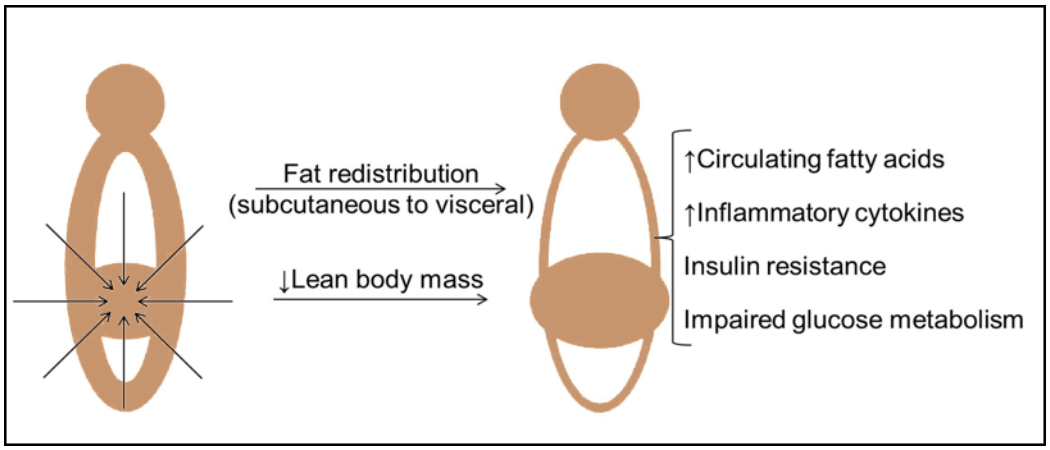

Figure 1: Changes in adipose tissue with ageing.

of non-endocrine disease are also common when coexistent with endocrine disease (e.g., angina in the setting of hyperthyroidism). In addition, there are alterations in hormones secondary to medications frequently prescribed in the elderly (e.g., hyperprolactinemia from phenothiazines).

Although not as abrupt as the decline in oestrogen levels during menopause, there are inexorable declines in testosterone ("andropause"), DHEA ("adrenopause"), and GH/IGF-I ("somatopause"). These changes, mediated by declines in LH/FSH, ACTH, and GH, respectively, are controlled at the level of the hypothalamus-pituitary complex (*Jones* and *Boelaert*, 2015).

AGE-RELATED CHANGES IN ADIPOSE TISSUE

Two major changes in adipose tissue that occur with ageing are the re-distribution of fat and the decrease in lean body mass (Figure 1). Adipose tissue redistributes from subcutaneous to visceral depots (*Kuk* et al., 2009). This redistribution is accompanied by an increase in circulating fatty acids and inflammatory cytokines as well as insulin resistance and impaired glucose metabolism (*Stout* et al., 2017). When older individuals are compared to younger ones, even after controlling for waist circumference, visceral fat increases and subcutaneous fat decreases (*Kuk* et al., 2009).

MUSCLE AGEING

The hallmark of muscle aging, sarcopenia, is operationally defined by thresholds of muscle loss, decreased muscle strength, and lower physical performance (*Locquet* et al., 2018). Risk of sarcopenia is very well predicted by the combination of age, grip strength, and calf circumference (*Locquet* et al., 2018). Loss of muscle mass as a proportion of body weight is almost universal with ageing and muscle loss in the lower extremities is greater than

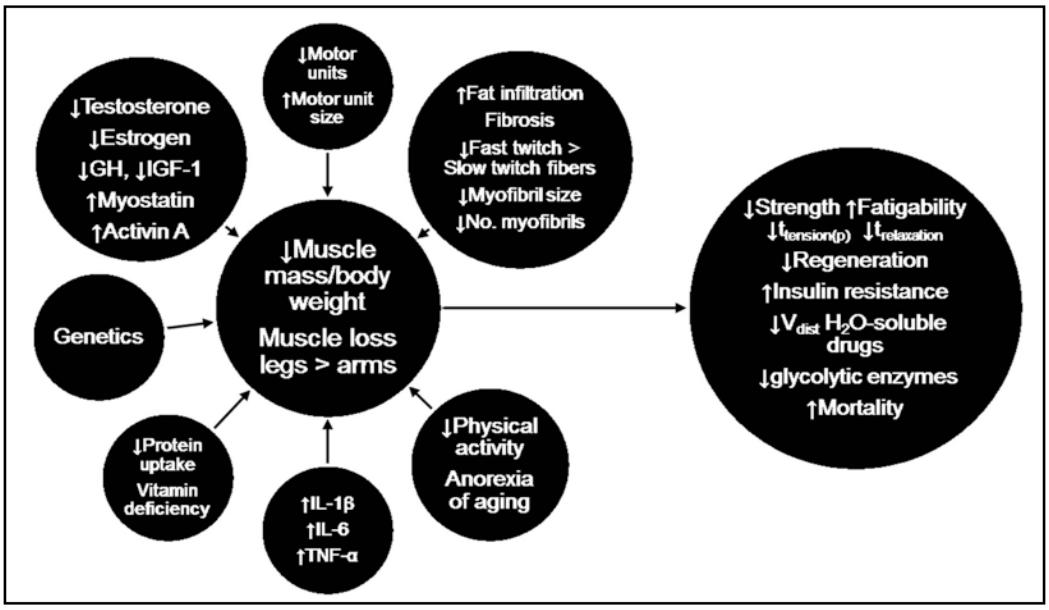

Figure 2: Contributing factors to muscle ageing (left) and consequences of sarcopenia (right).

that in the upper extremities. The loss of muscle mass is attributed to the decrease in motor unit number and size, lower physical activity common in older individuals, elevated inflammatory cytokines, diminished testosterone, oestrogen, and GH/IGF-I levels, elevations in myostatin and activin A, as well as nutritional deficits and genetic predisposition (Figure 2) (*Narici* and *Maffulli*, 2010). Histomorphologically, aged muscle shows increased fatty infiltration, fibrosis, decreased fast twitch > slow twitch fibers, smaller myofibril size, and fewer myofibrils (*Faulkner* et al., 2007).

Functional and clinical consequences of muscle ageing include decreased strength, increased fatigability, shorter time to peak tension and time to relaxation, decreased regenerative capacity, greater insulin resistance, lower volume of distribution for water-soluble drugs and decreased levels of glycolytic enzymes (*Narici* and *Maffulli*, 2010). Increased mortality is associated with sarcopenia (*Reinders* et al., 2015).

SKIN AGEING

Primary changes that occur with skin ageing include epidermal thinning, decreased vascularity, and the loss of rete pegs which project downward from the epidermis between the dermal papillae (*Montagna* and *Carlisle*, 1990). The elastin network of the skin is diminished, there is a loss of subcutaneous fat, and epidermal turnover is slowed (*Uitto*, 2008). Histologically, there is a loss of melanocytes, Langerhans' cells, sweat and sebaceous glands, as well as Meissner's and Pacinian corpuscles (*Yaar* and *Gilchrest*, 2001). Microarchitectural changes lead to poor nutrient transfer, a loss of protective lipids, and decreased area for heat transfer. Bleeding can more easily occur into dermal-epidermal space. Clinically, the ageing skin demonstrates an

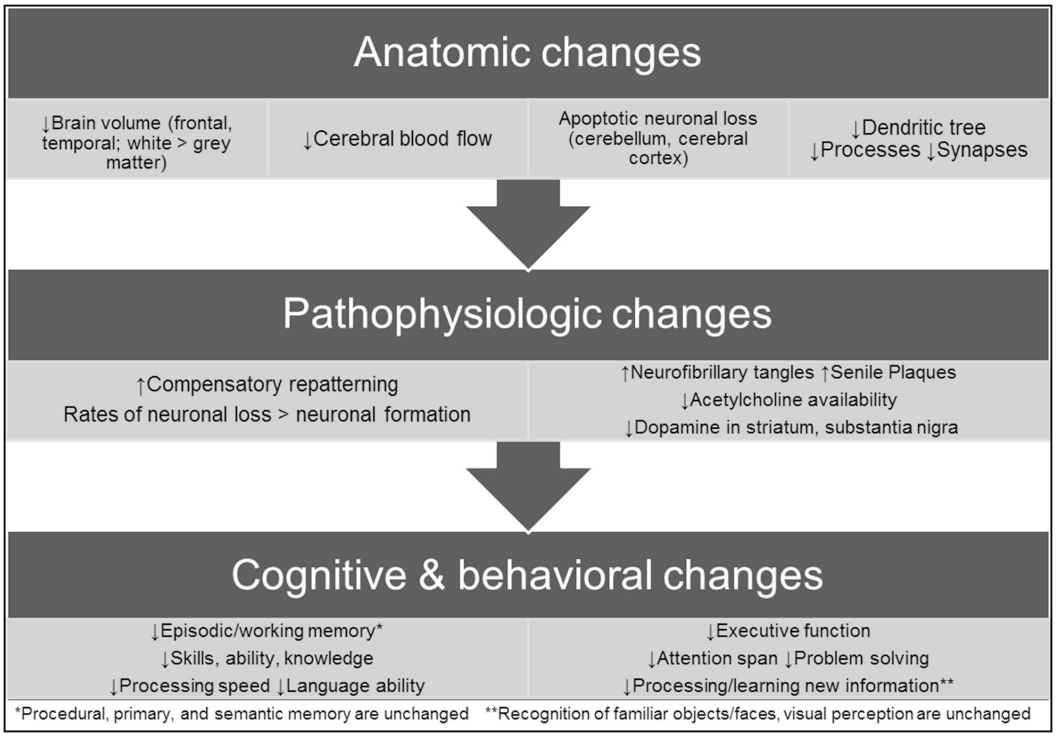

Figure 3: Ageing in the central nervous system by major anatomic, pathophysiologic, as well as cognitive and behavioural changes.

increased fragility to shear stress, slower wound healing, xerosis, reduced barrier function, compromised thermoregulation, wrinkling and sagging, decreased sensory perception, and purpura.

CENTRAL NERVOUS SYSTEM (CNS) AGEING

Key features of ageing in the CNS are outlined in Figure 3. Anatomically, the brain volume decreases, with predominant losses in the frontal and temporal lobes and with greater loss of white versus grey matter. Cerebral blood flow declines. Apoptotic neuronal loss becomes prominent in the cerebellum and cerebral cortex (*Dorszewska*, 2013). There is a reduction of the dendritic tree, loss of neuronal processes, and diminution of synapses (*van der Zee*, 2015).

Despite compensatory repatterning due to neuronal loss, the rate of neuronal loss exceeds that of neuronal formation. Pathological findings such as increased neurofibrillary tangles and senile plaques become more evident, although are not present to the extent that would be found in Alzheimer's disease (*Fjell* et al., 2014). There is reduced acetylcholine availability due to loss of cholinergic and muscarinic neurons, as well as lower synthesis and release of acetylcholine. Diminished levels of dopamine are found in the striatum and substantia nigra (*Klostermann* et al., 2012).

Cognitively, episodic and working memory declines (*Wilson* et al., 2002). There is a loss of specific skills and

knowledge with reduced processing speed and language ability. Executive function declines, as doses attention span, problem solving, as well as processing and learning new information. Procedural, primary, and semantic memories are unchanged with age. Recognition of familiar objects and faces and visual perception remain unaffected in healthy older adults.

SENSORY CHANGES WITH AGE

Vision

Periorbital tissues atrophy and lacrimal gland function, tear production, and goblet cell function are reduced with ageing (*Van Haeringen*, 1997). Despite decreased tear production, watery eyes are a common phenomenon since tissue atrophy leads to less effective drainage.

With ageing, the lens increases in thickness and hardness and the ciliary muscle decreases in strength; together, presbyopia or farsightedness results (*Strenk* et al., 2005). The lens also yellows and becomes more opaque with age, lessening colour discrimination. Increased light scatter due to lens alterations also reduces contrast. The resting pupil size and responsiveness decreases. In older individuals, the cornea yellows, undergoes focal and generalized thickening, and is more likely to have Hasall-Henle warts and especially arcus senilis (*Salvi* et al., 2006). There is a decrease in the production and drainage of the aqueous humour in the ageing eye, reduced speed and accuracy of pursuit and saccadic eye movements, and accumulation of scleral calcifications (Cogan's plaques) (*Salvi* et al., 2006) . There is a loss of retinal pigment epithelial cells which contributes to the decreases in number, size, and function of rods in the retina (*Liem* et al., 1991). Although the number of cones in the fovea is unchanged with age, their function is reduced. Vitreous floaters due to collagen condensations increase with age. Blood flow to the visual cortex is decreased.

Hearing

In the ageing ear, there is a loss of hair cells in the organ of Corti, decreased innervation of cochlear and auditory centres in the brain, stiffness and calcification of the basilar membrane of the sensory apparatus, thickening of the stria vascularis capillaries (source of endolymph), and degeneration of the spiral ligament (*Howarth* and *Shone*, 2006). Hearing loss due to these changes are usually of high frequency sound (presbycusis) and result in reduced speech discrimination, poor localization of the sound source, and decreased ability to discriminate between target sound and background noise (*Howarth* and *Shone*, 2006).

Taste and smell

There is a reduction in papillae on tongue and decreased taste sensitivity with ageing. Uneven gustatory deficiencies across the tongue exist, but the loss of taste in older individuals is due mostly to an impaired sense of olfaction (*Boyce* and *Shone*, 2006).

Detection thresholds for smelling are altered with age owing in part to a decrease in the number of sensing neurons (*Boyce* and *Shone*, 2006). This results in an underappreciated reduction in appetite, the extremes of which can be best appreciated in the observation that people with anosmia forget to eat.

AGEING OF THE GENITOURINARY SYSTEM

Bladder

Decreased innervation of the detrusor muscle and CNS changes lead to diminished detrusor contractility, lower maximum bladder capacity and flow rate, reduced ability to withhold voiding, and an increased post-void residual volume (*Elbadawi* et al., 1998). The constellation of these age-related alterations greatly increases the risk for urinary incontinence. The decline in oestrogen in women leads to a shorter urethral length, a reduced maximum urethral closure pressure, and less effective urethral barrier function (*Capobianco* et al., 2012); together, these factors predispose women to an increased risk of urinary tract infection.

Male reproductive system

Age-related neurologic, vascular, and endocrine changes in men lead to the greater stimulation required for erection, decreased spontaneous erections, diminished firmness of erections, lower force of ejaculation, smaller ejaculate volumes, and increased refractory times between erections (*Seftel*, 2005). Despite these changes, reports of lower sexual activity in older men are variable (*Lindau* and *Gavrilova*, 2010). However, an objective decline in male reproductive ability with advanced age is likely due to lower Leydig cell number, degeneration of the seminiferous tubules leading to diminished sperm production, an increase in sperm chromosomal abnormalities, reduced sperm motility, and a reduced ability of sperm to fertilize eggs (*Harris* et al., 2011). Enlargement of the prostate gland with ageing is common.

Female reproductive system

The loss of oocytes and ovarian dysfunction in older women is primarily responsible for the decline in oestrogen during the perimenopausal period and the reduction in implantation efficiency, decreased likelihood for pregnancy, lower number of live births, and poor success of *in vitro* fertilization (*Tarlatzis* and *Zepiridis*, 2003). Oestrogen deficiency causes diminished vaginal elasticity, reduced clitoral engorgement after stimulation, increased vaginal dryness and atrophy, as well as decreased cervicovaginal secretions (*Kingsberg*, 2002). Oestrogen deficiency is responsible for an increase in vaginal pH, which predisposes to colonization by enteric flora.

SYNDROMIC APPROACH

For many if not all system-specific ageing changes, multiple aetiologies are at play, including the influence of ageing changes in other physiologic systems. While characterizing ageing in a single system *in isolation* serves to simplify the task, it also minimizes the true nature of its complex regulation. The geriatric syndromes serve as examples of the complexity involved to fully explain common age-related changes. Geriatric syndromes have been operationally defined as multi-factorial, having multi-system involvement, and sharing risk factors such as older age, functional impairment, and impaired mobility (*Inouye* et al., 2007). Common geriatric syndromes include pressure ulcers, incontinence, falls, delirium, dementia, and osteoporosis.

Osteoporosis is the hallmark of bone ageing (*Chandra* et al., 2018) and will be used here as an illustration of the syndromic approach to describing physiologic ageing in the skeleton (Figure 4). Bone loss is ultimately the

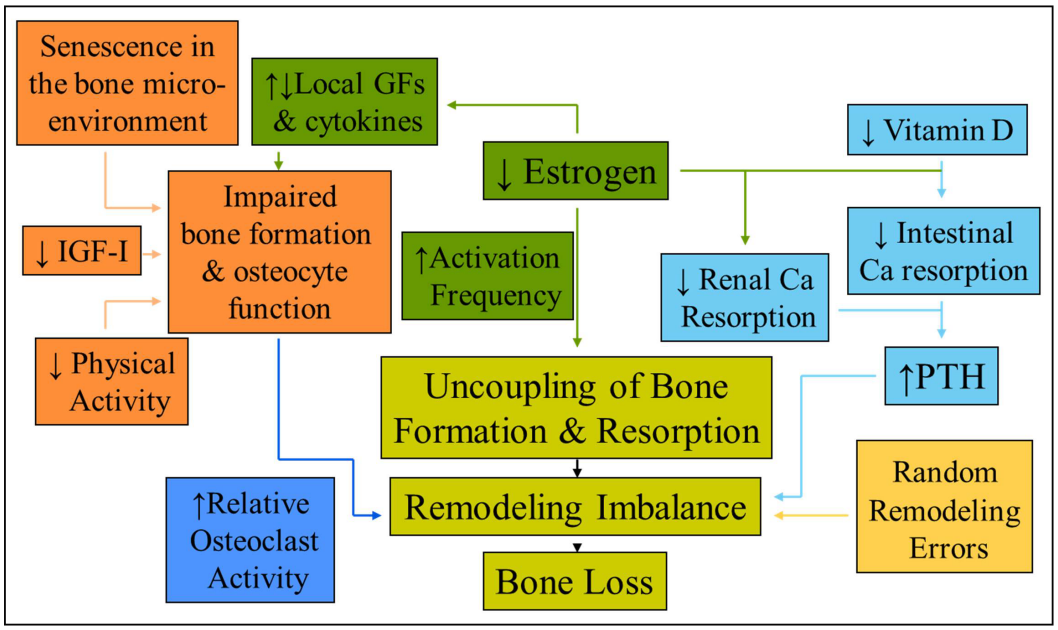

Figure 4: Osteoporosis as an example of a geriatric syndrome with multifactorial aetiologies for bone ageing.

result of a remodelling imbalance, where an uncoupling of bone formation to bone resorption causes a net loss of bone tissue. There are at least three major contributions to age-related bone loss: secondary hyperparathyroidism due to vitamin D deficiency and decreased renal calcium absorption, impaired bone formation and osteocyte function and, in both women and men, lower oestrogen levels.

Cell senescence in the bone micro-environment, declines in IGF-I levels, and decreased physical activity and response to mechanical loading contribute to poor bone formation. In the setting of unimpaired osteoclast function, there is a relative increase in bone resorption. With the decline in oestrogen, two key phenomena occur which contributes to bone loss; there is an increase in the activation frequency of osteoclasts (i.e., initiation of local resorption events) and there are alterations in local growth factors and cytokines favouring an inflammatory milieu with inhibitory effects on bone formation. There are also random remodelling errors that may increase with ageing.

RESILIENCE TO PHYSIOLOGIC AGEING

It is well established that psychosocial factors influence resilience to age-related social and behavioural stressors, but there is a limited understanding of human resilience in responses to physiologic or pathologic stressors (*Hadley* et al., 2017). It is clear, however, that low levels of resilience confer vulnerability to stressors (Figure 5). Specific physiologic resilient responses differ depending on both the stressor being exerted and the clinical or physiologic property to be maintained or restored.

There are growing examples of

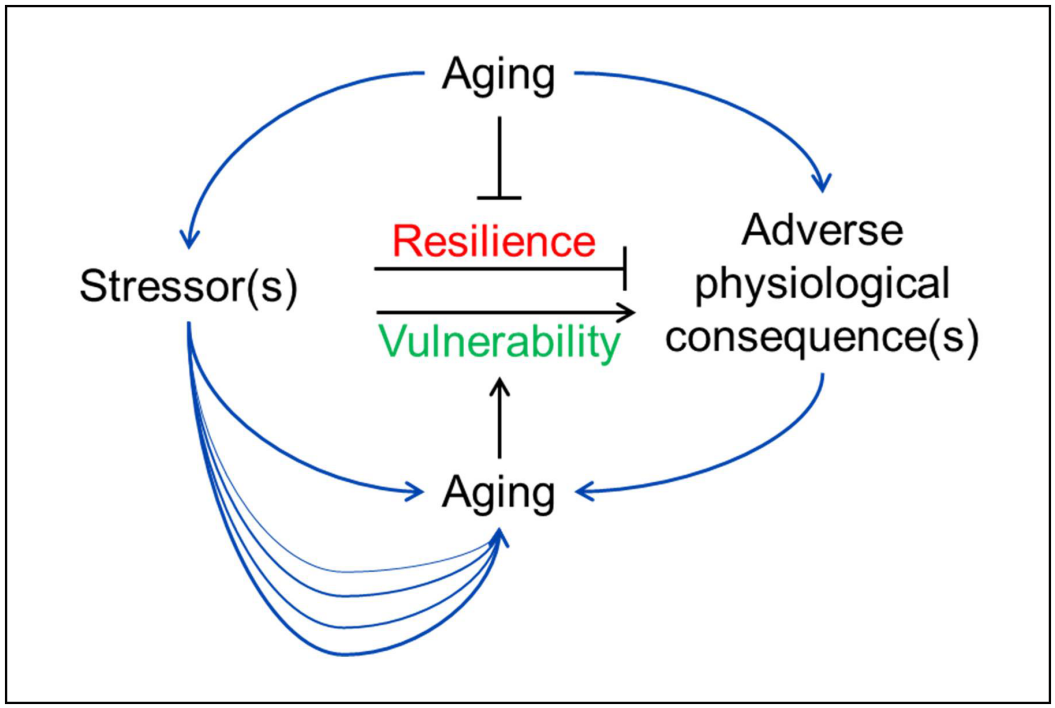

Figure 5: Relationships among stressors, aging, and resilience to adverse physiologic consequences.

resilient responses that are mediated by regulatory factors, which mitigate potential adverse consequences. Falls are common stressors in older individuals and can result in fractures, especially of the hip or wrist. Bone mineral density remains a very strong predictor of hip fracture up to 25 years after the measurement and preservation of femoral neck bone mass, even in individuals greater than age 75, does occur and minimizes the likelihood of fracture (*Black* et al., 2018).

Neuropathologic changes of Alzheimer disease (AD) uncommonly exist in isolation in the brains of older individuals, but are more often variably combined, or comorbid, with other lesions that underlie the dementia syndrome including vascular brain injury (VBI), Lewy body disease (LBD), and hippocampal sclerosis (HS). The frequency and severity of these neuropathologic changes in cognitively intact older individuals varies, with some showing a lesion burden considered sufficient evidence for dementia, a situation commonly referred to as apparent cognitive resilience. Here resilience to neuropathological stressors appears to be dependent on the presence of HS only, since cognitively intact individuals can have evidence for high lesion burden consistent with AD, VBI, and/or LBD at brain autopsy (*Latimer* et al., 2017).

COMMON MECHANISTIC APPROACH

There are common mechanisms thought to underlie physiologic ageing based on evidence in lower animal models which are now being confirmed

in non-human primates and in humans. These include chronic, low-grade, non-microbial inflammation, cellular senescence, accumulation of damaged macromolecules (DNA, proteins, carbohydrates, lipids), as well as stem and progenitor cell dysfunction (*Kirkland*, 2016).

In animal studies, targeting senescent cells using genetic or pharmacological approaches delays, prevents, or alleviates multiple age-related phenotypes associated with chronic diseases, geriatric syndromes, and loss of physiological resilience. In preclinical studies, reduction of senescent cell burden has successfully ameliorated frailty, cardiac dysfunction, vascular hyporeactivity and calcification, diabetes mellitus, liver steatosis, osteoporosis, vertebral disk degeneration, pulmonary fibrosis, and radiation-induced damage. Senolytic agents are being tested in proof-of-concept clinical trials based on these findings (*Kirkland* et al., 2017).

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AGEING AND THE MICROBIOME: AN AVENUE FOR INTERVENTION

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SUMMARY

Progress in ageing research has been impressive in recent years, leading to an increased understanding of the biological determinants of ageing and the identification of a number of interventions that might slow ageing in humans. Given that ageing is the largest risk factor for the chronic diseases that are increasing in prevalence globally, this raises the possibility that through the use of these interventions it will be feasible to slow ageing and prevent the onset of many diseases simultaneously. However, ageing is not generally considered as a disease and any intervention to slow ageing will necessitate a high safety profile to be used in healthy individuals. For this reason, it is important to consider non-pharmaceutical interventions, and modification of the microbiome is a promising avenue for exploration. Here I discuss how manipulation of the microbiome may impact ageing, highlighting data from animal models and human studies.

INTRODUCTION

Demographics are rapidly changing, leading to an increasing percentage of the population over the age of 65. In some countries that percentage will approach 40% in the near future. With ageing comes functional decline and disease onset. Most individuals over the age of 65 have one or more chronic diseases (e.g. neurologic, cardiovascular and metabolic syndromes, as well as cancers). While each of these diseases have identified risk factors, they pale in comparison to the risks associated with ageing itself (*Kennedy* et al., 2014). Targeting risks factors has proven effective in disease prevention; lowering cholesterol and glucose reduces onset and progression of cardiovascular and metabolic disease, respectively. What would happen if the biggest risk factor, ageing, was targeted?

Efforts to understand the molecular determinants of ageing have been underway for nearly a century and great progress has been made in the last few decades, largely through the use of non-vertebrate models such as yeast, worms and flies. Prior to these studies, many researchers proposed that ageing, due to its complexity, was highly difficult to modulate. Working with these organisms, however, researchers identified hundreds of genes that impact ageing and proposed several mechanisms to explain their action (*Longo* et al., 2012; *Uno* and *Nishida*, 2016; *Piper*

and *Partridge*, 2018). A surprising finding was that ageing pathways and mechanisms are likely conserved between these organisms, even though they are often quite divergent from an evolutionary perspective (*Smith* et al., 2008). Further extending this observation, more recent reports indicate that many of these same genes impact mammalian, and even possibly human, ageing (*Tian* et al., 2017).

Calorie (or dietary) restriction was shown to slow ageing in mice and rats over eight decades ago, and remains a hot topic for research today (*Balasubramanian* et al., 2017). The pathways modulated by nutrient limitation, including insulin/IGF and mTOR signalling, also modulate ageing and recent evidence has suggested that reducing mTOR activity may impact aspects of human ageing (*Mannick* et al., 2014). Interestingly, these interventions have been shown to alter the microbiome as well, raising the question as to whether their effects are modulated through the gut microbiota.

Here links between the microbiome and ageing are discussed using three short perspectives, with a focus on the gut. First, I detail changes in the gut microbiome that occur with human ageing, findings that might point to targeted modulation. Second, I outline the links between known interventions affecting ageing and alterations in the microbiome. Finally, I discuss the prospects of using targeted interventions to modulate microbiome composition as a means of slowing ageing. The intersection of two major fields of study, microbiome and ageing, offers great promise for novel discoveries and more importantly an avenue toward extending human healthspan, the disease free and highly functional period of life.

MICROBIOME ALTERATIONS DURING AGEING

Perhaps not surprisingly, there are extensive microbiome changes during the ageing process (*Kundu* et al., 2017). Diversity between individuals also increases with ageing (*Claesson* et al., 2011). One surprising finding was that the microbiome of residential dwelling elders was significantly different from those in long-term residential care (*Claesson* et al., 2011). Community dwelling elders have a more diverse microbiome, correlated with a diet higher in fibre and reduced levels of age-associated inflammatory factors, such as IL-6, TNF-α and C-reactive protein. Metagenomic analysis further indicated higher metabolism of butyrate and short-chain fatty acids in community dwellers, who were also healthier by several parameters. Microbiome changes are also associated with several diseases of ageing, including neurodegenerative conditions (*Kundu* et al., 2017). Finally, a recent study suggests that semi-supercentenarians (105-109 years) have a microbiome associated with health promotion, for instance enriched for taxa such as *Bifidobacterium*, *Christensenellaceae*, and *Akkermansia* (*Biagi* et al., 2010). None of these studies demonstrate a causal role for the microbiome in mediating ageing, they certainly raise that possibility. How easy it will be to modify the microbiome of elders, however, remains unclear.

Microbiome-related changes are also evident in ageing mice, with notable similarities and differences. One genus that declines in both human and mice is *Akkermansia*, which is associated with protection from a range of diseases (*Langille* et al., 2014). Germ-free mice are also a major tool for research and a

recent study suggests that these mice have enhanced lifespan (*Thevaranjan* et al., 2017), similar in some respects to that of *C. elegans* exposed to dead bacteria as a food source (*Thevaranjan* et al., 2017) or adult worms on plates deprived of bacteria entirely (*Kaeberlein* et al., 2006). Germ-free mice also experienced lower inflammation, which was increased when these mice were co-housed with old conventional animals (*Thevaranjan* et al., 2017). Numerous other changes have been detected, although some discrepancies exist between different mouse studies. By continuing to exploit the mouse model to understand the relationship between microbiome composition, ageing, and disease, it is almost certain that insights will be gained regarding human ageing and interventional strategies developed.

AGEING INTERVENTIONS AND THE MICROBIOME

A major step forward in ageing research has been the identification of dietary and small molecule-based interventions that slow ageing and extend healthspan in animal models. Among the most prominent of these are calorie restriction, metformin and rapamycin, all of which have been linked to changes in the microbiome. An advantage of the animal models is that it is easier to study the relationship between interventions that extend lifespan and microbiome changes. For instance, calorie restriction as expected is associated with significant changes in the microbiome, including an increase in bacterial species associated longer lifespan. Interestingly, both calorie restriction and intermittent fasting, which mimics the effects of calorie restriction, have been reported to extend lifespan in flies and this is associated with a reduced gut bacterial load (*Regan* et al., 2016; *Catterson* et al., 2018). The effect is more prominent in females, which are more prone to gut deterioration with age. Further analysis of the effects of calorie restriction and fasting will be important as these interventions enhance longevity and promote healthspan across a wide range of species.

Metformin is a widely used drug used to treat hyperglycaemia in the context of a range of metabolic conditions and has also been reported to extend lifespan in several animal models (*Barzilai* et al., 2016). Intriguingly, retrospective studies in humans suggest that diabetic patients taking metformin have a lower-than-expected mortality rate and moreover metformin may be protective for a range of other chronic conditions (*Bannister* et al., 2014). These findings together have suggested that metformin may deserve even more widespread use to extend human healthspan. A surprising finding regarding metformin's ageing effects came from *C. elegans*, where it was shown that lifespan extension by the drug required live bacteria as a food source (*Cabreiro* et al., 2013; *Heintz* and *Mair*, 2014). Axenic worms or those grown on dead bacteria do not respond to the drug. The mode of action was reported to be an inhibition of folate metabolism in *E. coli*, leading to reduced methionine. This is consistent with lifespan extension by methionine reduction in a variety of animal models, including worms. Several studies suggest that metformin affects the mammalian microbiome (*Forslund* et al., 2015; *Wu* et al., 2017; *Bauer* et al., 2018), but this relates to lifespan extension in mammals remains unknown.

Inhibition of the mTOR pathway is associated with lifespan and healthspan

extension in a range of animal models (*Kennedy* and *Lamming*, 2016), and preliminary data using rapalogs [a class of very specific mTOR inhibitors in which rapamycin is the founder (*Lamming* et al., 2013)] suggests similar effects may be possible in humans (*Mannick* et al., 2014, 2018). Acute rapamycin treatment in old mice impacts the faecal microbiome, with a notable increase in segmented filamentous bacteria. An earlier study in middle-aged mice reported modest effects of rapamycin on gut bacterial composition (*Hurez* et al., 2015). Whether these changes are important for the longevity effects of rapamycin remains unknown. A number of other drugs have been reported to extend lifespan in mice and they may have affected microbiome composition as well. For instance, acarbose, an α-glucosidase inhibitor that extends lifespan in male mice (*Strong* et al., 2016), alters microbiome and in turn its efficacy as an antidiabetic treatment may be dependent on microbiome composition (*Su* et al., 2015; *Gu* et al., 2017).

MICROBIOME INTERVENTIONS AND AGEING

Drugs are promising interventions for targeting ageing, but as stated this approach is fraught with regulatory hurdles. By contrast, microbiome-mediated interventions are likely less subject to regulatory concerns but research must be performed to determine to optimize the efficacy of any such approach (*Kundu* et al., 2017). Faecal microbiota transplants (FMTs) are perhaps the most promising to date, although the long-term effects of this approach remain controversial. At first blush, it would seem that to delay ageing, any intervention must be durable, or administered relatively frequently. Other approaches, including postbiotics and dietary approaches to modify bacterial populations, are probably even more primitive to date.

In particular, metagenomic studies that identify metabolite deficiencies in the gut of ageing people and corresponding changes in systemic metabolism offer great promise as it may be possible to correct these deficiencies with supplements or other approaches. For instance, healthier community dwelling elders were found to have enriched bacterial species for short chain fatty acid production, which may promote more youthful metabolism (*Claesson* et al., 2012). It should be noted that molecular changes associated with ageing are not always detrimental, and in some cases might be compensatory. A good example is testosterone, which declines with ageing in the male population. Testosterone supplementation to restore the hormone to youthful levels is not necessarily beneficial, and in fact is associated with increased risk of disease in some contexts (*Sansone* et al., 2017). Together, these observations call for more intensive studies in animal models and humans to (1) identify candidate microbiome-associated interventions that might extend healthspan and (2) test them effectively.

CONCLUSION

The possibility to slow human ageing and extend healthspan is a relatively recent occurrence, as this has only been relatively recently achieved in animal

models. The potential benefits of preventing multiple chronic diseases simultaneously and improving function later in age demand that this approach be explored. Meanwhile, the importance of gut bacterial species in human health and disease has emerged almost concurrently. To what extent do our residents age us, or keep us young? Merging these two concepts, ageing and microbiome research, offers great promise and requires intense investigation. Possibly we can live longer and healthier by training our residents to behave, or perhaps helping them train us to adopt healthier lifestyles?

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HOST-MICROBIOTA INTERACTIONS DURING AGEING

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SUMMARY

Host-associated microbial communities take part to several fundamental aspects of host biology, including development, nutrient absorption, immunity and pathogenesis. Throughout host life, microbes associated with external body surfaces vary in composition and function and can shift from commensal to pathogenic. However, whether these microbial communities can directly influence host ageing has remained elusive for a long time. Recent work in laboratory model organisms has revealed for the first time that commensal microbes can actively modulate host ageing and that microbial communities associated with young hosts can have a health-promoting action in middle-age individuals that leads to significant life span extension. These findings suggest new opportunities to identify systemic ageing-modulating mechanisms via the gut microbiota and will help design innovative anti-ageing interventions that could impact human health and ageing-associated diseases.

AGEING AND HOMEOSTASIS

Ageing is associated with pervasive functional decline and overall decrease in physiological fitness that lead to a progressive increase in the risk of disease and death. During ageing, homeostatic processes fail at multiple levels of biological complexity. DNA repair mechanisms become less efficient (*Vaidya* et al., 2014) and protein homeostasis, which ensures proteome stability, functionally declines at older age (*Morimoto* and *Cuervo*, 2009). Membrane and organelle composition, as well as cell physiology, undergoes dramatic changes during ageing. Ageing is typically associated with increased risk of uncontrolled cellular proliferation – leading to cancer (*Finkel* et al., 2007) – and to protective proliferation arrest – called cellular senescence (*Childs* et al., 2015). If on the one hand cellular senescence acutely reduces the risk of cancer, on the other hand it leads to inflammation and higher risk for disease and frailty (*Tchkonia* et al., 2013). Changes in the extracellular matrix composition, which is importantly involved in wound healing, stem cell functionality and longevity in experimental animal models (*Ewald* et al., 2015), additionally contribute to the age-dependent decline in organ and organism performance.

A combination of genetic and non-genetic factors affects the rate at which different organisms age and tune life expectancy in different species. Research done in model organisms has revealed key conserved molecular pathways, including the insulin-IGF1, the mTOR and AMPK pathways, which regulate ageing and life span across several species, from yeast to mammals (*Kenyon* et al., 1993; *Kapahi* et al., 2010; *Lapierre* and *Hansen*, 2012;

Weir et al., 2017). Environmental manipulations, including temperature changes, different diets and various forms of stress, also significantly impact ageing in several model organisms (*Liu* and *Walford*, 1966; *Weindruch* et al., 1986; *Libert* et al., 2007; *Ermolaeva* et al., 2013). Overall, ageing and life span are complex physiological outcomes of the genetically-encoded strategies that organisms evolved to maintain homeostasis throughout their lifetime in response to the continuous exposure to internal and external stimuli.

GUT MICROBIOTA CONTRIBUTE TO HOST PHYSIOLOGY

Complex microbial communities covering host surfaces occupy the interface between organisms and the external environment, from roots and leaves in plants to skin and mucosal surface including lungs and gut in animals. These microbial communities participate in a wide range of key biological processes, including nutrition, development (*Sommer* and *Backhed*, 2013; *Hill* et al., 2016), essential vitamin synthesis, metabolism (*Nicholson* et al., 2012), immune modulation (*Geva-Zatorsky* et al., 2017), defence against pathogens and disease. Host-associated microbiota constitute highly diverse and dynamic communities, able to modulate and buffer host physiology by promptly reacting to internal (e.g. hormonal and metabolic) and external (e.g. temperature, toxins, pathogens) stimuli. A large amount of circulating host metabolites in the host serum are synthesized by gut microbes (*Wikoff* et al., 2009), further supporting that host-associated microbiota are not passive passengers on host surfaces, but rather an integrative physiological partner that contributes to host homeostasis. Due to their essential contribution to virtually all host biological functions, it is reasonable to hypothesise that commensal microbes could be essential players during host ageing and could be targeted to design interventions aimed at modulating ageing and age-associated conditions.

GUT MICROBIOTA AND IMMUNE FUNCTION: A RECIPROCAL INFLUENCE

Mounting evidence indicates that the gut microbiota play a prominent role in shaping the immune system during development. In humans, new-borns acquire their initial resident gut microbiota from several sources, most prominently from the birth canal and from maternal skin during breast-feeding. These early-acquired bacterial consortia shape the structure of mature gut microbiota (*Dominguez-Bello* et al., 2010) and play an essential role for immunological development (*Round* and *Mazmanian*, 2009). Caesarean sections and formula feeding dramatically affect the gut microbial composition, and consequently impact proper immune system development, with potentially long-lasting effects for individual health (*Bokulich* et al., 2016). Gut bacteria interact with cells of both the innate and adaptive immune system in the gut mucosa and different metabolites synthesized by gut bacteria can induce pro or anti-inflammatory host responses (*Arpaia* and *Rudensky*, 2014). Epitopes derived from gut bacteria, both commensal and pathogenic, significantly shape the evolution of lymphocyte antibody and receptor

repertoires (*Zhao* and *Elson*, 2018). Consistently, specific-pathogen-free (SFP) raised mice have a much higher immunoglobulin (IgA) diversity compared to mono-colonized mice (*Lindner* et al., 2015).

While bacteria shape immune cells development and function, immune cells, in turn, play a key role in determining and controlling the composition and abundance of gut microbial communities. Pharmacologically immunosuppressed rats undergo a loss of commensal gut microbe diversity, which are generally associated with a healthy status (*Bhat* et al., 2017). Specific antibodies, i.e. IgAs, synthesized by intestinal plasma cells, are majorly secreted in the intestinal lumen and bind to a broad subset of bacteria to help maintain a healthy barrier function (*Bunker* et al., 2017). Under normal physiological conditions, IgAs target bacterial taxa that otherwise would contribute to pathogenesis (*Planer* et al., 2016). Conversely, specific pathogens, including influenza virus, succeed in infecting the host by precisely degrading human IgAs.

While bacteria influence immune system evolution, on the other hand the immune system acts as a selective force to shape the composition and function of gut microbes.

GUT MICROBIOTA IN AGEING AND DISEASE

Diet and lifestyle importantly affect differences in microbiota composition among individuals (*Rothschild* et al., 2018). The composition of host-associated gut microbiota undergoes dramatic changes in humans after birth, becomes stable during adulthood in non-pathological conditions, varies during pregnancy and then goes through drastic changes during ageing (*Kostic* et al., 2013). Although stable in composition during adulthood, microbiota has rapid functional metabolic oscillations during the day (*Thaiss* et al., 2016). Across many organisms, including laboratory flies, mice and humans, ageing is characterized by dramatic changes in the composition of the commensal gut microbiota, which could lead to dysbiosis and ultimately host demise (*Claesson* et al., 2012; *Guo* et al., 2014; *Clark* et al., 2015). While gut microbiota associated with healthy hosts are typically characterized by large bacterial taxonomic diversity, frailty and ageing are associated with loss of diversity and expansion of more pathogenic bacterial species. Studies across different human age cohorts have shown that large changes in the abundance of subdominant bacterial taxa in the gut are a hallmark of ageing. Moreover, exceptionally long-lived individuals, including supercentenarians, are characterized by the persistence of bacterial taxa associated with health (*Biagi* et al., 2016). While diversity-associated microbial taxa often decline during age, specific bacterial taxa, such as *Clostridiales*, are associated with malnutrition and increased frailty (*O'Toole* and *Jeffery*, 2015). In flies, reducing gut microbial dysbiosis by improving immune homeostasis promotes longer life span (*Guo* et al., 2015). In humans, after antibiotic treatment, pathogenic bacterial species, such as *Clostridium difficile* and *Enterococcus faecalis*, can restructure the gut microbial composition and cause severe chronic conditions that pose a major threat for public health (*Backhed* et al., 2012; *Milani* et al., 2016). In humans, faecal material transfer from healthy donors is successfully used in the clinic to resolve acute *Clostridium difficile* infections (*Lee* et al., 2016). Remarkably, transplanting microbes from obese individuals into

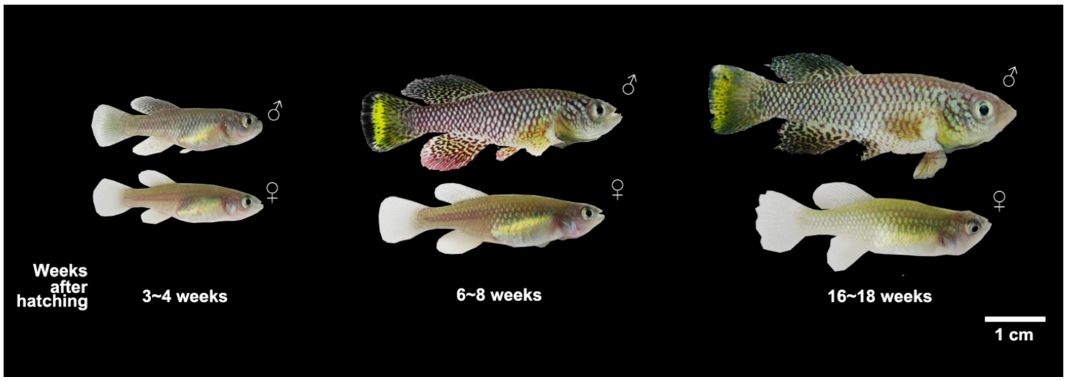

Figure 1: Ageing in the turquoise killifish. Reaching maturity as soon as 3-4 weeks post hatching, killifish display several ageing-related phenotypes by 16 weeks.

germ-free-raised mice leads to dramatic effects, including elevated adiposity and changes in fatty acid and amino acid metabolism, associated with systemic health (*Ridaura* et al., 2013). Overall, microbiota composition and function dramatically change during ageing and disease and manipulating the composition of the gut microbial communities via microbiota transplants has the opportunity to be a novel powerful intervention to impact the ageing process and improve systemic health.

GUT MICROBIOTA PLAY A CAUSAL ROLE IN MODULATING HOST AGEING AND LIFE SPAN

Despite evidence that the gut microbiota could dramatically modulate host metabolism and physiology, until recently it was not clear whether gut microbes could causally modulate host ageing and longevity. Recent work done in nematode worms, flies, fish and mice has shown that gut microbes can beneficially influence host ageing and life span, proving that specific components of this microbial consortium can *de facto* improve overall physiological fitness of the host (*Seidel* and *Valenzano*, 2018).

Work done in nematodes (*Caenorhabditis elegans*) has shown that worms feeding on different bacterial species and on varieties of *E. coli* strains different from the standard laboratory OP50 *E. coli* strain (*Girard* et al., 2007) live longer than standard-fed worms (*Sanchez-Blanco* et al., 2016; *Han* et al., 2017). A recent study in a short-lived fish has further shown in vertebrates that the gut microbiota is causally involved in modulating host ageing and life span, and that young-associated gut microbiota, transplanted to middle-age individuals, could lead to life span and health benefits (*Smith* et al., 2017). This study used the naturally short-lived turquoise killifish (*Nothobranchius furzeri*), which is the shortest-lived vertebrate raised in a laboratory setting (Figure 1), which includes captive strains with a median life span of about 4 months (*Cellerino* et al. 2016, *Valenzano* et al., 2017). For comparison, laboratory mice live 2.5–3 years and zebrafish can live until five years (*Kim* et al., 2016). Despite their short life span, turquoise killifish also display a wide range of age-related changes, including an increased occur-

rence of cancer, reduced regenerative capacity, increased cellular senescence, neurodegeneration and cognitive decline, making it a powerful new vertebrate model system to study ageing and age-related diseases (*Valenzano* et al., 2017). Turquoise killifish have complex gut microbiota, both in the wild and in captivity, similar in taxonomic diversity to mammals. During ageing, the overall microbial diversity of the resident gut microbiota decreases, while potentially pathogenic *Proteobacteria* become more prevalent, possibly contributing to host demise (*Smith* et al., 2017). After acute re-colonisation of the intestine of middle age individuals with gut microbiota from young donors, fish lived significantly longer, remained more active at old age, and kept highly diverse microbiota. Whether this gut microbial transfer influences immune function and whether its effect is sufficient to improve host health and therefore delay the ageing process is still an open question.

TURQUOISE KILLIFISH AS A MODEL TO STUDY VERTEBRATE IMMUNOSENESCENCE

Unlike invertebrate model organisms such as laboratory flies (*Drosophila*) and nematode worms (*Caenorhabditis elegans*), fish are equipped with an adaptive immune system consisting of both T and B-lymphocytes. B-lymphocytes undergo somatic recombination at the IgH (Immunoglobulin heavy chain) locus and generate an extremely diverse ($>10^9$) antibody repertoire, which enables to build complex immune responses towards extraneous agents, such as bacteria, viruses, etc. B-cells that bind antigens from microbes and viruses undergo amplification, affinity maturation and unfold a targeted immune response against them in a highly regulated process. Antibody repertoire diversity can be used as a proxy of the function of the whole B-lymphocyte compartment, with high diversity being better than low diversity, and antibody repertoire variation throughout ageing can be adopted as a biomarker of overall health status. Although it is known that upon ageing the B-cell repertoire declines (*Martin* et al., 2015), it is not clear whether this decline correlates with the decline in the gut microbial diversity. Currently, it is not clear what are the implications of the age-dependent decrease in immune function (e.g. decreased antibody repertoire diversity) for the changes in microbial diversity and function that occur during ageing. Age-dependent immune dysfunction could lead to proliferation of pathogenic bacteria that are already present in the young gut microbiota, eliciting bacterial community dynamics that favour more proliferative and pathogenic bacterial taxa over commensal and slow-replicating taxa. Alternatively, specific bacterial strains – within a specific subset of bacterial species – could evolve within the gut to escape immune attacks, independently of immune functional decline associated with ageing. Bacterial evolution towards increased pathogenicity could, in turn, lead to host damage and systemic functional decline. As a third alternative, age-dependent host immune dysfunction and bacterial evolution could occur simultaneously, leading to increased pathogenicity of gut bacteria and age-related diseases. The characterization of the sequence of the IgH locus in short-lived vertebrate species, such as the turquoise killifish, will enable to

study in detail whether the immunoglobulin diversity and abundance change during ageing and whether such changes are influenced by anti-ageing interventions, such as transplants of young-associated gut microbiota. Additionally, applying high-throughput approaches to study the immunoglobulin repertoire, such as the recently developed IgSeq method (*Weinstein* et al., 2009), will enable to functionally compare immunoglobulin diversity in the gut with commensal and pathogenic microbial diversity at different ages. High-resolution characterisation of immune system and microbiome changes during host life will lead to understand whether immune senescence precedes the loss of microbial diversity or whether age-dependent loss of microbial diversity in the gut anticipates immune senescence. The use of a short-lived and experimentally tractable vertebrate model organism, such as the turquoise killifish, will hence be instrumental to answer key questions more rapidly than in longer-lived vertebrate model organisms, such as zebrafish and mice.

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CONTRASTING CONSERVED AND TISSUE-SPECIFIC RESPONSES TO AGEING IN VERTEBRATES

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SUMMARY

Ageing is the most important risk factor for diseases accounting for more than 90% of mortality in western countries. While genomic damage as the root cause of cancer is well established, the primary causes of most degenerative diseases of ageing such as cardiovascular diseases, neurodegenerative diseases and type 2 diabetes are still unknown. In a previous work, we have shown that there is a common core of ageing-associated gene regulation, conserved across vertebrate species and tissues.

In this work, we explored the association between this conserved core signature of ageing and the actual manifestation of ageing in individual tissues in more detail. To this end, we characterized transcriptomic signatures of ageing in individual tissues and investigated the contribution of the microbiome as a potential driver of ageing-associated inflammation. While core versus tissue-specific signatures showed only small differences in phylogenetic conservation or network connectivity, a considerable percentage of genes was found exclusively in tissue-specific signatures. However, on the functional level, tissue-specific signatures showed a very high overlap with the core signature of ageing. Moreover, cancer-regulated genes were enriched among genes of the core signature while they were depleted among the tissue-specific signatures. In contrast, for genes deregulated in degenerative diseases, we observed the tendency of an enrichment amongst genes of the tissue-specific signatures. Through comparisons of ageing signatures to genes regulated in response to microbial colonization, we observed a significant overlap with genes of the core signature of ageing.

Overall these results indicate that tissue ageing on the transcriptomic level is mostly driven by the core signature of ageing and that the microbiome is a potential modulator of this signature.

INTRODUCTION

Ageing is associated with a continuous functional decline and is recognized as a dominant factor driving the pathology of diseases contributing to the majority of mortality in western countries (*Lopez* et al., 2006; *López-Otín* et al., 2013). There is a remarkable conservation of ageing-associated pathologies such as cancer, cardiovascular disease and cognitive decline across vertebrates (*Dean* et al., 1981; *Genade* et al., 2005; *Pettan-Brewer* and *Treuting*, 2011).

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However, despite a large number of studies investigating ageing from the molecular (*López-Otín* et al., 2013) to the population level (*Jones* et al., 2014), a coherent picture of the processes that are driving ageing and their individual contribution to the pathogenesis of ageing diseases is just emerging (*López-Otín* et al., 2013). An often-observed feature of ageing is a low-grade inflammatory phenotype ("inflammaging") (*Chung* et al., 2009) that is characterized by a minor activation of the immune system even in absence of a detectable pathogen (*Franceschi* and *Campisi*, 2014). Inflammaging is an important risk factor for morbidity and mortality in the elderly (*Franceschi* et al., 2000).

A key problem in the study of the ageing phenotype is its remarkable complexity which makes a clear distinction between causal and consequential pathological changes a challenging task (*López-Otín* et al., 2013; *Bousquet* et al., 2014; *Franceschi* and *Campisi*, 2014; *Castellani* et al., 2016). In particular, while genomic mutations as a key event in cancer are well established (*Valko* et al., 2004), the root causes of degenerative ageing diseases such as cardiovascular diseases or neurodegenerative diseases have yet to be identified (*Carretero* and *Oparil*, 2000; *Galkina* and *Ley*, 2009; *Dexter* and *Jenner*, 2013; *Reitz* and *Mayeux*, 2014). One contributing factor is that ageing diseases are most often studied in isolation in the particular organ system they affect (*Castellani* et al., 2016), while extensive comorbidities between ageing diseases (*Fried* et al., 1999) hint at general underlying mechanisms not restricted to individual organ systems (*Masoro*, 2005; *Bousquet* et al., 2014).

In order to elucidate common pathomechanisms underlying ageing diseases, we recently have analysed a comprehensive next-generation sequencing data set of vertebrate ageing covering four organisms (human, mouse, zebrafish and the killifish *Nothobranchius furzeri*) across up to four tissues (brain, liver, skin, blood) (*Aramillo Irizar* et al., 2018). Using a novel method assessing differential activity directly on a functional level, we identified a highly conserved core of ageing-associated transcriptional regulation including an induction of inflammatory processes and a down-regulation of cell-cycle as well as developmental processes. While many of these processes have been implicated in ageing pathologies in individual species before (*Lee* et al., 2000; *Teta* et al., 2005; *Chung* et al., 2009), we found that they are part of a conserved core of ageing-associated regulation in the studied vertebrates.

By investigating the association between transcriptional signatures of ageing with differential gene expression observed in ageing related diseases, we found that ageing opposes transcriptional changes of cancer but is aligned with alterations in degenerative disorders. Intriguingly, these results are in line with epidemiological data showing a peak in cancer incidence between 70 to 80 years of age (*Harding* et al., 2012), while the incidence of degenerative diseases increases up to the oldest age groups (*Berzlanovich* et al., 2005; *Harding* et al., 2012). Thus, ageing moves the transcriptome away from cancer but brings it closer to that of degenerative diseases. On the functional level, we found that immune- and cell cycle-related processes most strongly contributed to this antagonism, which is supported by their central role in ageing pathologies (*Campisi*, 2013; *Jurk* et al., 2014). We observed a similar antagonism on the genetic level whereby for the majority of shared risk loci between cancer and degenerative diseases the allele that predisposed to

cancer protected against degenerative diseases and vice versa (*Aramillo Irizar* et al., 2018). We found the strongest signals close to central regulators of immune function and cellular senescence such as the P16/Ink4A locus, a master regulator of cellular senescence (*Congrains* et al., 2013), the Lnk/SH2B3 protein, a key regulator of cytokine signalling (*Devallière* and *Charreau*, 2011), and VAMP8 a central regulator of autophagy (*Diao* and *Liu*, 2016).

Besides ageing-associated changes in the host, ageing and inflammation might also be triggered by changes in microbiome composition in the elderly. Bacterial composition changes continuously throughout lifetime (*O'Toole* and *Jeffery*, 2015) and microbial diversity continuously becomes richer in humans with increasing age (*Lozupone* et al., 2012). Diseases associated with ageing often involve or are preceded by inflammaging, which can be aggravated by changes in the microbial composition (*Fransen* et al., 2017). A microbiome transfer experiment from old to young germ-free mice led to the upregulation of TNF-α and a dysregulation of pathways involved in the immune response. Inflammaging supporting bacteria were enriched after that treatment, including a higher amount of *Akkermansia*, TM7 bacteria, and *Proteobacteria* (*Thevaranjan* et al., 2018). A similar outcome can be observed when germ-free and old mice are co-housed (*Fransen* et al., 2017). Common inflammaging biomarkers, such as circulating pro-inflammatory cytokines, cannot be detected in germ-free mice and treating old mice with anti-TNF could alleviate microbiome-driven inflammation (*Thevaranjan* et al., 2018). In addition, in the ageing microbiome, less bacteria are able to synthesize β-glucuronidases, which are key modulators of epithelial cell toxicity caused by drugs (*Langille* et al., 2014). Moreover, the amount of monosaccharide-utilizing compared to polysaccharide-using bacteria positively correlates with age in mice (*Langille* et al., 2014).

The immune system of the host can be altered by a modified microbial composition caused by environmental factors including lifestyle and diet. As consequence, a disturbed gut homeostasis can increase the risk for diseases (*Langille* et al., 2014; *Fulde* et al., 2018). This is especially the case in elderly humans, in which poor health is associated with imbalances in the microbiome (*Claesson* et al., 2012). Thus, species distribution in the microbial flora is strongly associated with phenotypes of the host such as inflammation, the ability of independent living, sarcopenia as well as geriatric depression. For instance, independently living human individuals are characterized by a higher number of bacteria, which are able to perform biosynthesis of short chain fatty acids (*Claesson* et al., 2012). Taken together, host-microbiome interactions are potential key modulators of health and contribute to the physical conditions of the host.

While we have characterized the conserved transcriptomic signature of ageing in great detail in our previous work, we did not explore the actual manifestation of this signature in individual tissues (*Aramillo Irizar* et al., 2018). Thus, the aim of this work is to investigate conserved tissue-specific ageing in more detail. We aim to investigate two central hypotheses in ageing research in more detail, 1) whether there is a common mechanism underlying ageing in all tissues, which would point to a common driver of ageing (for instance the microbiome) or 2) whether ageing on the organismal level is basically the sum of functional deterioration in individual tissues. In the first

case, we would expect that tissue-specific ageing presents just a tissue-specific manifestation of the changes we observe in the core ageing signature. In the second case, we would expect strong differences in ageing between tissues and the core signature representing the least common denominator. Moreover, we explored the potential role of the microbiome as an important trigger for ageing-associated inflammation and thereby as a potential key driver of the observed transcriptomic signature of ageing.

MATERIAL AND METHODS

Data

To identify sets of conserved ageing-associated genes, we used a similar approach as described in a previous study, in which we searched for commonly regulated processes across species (*Aramillo Irizar* et al., 2018). More specifically, we used expression values of genes that were orthologue across all the considered four species. We determined orthologues across all four species (zebrafish, killifish, human and mouse) using the R-package orthology. We considered only genes with an orthologue in all four species which had detectable expression across all studied tissues and species (RPKM>0). After removing genes that did not pass the ANOVA-based testing procedure 3748 genes remained [see the Methods section of (*Aramillo Irizar* et al., 2018) for details]. In order to reduce bias due to different reference gene sets, all analyses described in this work were performed using these 3748 genes as basis. The core ageing signature comprised genes, which were conserved over all tissues and species. Genes with a conserved pattern for all species but only one tissue were assigned to the tissue ageing signatures. The specific ageing signatures contained all genes, which were associated with ageing only for one tissue in one species.

Differentially expressed genes were calculated for 13 cancer types investigated in the consortia 'International Cancer Genome Consortium' (ICGC) (Hudson et al., 2010) and 'The Cancer Genome Atlas' (TCGA). The following tissues were considered: lung (LUAD-US), breast (BRCA-US), prostate (PRAD-US), uterus (UCEC-US), kidney (KIRC-US), head and neck (HNSC-US), colorectal (COAD-US), liver (LIHC-US), bladder (BLCA-US), skin (SKCM-US), cervix (CESC-US), pancreas (PAAD-US), and ovary (OV-US). Read counts per gene were downloaded from the ICGC data portal (https://dcc.icgc.org/) (*International Cancer Genome Consortium* et al., 2010). Sample groups were compared with DeSeq2 v1.8.2. Thereby, gene outliers were replaced with trimmed mean value. All other disease data sets were collected from published studies and processed as described by *Aramillo Irizar* et al. (2018). Genes differentially expressed between germ-free and conventionally raised mice were taken from *Pan* et al. (2018). Only transcription data of adult mice (12 to 16 weeks of age) were considered in our study.

Enrichment analyses

Gene set enrichment analyses for KEGG pathways and transcription factor binding sites (TFBS) were performed with the online tool innateDB (*Lynn* et al., 2008). To test for significance, a hypergeometric test was applied. Gene set enrichment analyses for Gene Ontology (GO) terms were conducted with the online tool g:profiler (*Reimand* et al., 2016). Exclusively the GO class "Biological processes" was considered. All p-values were corrected

for multiple testing using the Benjamini Hochberg approach (*Benjamini* and *Hochberg*, 1995). Protein-protein interaction networks were created with the R package igraph v1.2.1 based on the String database v10 using medium confidence for connection predictions.

Enrichment of ageing signature genes in disease-associated genes

The enrichment score was calculated as [(<number of genes in ageing signature> / <number of disease associated genes>) / (<number of genes in ageing signature> / <number of all investigated genes>)]. Thereby, the regulation direction was not considered.

Calculation of evolutionary conservation per gene

The latest Version (May 8th, 2015) of phylogenetic p-values (phyloP) from the PHAST software (*Pollard* et al., 2010) for multiple alignments of 99 vertebrate genomes against the human genome (*Siepel* et al., 2005) were obtained from the University of California, Santa Cruz's webserver under the address: "http://hgdownload.soe.ucsc.edu/goldenPath/hg38/phyloP100way/". We evaluated phyloP-100-way genetic conservation scores for every single base position of the signatures' genes. The phyloP scores of genes found in any of our ageing signature were averaged over all base-pairs of that gene, leaving us with one mean phyloP score per gene. Chromosome, gene and exon structures were based on the latest version of the human genome (hg38). The mean scores per gene were then compared across signatures.

RESULTS

While we determined commonly differentially regulated processes in our previous work (*Aramillo Irizar* et al., 2018), we applied this approach directly on the gene expression level to identify genes that show a conserved regulation across four vertebrate species (*Homo sapiens*, *Mus musculus*, *Danio rerio* and *Nothobranchius furzeri*) and across tissues (blood, brain, liver and skin). Thus, we obtained a core signature of ageing comprising 985 genes and tissue specific signatures comprising 789 genes for blood, 1423 genes for brain, 826 genes for liver and 1281 genes for skin (referred to as tissue signatures). Moreover, we compared those signatures to differentially expressed genes from ageing tissues in mice and humans (referred to as specific signatures) (*Aramillo Irizar* et al., 2018). Following the two central hypotheses, we investigated the overlap between the core and the tissue signatures of ageing (Figure 1). We found that the signatures of blood and liver were smaller in size than the core signature, while the brain and skin signatures were larger. Tissue signatures showed an average overlap of 38 % with the core genes. Thus, there is a large overlap of the tissue signatures with the overall core signature of ageing. Considering the specific signatures, we found that they showed an average overlap of 61 % with either core or tissue signatures demonstrating that the tissue-specific manifestation of ageing in individual species shows a high concordance with conserved ageing in the corresponding tissue across species (Figure 1).

Conservation and connectivity of the individual ageing signatures

In a first step, we investigated the phylogenetic conservation of genes belonging to the individual signatures. Please

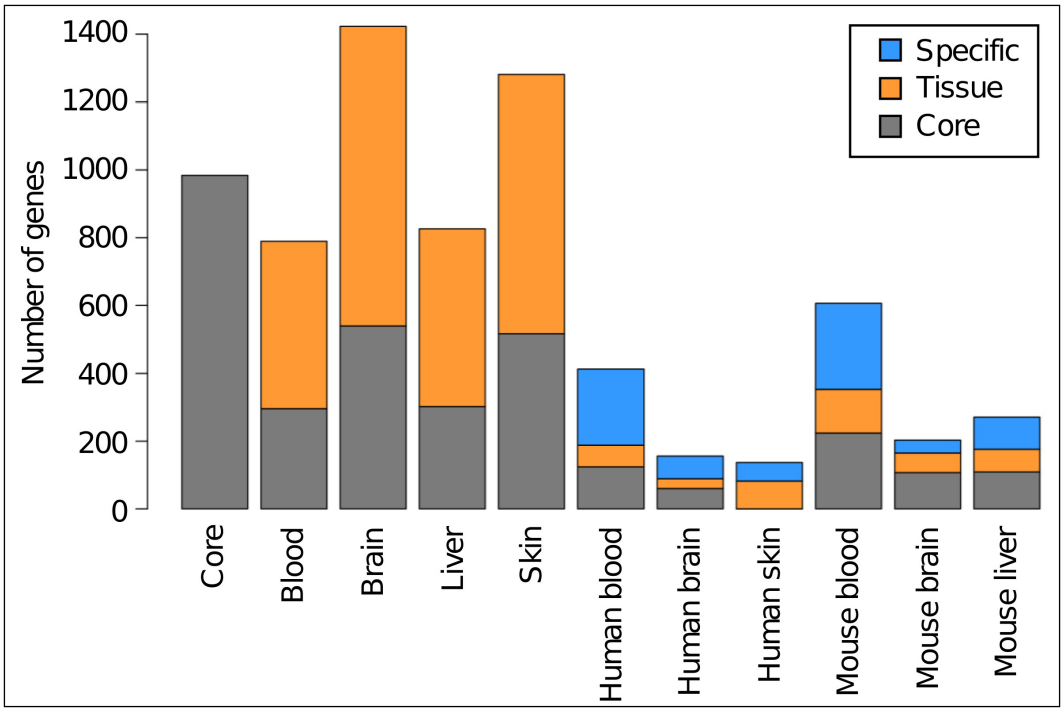

Figure 1: Number of genes involved in each ageing signature. A large proportion of the tissue ageing signature and the specific ageing signature is already enclosed in a more general ageing signature.

note that when considering tissue signatures, we excluded genes also belonging to the core signature. We found no significant differences regarding the phylogenetic conservation between the core signature and the tissue signatures (Figure 2A). Subsequently, we investigated the network context of the genes belonging to the ageing signatures based on their connectivity in the String database (*Szklarczyk* et al., 2015). Only interactions between genes belonging to one of the ageing signatures were considered. We found that genes belonging to the core signature tended to have fewer connections to other genes than the tissue signatures (Figure 2B-D). This was especially reflected in a higher number of nodes (= genes) with at least 50 different interaction partners, the so-called hub genes, in the tissue ageing signatures (Figure 2C). Also, from the ten proteins with highest connectivity, only one belonged to the core ageing signature (108 interaction partners). However, with 87 % the vast majority of proteins of the core signature remained unconnected.

Functional characterization of core vs. tissue-specific ageing

Next, we functionally characterized the genes belonging to individual tissues. We determined significantly enriched processes as well as enriched transcription factor binding sites in the individual ageing signatures.

Following the results from our previous work, particularly immune related pathways and cell cycle processes were enriched among the different ageing signatures. This is also in agreement with the central role of these

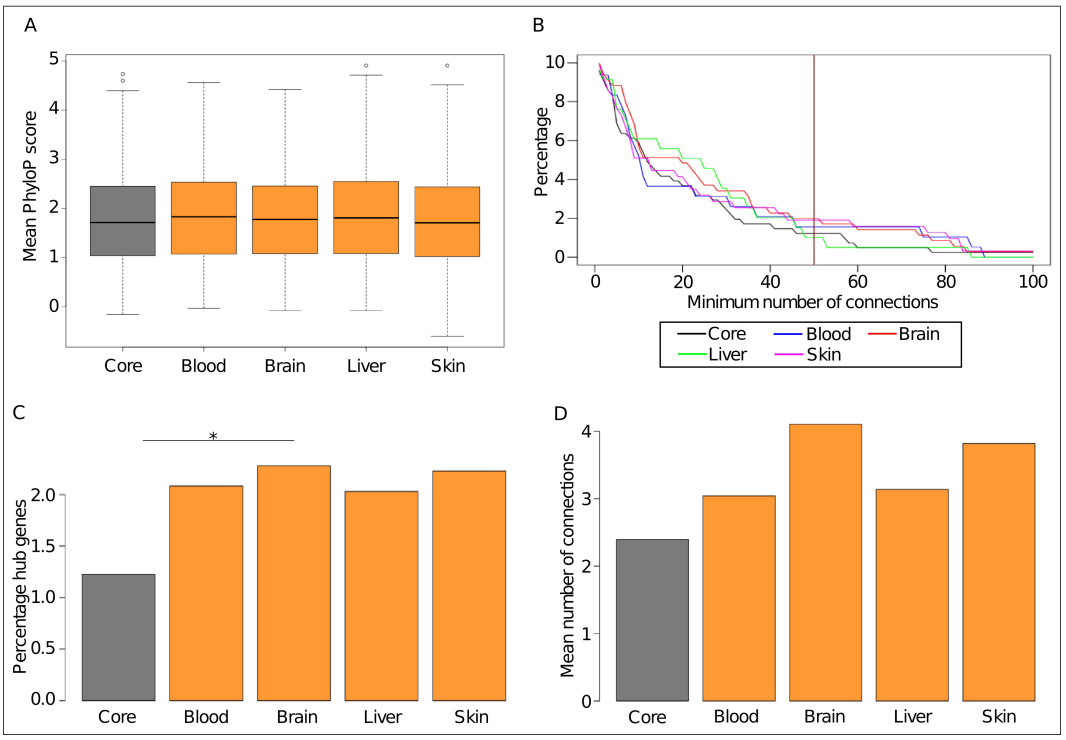

Figure 2: Characteristics of ageing signatures. (A) Genetic conservation of age-linked genes across species. The phyloP scores of genes found in the ageing signatures were averaged over all base-pairs of a gene, based on the human reference (hg38), and compared across signatures. (B) Number of connections within the interaction network based on all ageing-associated genes (core, tissue, and specific signature). Number of connections to all other nodes (independent of the signature) were counted. For better clarity, the y-axis is constrained to a plotting range of 0 % to 10 % and the x-axis to a maximal number of 100 connections (maximal number of connections is 309, three genes had more than 100 connections across all signatures). (C) Number of hub genes (genes with more than 50 connections) within the network described in (B). (D) Average number of connections within the network described in (B).

processes in ageing pathologies (*Chung* et al., 2009; *Campisi*, 2013). We performed gene set enrichment analyses based on gene ontology (GO) terms using once the complete tissue signatures and once the tissue signatures excluding genes of the core signature. We found the highest number of enriched processes for the core signature even in comparison to the brain and skin signatures which contained more genes (Figure 3A). Importantly, we found that most of the enriched processes in the tissue signatures were also enriched in the core signature. This was true both, when considering tissue signature genes not belonging to the core signature only, and when considering the full gene sets. Thus, a mean of 65.2 % of the functional groups (GO terms) enriched in the tissue signature were also enriched in the core signature when considering only genes specific to the tissue signature and 70.6 % when considering the entire gene set.

Testing for the enrichment of transcription factor binding sites (TFBS), we found an enrichment of transcription factors in the core signature, known to play a central role in the pathogenesis of ageing diseases, such as HIF-1 (involved in ischemic disease,

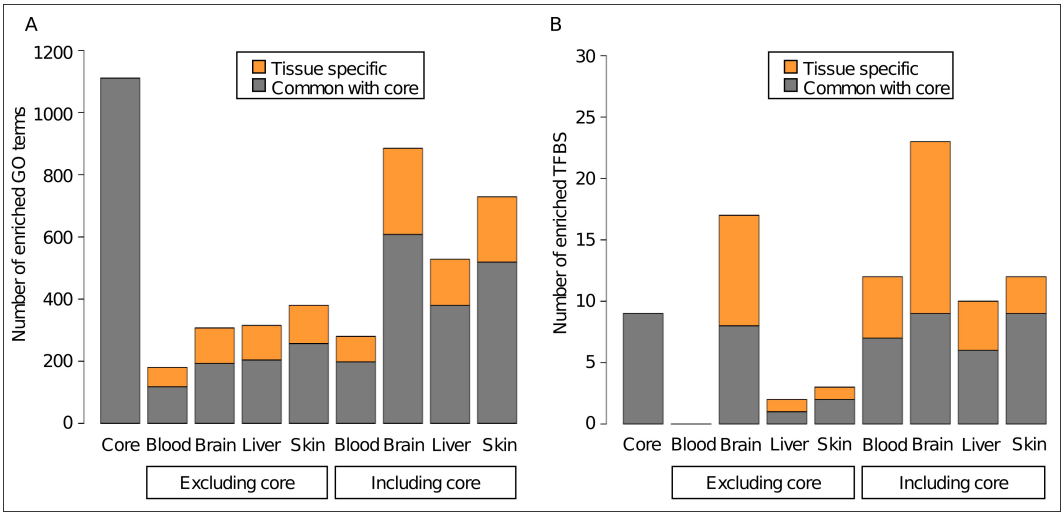

Figure 3: (A) Functional enrichment analysis using gene ontology (GO) cellular function terms. Number of GO terms enriched for each ageing signature. (B) Transcription factor enrichment analysis. Number of TFBS enriched for each ageing signature.

tumour angiogenesis), E2F-4 DP-2 (involved in tumour suppression, colorectal cancer), and E3F1 DP-2 (involved in tumour suppression).We observed a larger number of enriched transcription factors for the complete versus the reduced tissue signatures (Figure 3B). However, for all tissues except brain the enriched transcription factors showed a strong overlap with the core ageing signature: on average 54.6 % of the transcription factors enriched in the reduced tissue signatures were also enriched in the core signature and on average 58.1 % of transcription factors were enriched in the full gene sets.

Disease-specific characterization of ageing signatures

Since ageing is the most important risk factor for human disease (*Lopez* et al., 2006; *López-Otín* et al., 2013), we determined the enrichment of disease-regulated gene sets with genes belonging to the individual signatures of ageing. Specifically, we investigated to which extent genes, which are differentially expressed in cancer or ageing-associated degenerative diseases, showed an enrichment among genes belonging to the core or the tissue-specific signatures (Figure 4). While cancer-associated genes showed a significant enrichment among genes forming the core signature of ageing (Fisher's exact test p-value = 0.0461), they showed a significant depletion among the tissue-specific signatures (Fisher's exact test p-value = 0.0187). In contrast, for degenerative diseases the enrichment among genes belonging to the core ageing signature was less pronounced, while we observed a larger number of cases of enrichment among gene sets belonging to the tissue-specific ageing signature. In this context it is also important to emphasize that cancer-associated genes exclusively originated from the affected tissue while deregulated genes from degenerative diseases originated mostly from blood expression data [cf. (*Aramillo Irizar* et al., 2018) for details]. Thus, the comparison to the blood signature is likely most representative for the degenerative disease signatures.

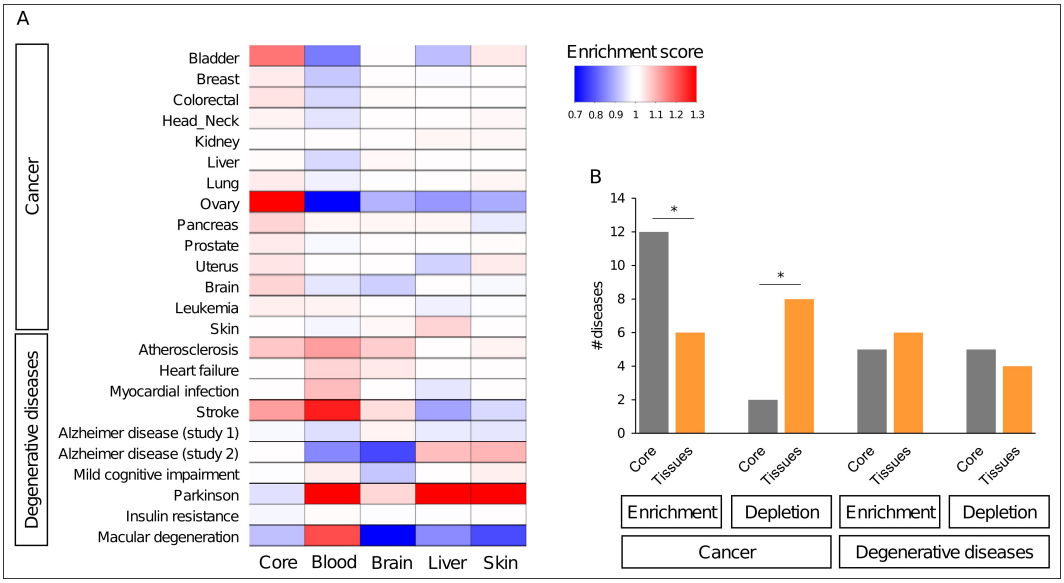

Figure 4: (A) Enrichment of genes belonging to the individual signatures among genes deregulated in different ageing diseases. (B) Frequency of enrichment of disease-deregulated genes in core and tissue signatures. For the three tissue signatures, average values across the four tissues are shown.

The microbiome is a modulator of the core signature of ageing

Next, we investigated to which extent the different ageing signatures were associated with the host response to microbial colonization. Hence, we compared the signatures to gene expression differences in colon of adult mice possessing a microbiome compared to germ-free mice (*Pan* et al., 2018) (Figure 5). Of the 3748 genes considered for the ageing signatures, 355 showed a differential expression in germ-free versus conventionally raised mice. We found that in particular the core signature of ageing showed an enrichment of genes differentially expressed during microbial colonization (Fisher's exact test p-value = 0.022) while there was no enrichment of the other signatures. In contrast, the core signature of ageing showed a significant enrichment of microbiome-regulated genes in comparison to brain and liver signatures. Intriguingly, comparing the direction of changes between colonized mice versus germ-free mice with the direction of changes observed in the ageing signature, 68 % of the genes belonging to the core signature showed an opposing direction of regulation during colonization.

DISCUSSION

While we identified a common signature of ageing in our previous work (*Aramillo Irizar* et al., 2018), we here explored the tissue-specific representation of this signature. Specifically, we investigated two partially competing hypotheses in ageing research that 1) ageing is driven by a common underlying process across all tissues or 2) that ageing on the organismal level is

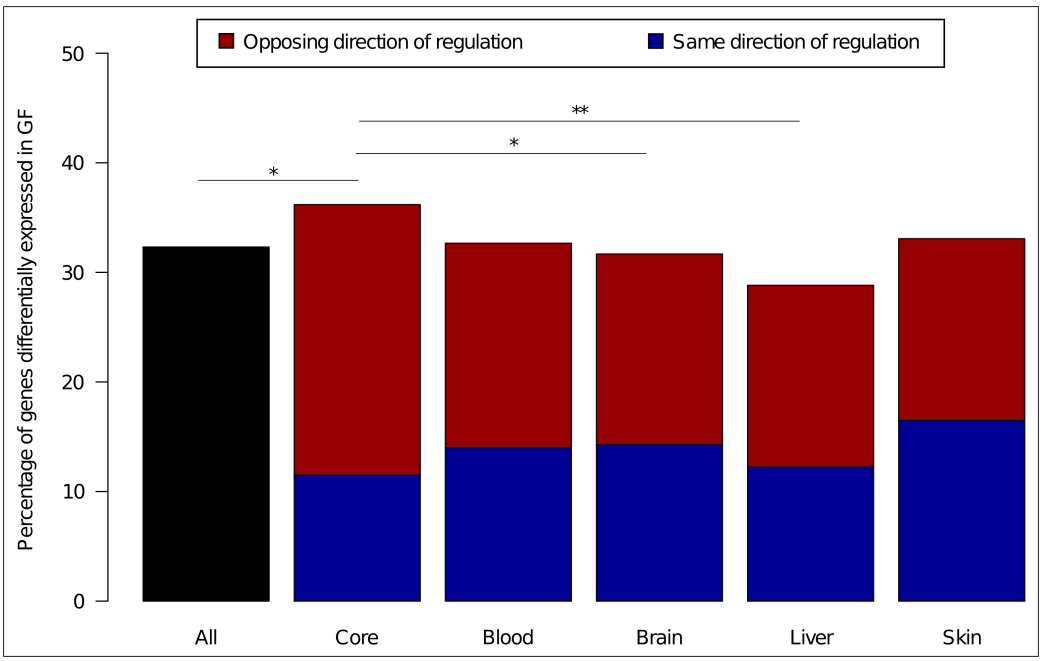

Figure 5: Comparison between ageing signatures and differentially expressed genes in response to microbial colonization. "All" represents the total percentage of all considered genes differentially expressed upon microbial colonization. For each signature the fraction of genes showing the same or opposing direction of regulation are indicated. "Same direction of regulation" corresponds to genes that pointed in the same direction of response in germfree versus colonized mice like in young vs. old mice.

mostly a consequence of functional deteriorations in individual organs.

While we found mostly no differences in phylogenetic conservation between the core and tissue-specific signatures of ageing, the tissue-specific signatures tended to show a stronger degree of interactions. On the functional and the regulatory level tissue-specific signatures showed a very strong overlap to the core signature of ageing: Most processes or transcription factor binding sites enriched for genes belonging to the tissue ageing signatures showed also an enrichment for the genes of the core ageing signature. This supports the hypothesis that ageing in individual tissues probably just represents different tissue-level manifestations of the same underlying processes driving the core ageing signature. On the molecular level, the observed differences in the tissue-specific signatures compared to the core ageing signature represent probably just tissue-specific downstream consequences of a process' deregulations by the core ageing signature. These downstream consequences could arise, for instance, due to the induction of tissue-specific compensatory processes or due to a reallocation of cellular resources away from tissue-specific processes to processes that need to be induced as part of the core ageing signature.

Testing for enrichment of disease-regulated genes among the individual signatures revealed that cancer-regulated genes tend to show a stronger enrichment among genes belonging to the core signature of ageing compared to the tissue-specific signatures of ageing. In contrast, degenerative diseases of ageing did not show this tendency.

In our previous work we hypothesized that the antagonism between cancer and degenerative diseases might be driven by an accumulation of genomic damage with age (*Aramillo Irizar* et al., 2018). This accumulation of damage leads to an induction of processes aimed at the suppression of potentially malignant cells which, while to some extent preventing the proliferation of potential cancer cells, drives tissue degeneration and thereby degenerative diseases as a side effect. This hypothesis is well in line with our observation of the enrichment of disease-specific gene sets in the individual ageing signatures: While cancer-deregulated genes as a potential causative factor for the ageing signature are enriched among the core signature of ageing, genes deregulated in degenerative diseases do not show this tendency.

We moreover tested the influence of the microbiome as a potential driver of the core ageing signature. The core ageing signature was significantly enriched for genes differentially expressed between germ-free and conventionally raised mice, whereas no enrichment was found with the tissue-specific signatures of ageing. Importantly, microbial colonization was associated with an expression signature opposing the direction of regulation observed in the core signature of ageing. Microbial colonization seems to lead to a rejuvenation of the colon which is likely explained by a larger colon mass in conventional versus germ-free animals (*Wostmann*, 1981) thus requiring a stronger proliferation while the core signature of ageing is associated with an inhibition of cellular proliferation. These observations are in some contrast to previous observations about an extended lifespan of germ-free animals (*Thevaranjan* et al., 2018). However, the lifespan-effect is context-dependent. Thus, under caloric restriction germ-free mice show a shortened lifespan (*Tazume* et al., 1991). Moreover, to our knowledge lifespan effects of microbial colonization (i.e. after maturation) have not been tested yet.

Overall, these analyses tend to favour the first hypothesis postulating that ageing is driven by a common process across organs. The tissue-specific manifestation of disease-processes potentially represent downstream consequences of a deregulation of cellular processes in response to the core signature of ageing. Specifically, on a functional and regulatory level the tissue-specific signatures of ageing showed a very high degree of similarity to the core signature of ageing. Moreover, the enrichment of cancer-deregulated genes in the core signature of ageing and a depletion among tissue-specific signatures strongly supports our hypothesis that processes that are actually geared to suppress the proliferation of potential cancer cells are one of the main drivers of ageing-associated regulation conserved across tissues. This is also supported by the enrichment of genes deregulated in degenerative diseases in the tissue-specific signatures, which we suppose arose as a manifestation of the downstream consequences of the core signature in the individual tissues.

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AGEING AND THE MICROBIOME – LESSONS FROM NON-SENESCENT MODELS

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SUMMARY

Research on the biological processes of ageing is at the brink of a revolution with respect to our understanding of its underlying mechanisms as well as our ability to prevent and cure a wide variety of age-related pathologies. Ageing is ordinarily recognized as a process that results from the combined influence of genetic and epigenetic determinants, life-style associated factors and external events. Studies using model organisms such as budding and fission yeasts, the nematode *Caenorhabditis elegans*, zebrafish and mice have generated significant insight into (epi)genetic pathways involved in ageing. However, developing an integrated understanding of these diverse processes and, thereby, achieving insight into the causes and mechanisms of the ageing process, remain a challenge. Here we review recent studies in non-senescent *Hydra* which demonstrate that a continuously high activity of the transcription factor FoxO contributes to continuous stem cell proliferation and at the same time also supports robust colonization of epithelia with a stable microbiome.

THE SIGNATURE OF AGEING

Numerous studies show that ageing is not a series of stochastic defects but a directed biological program that has a conserved genetic background (*Hitt* et al., 1999; *Kenyon*, 2010; *Jeck* et al., 2012; *López-Otín* et al., 2013) and is dependent on both genetic and environmental factors (*López-Otín* et al., 2013; *Dato* et al., 2017). Remarkably, only two proteins have been consistently associated with human ageing and longevity – Apolipoprotein E (Apo-E) and forkhead-box protein O3 (FoxO3). The genes were initially discovered in candidate gene studies of long-lived French (Apo-E) (*Schächter* et al., 1994) and long-lived Japanese/Okinawans in Hawaii (FoxO3) (*Willcox* et al., 2008), by comparing selected candidate gene frequency between long-lived cases (mainly centenarians) and shorter-lived controls. These findings were subsequently replicated in numerous populations worldwide (*Morris* et al., 2015). Genome-wide association studies (GWAS) utilizing larger numbers of cases and controls confirmed these genes as impacting human longevity but no other candidates have emerged with widespread replication (*Broer* et al., 2015). Apo-E plays an important role in clearance of cholesterol and lipoproteins from the circulatory system in vertebrates (*Phillips*, 2014). The gene consists of three alleles – the 'protective' allele ε2, the most common 'neutral' allele ε3 and the 'risk' allele ε4. While ε2 is associated with reduced risk for CVD and dementia as well as with increased life

span, ε4 contributes to opposite effects (*Schächter* et al., 1994; *Bennet* et al., 2007; *Nebel* et al., 2011; *Lindahl-Jacobsen* et al., 2013). FoxO is a conserved transcription factor that shuttles between cytoplasm and nucleus (*Van Der Heide* et al., 2004) and binds directly to the regulatory sequences of its target genes (*Webb* et al., 2016). Four mammalian members of the FoxO family are described, FoxO1, FoxO3, FoxO4 and FoxO6. While FoxO6 is rather specific for neurons, the other FoxOs are expressed in most tissues and are causally linked to cell survival, cellular proliferation and DNA damage repair response (*Monsalve* and *Olmos*, 2011). In humans, a 1.5 Kb region of chromosome 6, in or near intron 2, contains dozens of non-coding *foxO3* single nucleotide polymorphisms (SNPs), in high linkage disequilibrium (close association) (*Donlon* et al., 2012). These *foxO3* SNPs vary in frequency by ethnicity, but one or more SNPs are directly associated with life span in all populations. This finding has been observed in case-control studies of centenarians and shorter-lived controls as well as cohort studies of older individuals (*Broer* et al., 2015; *Santos-Lozano* et al., 2016). Furthermore, since the FoxO protein and its target sites are highly conserved, analyses of human, mouse, *C. elegans* and *Drosophila* homologs recently uncovered FoxO as being a central hub in regulating a network of ageing-related genes (*Webb* et al., 2016). Functional analyses in model organisms verified that the level of FoxO expression is indeed directly linked to life span without detectable costs for the individuals (*Kenyon* et al., 1993; *Hwangbo* et al., 2004; *Schaible* and *Sussman*, 2013). Several human tissues experience synchronized changes in gene expression during the ageing process which were shown to be a direct cause of age-related diseases (*Yang* et al., 2015). Furthermore, also the immune system is affected by the ageing process. "Immunosenescence" describes the deteriorating function of the immune system which results in increased susceptibility of elderly populations to infection, autoimmune diseases and cancer (*Pawalec*, 1999; *Castle*, 2000). The breakdown of mechanical barriers like the epithelia of lung, skin or intestinal tract leads to increased incidences of pathogenic invasion (*Gomez* et al., 2005). Combined with the declined functionality of immune cells and dysregulation of central components of innate immunity, elderly individuals observe a lower effectiveness in coping with the increased inflammation status, consequently resulting in higher morbidity and mortality (*Franceschi* et al., 2005; *Rosenstiel* et al., 2008). Although the understanding of FoxO signalling antagonizing ageing appears crucial to explain life span and health span in humans, the causative role of FoxO can only be addressed in model organisms.

MICROBES MATTER DURING THE AGEING PROCESS

All living beings are metaorganisms, associated with myriads of viruses, archaea and bacteria (*Zilber-Rosenberg* and *Rosenberg*, 2008; *Bosch* and *McFall-Ngai*, 2011). High throughput sequencing studies on humans showed that all epithelia are colonized with different, distinct microbial communities (*Eckberg* et al., 2005; *Grice* and *Segre*, 2011). Furthermore, many severe and chronic diseases, *e.g.* irritable bowel syndrome, inflammatory bowel disease, allergy or asthma, are associated with an altered bacterial

composition on the affected site (*Khosravi* and *Mazmanian*, 2013; *Fujimura* and *Lynch*, 2015). Studies in model organisms show that symbiotic bacteria can provide essential amino acids to the host (*Sandström* and *Moran*, 1999; *Shigenobu* et al., 2000), detoxify harmful substances (*Chaucheyras-Durand* et al., 2010) or degrade complex carbohydrates and thereby make them accessible to the host (*Warnecke* et al., 2007; *Keeney* and *Finlay*, 2011). Additionally, the composition of the gut microbiota regulates the nutrition uptake and is involved in weight control in mice and humans (*Ley* et al., 2005, 2006). Therefore, an organism's health status is reliant on an intact gut microflora (*Goodman* et al., 2009; *Blottière* et al., 2013; *Khosravi* and *Mazmanian*, 2013). Recent research also underlines the importance of bacterial colonization on host behaviour (*Vuong* et al., 2017) as well as fundamental developmental processes involving cell proliferation and immune system maturation (*Rakoff-Nahoum* et al., 2004; *Mazmanian* et al., 2005; *Bates* et al., 2006; *Cheesman* et al., 2011; *Hill* et al., 2016).

Interestingly, the human microbiome is not stable throughout life but experiences significant changes with age. First inoculation occurs during birth as new-borns are colonized with birth canal specific taxa (*Dominguez-Bello* et al., 2016). Disruption of this transmission via caesarean section or prenatal use of antibiotics may increase the risk of coeliac disease, asthma or obesity (*Couzin-Frankel* et al., 2010; *Decker* et al., 2010; *Ajslev* et al., 2011; *Metsälä* et al., 2015) and therefore have a huge impact on the individual's health. The intestinal microbiota resembles an adult stage already at the age of three (*Yatsunenko* et al., 2012), while the community of the skin microbes still undergoes drastic changes in puberty before it reaches a robust state (*Oh* et al., 2012). Over adulthood the bacterial communities are very stable but start to change again in elderly cohorts. The ageing process affects the structure of the human gut microbiota which results in a decrease in species diversity and a higher risk of pathogenic infections (*Keller* and *Surawicz*, 2014). In model organisms the structure of the bacterial community is discussed to have a direct influence on the ageing process as it can be protective against pathogens, shapes the nutrient landscape and affects the inflammation status (*Heintz* and *Mair*, 2014). In the short-lived African turquoise killifish the microbial community of young donors is able to extend life span in older individuals by preventing the decrease in microbial diversity associated with host ageing (*Smith* et al., 2017). Concordantly, latest research in *C. elegans* describes the positive impact of certain bacterial genotypes on host life span and health as non-essential bacterial compounds, *e.g.* colanic acid, are able to regulate mitochondrial dynamics and unfolded protein response (UPR^{mt}) (*Han* et al., 2017).

NON-SENESCENT MODEL ORGANISMS TO STUDY THE INTERACTION BETWEEN FOXO AND MICROBES

While most ageing research has been done in short-living models, some animals show no ageing phenotype and are considered as non-senescent. The freshwater polyp *Hydra* (Figure 1A) has an estimated life span that exceeds 1400 years (*Jones* et al., 2014). It belongs to the class of *Hydrozoa* within

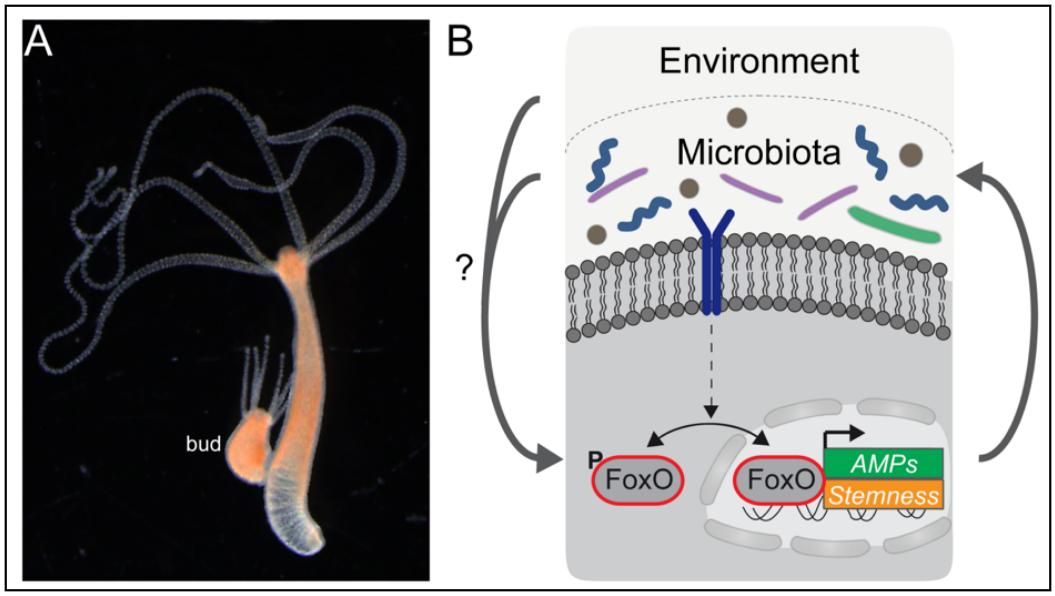

Figure 1: Conserved transcription factor FoxO controls non-senescence and innate immunity in Hydra. (A) Non-senescent Hydra. Continuous proliferation of the stem cell compartment provides the potential of an unlimited life span and clonal growth by asexual budding. (B) Model depicting the transcription factor FoxO as the key regulator of tissue maintenance and as link to the innate immune system. In response to environmental or bacterial signals FoxO can be shuttled between the transcriptionally inactive state in the cytoplasm and the active form in the nucleus. FoxO targets include genes of tissue maintenance and AMPs that directly loop back to the associated microbes. Therefore, FoxO is not only an ageing antagonist but could also be involved in maintenance of the metaorganism (from: *Mortzfeld* and *Bosch*, 2017).

the phylum of *Cnidaria*, the sister group of bilaterians, and therefore holds a basal position in the animal tree of life (*Martindale* et al., 2002). Due to three everlasting stem cell lineages that give rise to about 20 cell types (*Bosch*, 2009), the animals are able to continuously reproduce asexually via budding (Figure 1A). A single homolog of the longevity gene *foxO* was found to be expressed in all three stem cell lineages in *Hydra* (*Hemmrich* et al., 2012). While earlier *Hydra* studies associated the transcription factor with apoptosis (*Lasi* et al., 2010) and stress resistance (*Bridge* et al., 2010), functional analyses uncovered FoxO's role in both stem cell regulation and innate immunity (*Boehm* et al., 2012). *In situ* hybridizations of the cnidarians *Clytia* and *Hydra* revealed *foxO* expression in the area of cell proliferation (*Chevalier* et al., 2006; *Boehm* et al, 2012). Epithelial FoxO deficiency for the latter resulted in a significant increase of differentiated cell mass in relation to the stem cell compartment which resembles the ageing phenotype in senescent animals. Inversely, an overexpression in the interstitial stem cell lineage caused not only an increase in cell proliferation, but also expression of stem cell marker genes in terminally differentiated cells (*Boehm* et al., 2012). FoxO's capacity in other model organisms [*Drosophila* (*Hwangbo* et al., 2004) and *C. elegans* (*Kenyon* et al., 1993)] to delay the onset of age-related processes and to extend life span underlines its conserved potential in stem cell control and tissue maintenance, especially in the absence of classical stem cell

factors like Oct-3/4 or SOX2 (*Chapman* et al., 2010). Remarkably, the changes in tissue homeostasis were accompanied by altered expression of antimicrobial peptides (AMPs), effectors of the innate immune system.

Microbial colonization has been studied intensively in cnidarians as they have a huge impact on marine ecology but also offer a great opportunity to study bacteria-host interactions in organisms of lower complexity. Intracellular photosynthetic dinoflagellates (*e.g. Symbiodinium*) allow corals to populate nutrient-poor environments (*Muscatine* and *Porter*, 1977), however, recent research has shown that basal metazoans in addition to eukaryotic photobionts also harbour extracellular, species-specific bacterial communities (*Rohwer* et al., 2002; *Littman* et al., 2009; *Franzenburg* et al., 2013a). In the case of *Hydra* even closely related species are colonized by specific microbiomes that mirror the phylogenetic relatedness between their host species (phylosymbiosis; *Brooks* et al., 2016). The species-specific microbiomes in cnidarians are extremely stable and still resemble the ones of animals from the wild even after years of culturing under laboratory conditions involving artificial water and feeding (*Fraune* and *Bosch*, 2007; *Franzenburg* et al., 2013a; *Mortzfeld* et al., 2016). The acquisition of bacterial symbionts occurs during early development and establishes in a robust, reproducible pattern (*Franzenburg* et al., 2013b; *Mortzfeld* et al., 2016). Specific bacterial taxa may not only be acquired horizontally (*Apprill* et al., 2009; *Sharp* et al., 2010) but in some cases also via vertical transmission (parent to offspring) (*Fraune* et al., 2010; *Sharp* et al., 2012). Certain taxa of free-living bacteria can even be attracted by the host using chemotactic gradients (*Tout* et al., 2015). Underlining the importance of the microbiome, a study with germ-free animals showed that *Hydra* is protected from fungal infections by its bacterial colonizers. Interestingly, recolonization experiments proofed that a combination of the two most abundant bacteria of the naturally occurring microbiota [*Curvibacter sp.* 75% and *Duganella sp.* 10% (*Franzenburg* et al., 2013a)] was most protective, while monoassociations resulted in insufficient effects (*Fraune* et al., 2015).

AGEING ANTAGONIST FOXO CONTROLS BOTH STEM CELLS AND MICROBES

Besides its well-known conserved function as major tissue regulator and ageing antagonist, FoxO modulates the innate immune system in various model organisms including mouse (*Seiler* et al., 2013), *Drosophila* (*Becker* et al., 2010), *C. elegans* (*Libina* et al., 2003) and *Hydra* (*Boehm* et al., 2012). In mice FoxO signalling has been shown to reduce susceptibility to bacterial infections by reducing oxidative stress and induction of inflammatory cytokines (*Joseph* et al., 2016). Furthermore, FoxO transcription factors directly regulate TLR3-mediated innate immune responses as well as the expression of AMPs and thereby contribute to pathogen clearance in the respiratory tract (*Seiler* et al., 2013). Also in *Drosophila* AMPs are well-known effector molecules of the innate immune system and important regulators of the bacterial colonizers. Here, oral bacterial infection induces FoxO activity in the intestine, while impaired FoxO signalling decreases resistance to

intestinal infections. The inability to raise the expression level of AMPs leads to an elevated bacterial load and a decline in survival (*Fink* et al., 2015). In *Hydra*, the microbiome is selectively assembled by a species-specific combination of AMPs which are predominantly expressed in epithelial cells (*Fraune* et al., 2010; *Franzenburg* et al., 2013a). FoxO-deficient *Hydra* polyps show in addition to defects in stem cell maintenance a severe change of the immune status and drastically altered expression of AMPs (*Boehm* et al., 2012). Remarkably, loss of tissue homeostasis as well as AMP-deficiency compromise the ability to select for microbial communities resembling the polyps' native microbiota (*Fraune* et al., 2009; *Franzenburg* et al., 2013a). Interestingly, a recent study indicates that FoxO signalling may also be involved in the establishment of symbiosis in the early developmental phase of the sea anemone *Aiptasia* (*Wolfowicz* et al., 2016).

Taken together, the conserved transcription factor FoxO appears to combine two functions crucially involved in ageing and health in metazoans (Figure 1B): FoxO is responsible for stem cell regulation, including tissue maintenance and renewal, and controls the innate immune system. In response to environmental (including bacterial) signals, FoxO switches between a transcriptionally inactive state in the cytoplasm and an active form in the nucleus thereby serving as an intracellular control board for environmental signals. The capabilities of the FoxO transcription factor to extend life span and control effectors of the immune system demonstrate a strong and unique mechanism of cross-regulation of tissue homeostasis and innate immunity.

By exploring epithelial FoxO loss-of-function mutants, we recently made two important discoveries (*Mortzfeld* et al., 2018). First, deficiency in FoxO signalling leads to dysregulation of multiple AMP families. Most genes encoding epithelially expressed AMP families including Hydramacin, Kazal and Arminin respond with downregulation to FoxO-deficiency. Only one gene (contig 45266) was found to be upregulated in FoxO deficient animals, suggesting a mainly activating function of FoxO signalling on AMP expression and innate immunity. Second, FoxO loss-of-function polyps were more susceptible to colonization of foreign bacteria and impaired in selection for bacteria resembling the native microbiome. Therefore, FoxO-induced decrease in AMP expression is correlating with differences in microbial colonization and highlights the inhibitory action of AMPs against non-commensal bacteria. In a state of intact FoxO signalling secretion of numerous AMP families provides a highly selective milieu and shapes the microbial composition in a species-specific manner (Figure 1B). FoxO-deficiency reduces the expression of AMPs, which results in a decreased selection pressure on colonizing taxa and in establishment of higher abundances of foreign bacteria in the community. Consequently, especially during the process of colonization, reduced expression of FoxO compromises the resilience of the microbiome.

CONCLUSIONS

Stem cell maintenance and immunity are two of the prominent areas in ageing research. Observations in different organisms indicate that, in contrast

to the essentially static genome, the microbiome is rather dynamic throughout life history. Our observations in non-senescent *Hydra* add support to the view that (i) there is a need to consider the holobiotic nature of an organism when thinking about longevity, that (ii) the microbial environment matters in the context of senescence and contributes to complex processes such as ageing; and that (iii) the hub regulator FoxO presents a direct link between age-related processes and microbial colonization. In the newly discovered world of metaorganisms, stem cell proliferation and immunity are part of a global program, which seeks to fuse stem cell biology with ecological concepts and the rules governing the interactions between an organism and its microbial environment (*Mortzfeld* and *Bosch*, 2017; *Mortzfeld* et al., 2018).

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AGEING AND GUT TO BRAIN SIGNALLING

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INTRODUCTION

There are several major gut-brain communication pathways. These include signalling via the vagus and spinal nerves, immune factors via cytokines, endocrine signalling via gut hormones, and microbial products that reach the brain via bloodstream (*Holzer* and *Farzi*, 2014). In this brief essay, the focus is on the effects of ageing on gut to brain signalling via the vagus.

AGEING GENERAL

There is a general functional decline in all mammalian physiological systems in advanced old age. For the intestine, this includes a decrease in motility, small intestine permeability and mucosal defence (*Calvani*, 2018). In addition, there are associated changes in lifestyle and dietary habits, reduced somatic mobility and increased probability of hospitalisation and medication use (*Han* et al., 2017). There may also be deficits in memory and increases in anxiety in humans (*Han* et al., 2017) or mice (*Scott* et al., 2017). Other age-related changes include alterations in the HPA axis with decreased negative feedback mechanisms and increased basal glucocorticoid release (*Calvani* et al., 2018). Circadian rhythm disruption and altered sleep/wake cycles have been also been reported to increase in prevalence in old age (*Hood* and *Amir*, 2017; *Calvani* et al., 2018). The microbiome and gut to brain signalling has increasingly been focused on in the ageing research field because of the changing nature of the microbiome during the lifespan and recognition that how the intestine signals to the enteric and central nervous systems alters during ageing. Thus, to understand how the microbiome influences ageing and devise potential remedial interventions it is necessary to understand the gut to brain neural signalling pathway.

MICROBIOME

Eli Metchnikoff proposed that people in Eastern Europe live longer because they consume lactic acid bacteria (*Metchnikoff*, 1908). The link between microbiota and longevity is also supported by the observation that germ-free mice live longer than conventional controls (*Dinan* and *Cryan*, 2017). This association does not mean that the intestinal microbes themselves age. However, here is a decrease in intestinal microbial diversity in the elderly with, for example, an increase in *Bifidobacteria,* but a decrease in their relative proportion (*Claesson* et al., 2011; *Dinan* and *Cryan*, 2017; *Calvani*

et al., 2018). A confounding factor is that age-related alterations in diet can lead to changes in the resident microbiome (*David* et al., 2014). It is at present not established entirely whether changes in microbiota are the cause or result of host ageing.

There is some experimental evidence that for the nematode *Caenorhabditis elegans*, intestinal microbes might manipulate lifespan. The prolongevity effects of metformin in *C. elegans* appear to depend on the suppression of folate metabolism by the worm's intestinal *E. coli* bacteria (*Cabreiro* et al., 2013). In the absence of intestinal *E. coli*, the *C. elegans'* lifespan was extended, but metformin reduced its lifespan. In addition, the effect of supplementary *E. coli* was dependent on the strain used; metformin resistant E. *coli* produce short lifespans in response to metformin, but metformin sensitive *E. coli* strains impaired folate metabolism and slowed ageing (*Cabreiro* et al., 2013). In a separate *C. elegans* model, genetic manipulation of *E. coli* to increase their secretion of the polysaccharide colanic acid increased the worm's lifespan by action on the host's mitochondrial homeostasis (increases fragmentation) and unfolded protein response. Purified colanic acid alone was sufficient to promote longevity (*Han* et al., 2017). Dietary colanic acid also increased lifespan of *Drosophila melanogaster* (*Han* et al., 2017). In other experiments, it was shown that *C. elegans* fed on NO-deficient bacteria have a reduced lifespan whereas worms exposed to NO donors had extended lifespans (*Gusarov* et al., 2013). These remarkable results imply that, for *C. elegans* (and possibly for *Drosophila*); intestinal microbes have a causal effect on the host lifespan.

The resident and transient gut microbiota can also influence gut to brain signalling via neuroactive molecules that they generate and release. Intestinal bacteria can release a variety of neurotransmitters including GABA, noradrenaline, dopamine, acetylcholine and 5-HT. These neurotransmitters can cross the mucosal layer to act on neuronal fibres innervating the epithelium (*Dinan* and *Cryan*, 2017; Calvani *et al.*, 2018). Short-chain fatty acids produced by intestinal microbes may affect the enteric nervous system or brain directly or with respect to butyrate via epigenetic modulation through histone deacetylases (Dinan and Cryan, 2017; *Calvani* et al., 2018). Reduced levels of short-chain fatty acids associated with old age have been associated with increased susceptibility to intestinal inflammatory disorders, and it has been speculated that short-chain fatty acid availability may affect human longevity (*Nagpal* et al., 2018).

INFLAMMATION

Ageing is associated with raised background inflammation (inflammageing). Inflammageing is associated with increased risks for morbidity and mortality and age-related diseases (*Franceschi* and *Campisi*, 2014). The increased background inflammation appears to be accompanied by reduced inflammatory response to acute challenges (*Franceschi* and *Campisi*, 2014) perhaps because the acute inflammatory response starts off from a higher background level to a given ceiling. A counterpoint is that chronic inflammation in the very elderly may have an adaptive role possibly underlying tissue remodelling; for example, healthy centenarians can have an increased background inflammation with associated hypercoagulability, but this may be

balanced by other anti-inflammatory mechanisms (*Franceschi* and *Campisi*, 2014).

It has been reported that intestinal commensal bacteria that maintain immune tolerance tend to be reduced in aged people, but opportunistic bacteria that stimulate intestinal inflammation are often increased in number (*Nagpal* et al., 2018). The old age inflammatory phenotype can be manipulated experimentally. For example, when the resident microbiome was transplanted from old to young germ-free mice, there was increased inflammation in the intestines, increased leakage of bacterial components into the circulation and increased T-cell activation systemically (*Fransen* et al., 2017). The importance of inflammageing in gut function is illustrated by the IP injection of the tumour necrosis factor alpha antagonist etanercept in aged mice, which reversed the old age related decrease in colon serotonin transporter and slowing of intestinal motility (*Patel* et al., 2017).

Inflammageing is manifest in the nervous system through the activation of microglia (the tissue macrophages of the brain) and may be associated with neurodegenerative pathologies such as Parkinson's disease (*Jyothi* et al., 2015). The roles of microglia are complex with metabolic and neuroprotective functions, but in old age the microglial sensome can shift from being mainly protective to favouring neuroinflammation (*Hickman* et al., 2013). Microglia activation and/or development are influenced by the gut microbiome (*Erny* et al., 2015), since germ-free mice have defective and immature microglia, and antibiotic treated mice also had immature microglia. Reconstitution of microbiota from donor specific pathogen free mice restored microglia function (*Erny* et al., 2015). The influence that the microbiome appears to have on brain microglia might explain, at least to some extent, the shift in old age to a pro-inflammatory microglial phenotype.

GUT MOTILITY

Chronic constipation, with or without incontinence, tends to increase in prevalence with old age (*Higgins* and *Johanson*, 2004; *De Giorgio* et al., 2015; *Ranson* and *Saffrey*, 2015). In north America, and after age 65 y, 16 or 26% (male vs female) have reported chronic constipation and this rose to 26 or 34% at the 84th year (*De Giorgio* et al., 2015). Beyond psychosocial and economic factors, it is believed that ageing of the gut itself can underlie these numbers.

Changes in gastrointestinal function with ageing occur in both humans and animal models and the upper and lower gut are most at risk. The oesophagus, stomach, colon, and rectum are targeted with difficulties in swallowing and defaecation; there is also an increase in intra-colonic pressure (reviewed in *Hall*, 2002; *Wade*, 2002; *Soenen* et al., 2016). Aged rats have fewer colonic migrating motor complexes as measured by electrodes in fasting conscious animals (*Metugriachuk* et al., 2006). Similarly, aged (2 y old) mice have reduced total faecal output with decreased water content in the pellets compared to their younger (3 mo) counterparts (*Patel* et al., 2012). In particular, there was a decrease in velocity of epoxy coated pellet movement in the colon and an increase in impaction (*Patel* et al., 2012).

MYENTERIC PLEXUS

Since the enteric nervous system (ENS) controls gut motility and secretion, it is reasonable to ask whether the increased prevalence of constipation in the elderly is accompanied by alterations or reductions in number of enteric neurons. Indeed, reductions in the number of myenteric neurons have been reported for the elderly. Along the gut there is an apparent 34% decrease in the number of myenteric neurons in the elderly, with the largest reduction (>38%) reported for the duodenum (*de Souza* et al., 1993). In the human colon myenteric plexus, there was a decrease in the number of choline acetyltransferase positive neurons while neuronal nitric oxide positive neurons did not decline in number (*Bernard* et al., 2009). *Hanani* et al. (2004) reported an increase with age in the proportion of colonic myenteric ganglia with cavities and a decrease in the proportion of normal ganglia.

Similar reductions in myenteric neurons have been reported for animals. Moreover, numerous animal studies suggest that the ENS is more susceptible to age-related degeneration than other nervous systems (*Saffrey*, 2013). Aged Fisher 344 rats showed significant reductions in the number of myenteric neurons (and associated glia) in both small and large intestines, except for the rectum (*Phillips* et al., 2004). The same was true for mice when young (3 mo) were compared to old (24 mo) ones (*El-Salhy* et al., 1999). An analogous reduction in neurons (5 vs 25 mo old) has been reported for the myenteric and submucous plexuses in the small intestine of the guinea pig (*Phillips* and *Powley*, 2007; *Zanesco* and *Souza*, 2011).

The enteric nervous system is a complete nervous system in the sense that it contains primary afferent, inter- and motor neurons. Also, peristalsis and mixing occur *ex vivo* after all nervous connections with the extrinsic nervous systems have been severed, demonstrating that the enteric nervous system can function independently. There are several functional classes of specialised neurons within the ENS subserving different functions (*Kunze* and *Furness*, 1999), but only one class, intrinsic primary afferent neurons (IPANs), is both chemo- and mechanosensitive and serves as an intramural gatekeeper relaying more than two thirds of signals originating from luminal contents to the afferent vagus nerve (*Perez-Burgos* et al., 2014). The remainder (< one third) of afferent chemoceptive vagal signals derive from direct innervation of the intestinal epithelium by the vagus. These considerations make it important to ask, with respect to gut to brain signalling, whether there are enteric neurons particularly sensitive or resistant to the effects of ageing.

Cholinergic (choline acetyltransferase (ChAT) immunopositive) enteric neurons appear to be especially vulnerable to ageing-related decrease when compared to other histochemical phenotypes (*Camilleri* et al., 2008). The number of ChAT positive human myenteric neurons decreased with age while nNOS neurons do not appear to change (*Bernard* et al., 2009). In rodents too, ChAT positive were preferentially lost (after 12 mo of age) with nicotinamideadenine dinucleuotide phosphate diaphorase positive neurons (nNOS containing) being spared (*Phillips* et al., 2003; *Phillips* and *Powley*, 2007). Intriguingly, this loss of enteric cholinergic neurons is paralleled by a preferential decrease in cholinergic innervation in the aged brain (*Casu* et al., 2002). However, enteric

cholinergic neurons belong to several functional classes including IPANs, excitatory motor neurons and interneurons, and the question arises which of these classes are the most vulnerable.

It has been suggested that IPANs are most susceptible to neurodegeneration with age (*Wade*, 2002; *Wade* and *Cowen*, 2004), exhibiting degeneration to a greater extent than other phenotypes, e.g. serotonergic interneurons or nitrergic inhibitory motor neurones. The evidence for this is incomplete, relying mainly on the decrease in old age of vitamin D-dependent 28 kDa calcium binding protein (calbindin) positive neurons or the decrease in substance P positive neurites in the guinea-pig or rodent myenteric plexus (*Wade*, 2002; *Wade* and *Cowen*, 2004). Because IPANs are positive for substance P, choline acetyltransferase (*Phillips* et al., 2003) and calbindin there is at least circumstantial evidence that IPANs may be principally sensitive to the effects of ageing.

Ageing also affects the neurites (processes) of myenteric neurons. Age-related development of dystrophic nerve fibers alters the shape of human (*Hanani* et al., 2004) and guinea pig enteric ganglia (*Abalo* et al., 2007), presumably due to the presence of swollen fibres. In aged mice (>18 mo old) calbindin containing and nNOS immunoreactive neurons developed multiple swollen processes when compared to young mice (3 mo) (*Gamage* et al., 2013). Other signs of degeneration and pathology include the accumulation in guinea pig or rat of lipofuscin (pigment granules composed of lipid-containing residues of lysosomal digestion) (*Phillips* et al., 2004; *Abalo* et al., 2007) and the presence of α-synuclein immunoreactive aggregates and hyperphosphorylated microtubule-associated Tau protein (*Phillips* et al., 2009). It should be noted that glial cells and interstitial cells of Cajal also decrease with age, although neuronal reduction seems to occur first (*Phillips* and *Powley*, 2007; *Camilleri* et al., 2008; *Saffrey*, 2013, 2014).

Despite neural loss and degeneration, the ageing gut and the enteric nervous system have a significant functional reserve since intestinal motility, although reduced, appears largely intact until the animal is very old (*Saffrey*, 2013).

EXTRINSIC AFFERENTS

The intestine is innervated by nerve fibres whose cell bodies lie outside the gut wall. These fibres run within mesenteric neurovascular bundles that branch off arterial arcades supplying intestinal segments. The nerve bundles contain vagal, and spinal and sympathetic nerve fibres.

Single unit (extracellular action potentials from single nerve fibres) mesenteric nerve fibre recordings have been made using *ex vivo* segments of human ileum or sigmoid colon (*Yu* et al., 2016). For these recordings, background firing rates correlated negatively with age (from 24 to 77 y old), and number of single units showing burst firing patterns also decline with age. There was also a decrease in the number of substance P containing nerve fibres (presumably either intrinsic or extrinsic) innervating the luminal mucosa (*Yu* et al., 2016). Paradoxically, there was also an increase in the density of mucosal mast cells and ileal enterochromaffin cell numbers with age, which the authors attributed to a compensatory mechanism for the

sensory neurodegeneration (*Yu* et al., 2016). These results parallel others that report decreased intestinal visceral pain perception in old age (*Lasch* et al., 1997; *Lagier* et al., 1999).

The diminished mesenteric nerve fibre discharge seen in elderly humans has been replicated in ageing mice. Single and multiunit spike recordings have been made from the mesenteric nerve of young and aged (3 mo vs 12 and 24 mo old) C57bl/6 mice (*Keating* et al., 2016). Baseline mesenteric multiunit spiking activity was significantly decreased for both jejunal and colonic mesenteric afferent fibres in old (24 mo) compared to young (3 mo) mice. Mesenteric nerve fibre responses to intraluminal fluid distension were also reduced in old mice. The TRPV1 receptor is also a mechanoreceptor in the intestine mediating the response of mesenteric afferents to fluid distension (*Rong* et al., 2004). It is therefore of interest that intestinal TRPV1 receptor expression was diminished in the older mice (*Keating* et al., 2016).

There appears to be little functional data on the effects of ageing on the afferent vagus compared to the mixed mesenteric nerve. There are, however, reports in the literature that the vagus nerve of old animals displays degenerative anatomical changes. In aged rats, vagal afferents appear to have swollen varicosities in fibres innervating the myenteric plexus, smooth muscle and mucosa (*Phillips* and *Powley*, 2007). It is not established that there is an actual decrease in the number of vagal fibre endings supplying the myenteric plexus but there are dystopic changes including dilations and swellings (*Phillips* et al., 2010) in the NIH Fisher 344 rat model of ageing. The extent of the terminal arbors is also reduced compared to young rats. Similar degenerative changes are seen in vagal afferents to mucosal villi and the musculature (*Phillips* et al., 2010). It is thought that this reduction or the pathological changes in vagal innervation results from a decrease in trophic factors released from the targets of vagal innervation (*Phillips* et al., 2010) in particular NT-4 (*Phillips* et al., 2010).

Sympathetic nerve fibres originate from prevertebral sympathetic ganglia to innervate the ENS, intestinal arteries and smooth muscle (*Phillips* and *Powley*, 2007). Tyrosine hydroxylase staining can be used to identify the sympathetic innervation of the intestine, and positive fibres that supply the myenteric and submucous plexuses of aged (24 mo old) rats display axonopathies such as swollen axons and small sparse terminals (*Phillips* and *Powley*, 2007). Functionally, old age has been associated with decreased ability of the sympathetic nervous system to adapt to environmental or interoception of stimuli (*Hotta* and *Uchida*, 2010).

INTRAMURAL GATEKEEPER

90 to 95% of sensory neuron processes innervating the intestinal epithelium arise from the ENS, with the rest originating from neurons whose somata are located outside the intestine (*Keast* et al., 1984; *Ekblad* et al., 1987). In agreement with this anatomical data on epithelial innervation density, is the recent discovery that more than two thirds of vagal afferent signals evoked by a luminal probiotic is relayed to the vagus via the enteric neurons (*Perez-Burgos* et al., 2014). Neuroactive luminal molecules first excite juxtaepithelial neurites belonging to IPANs whose cell bodies are located within

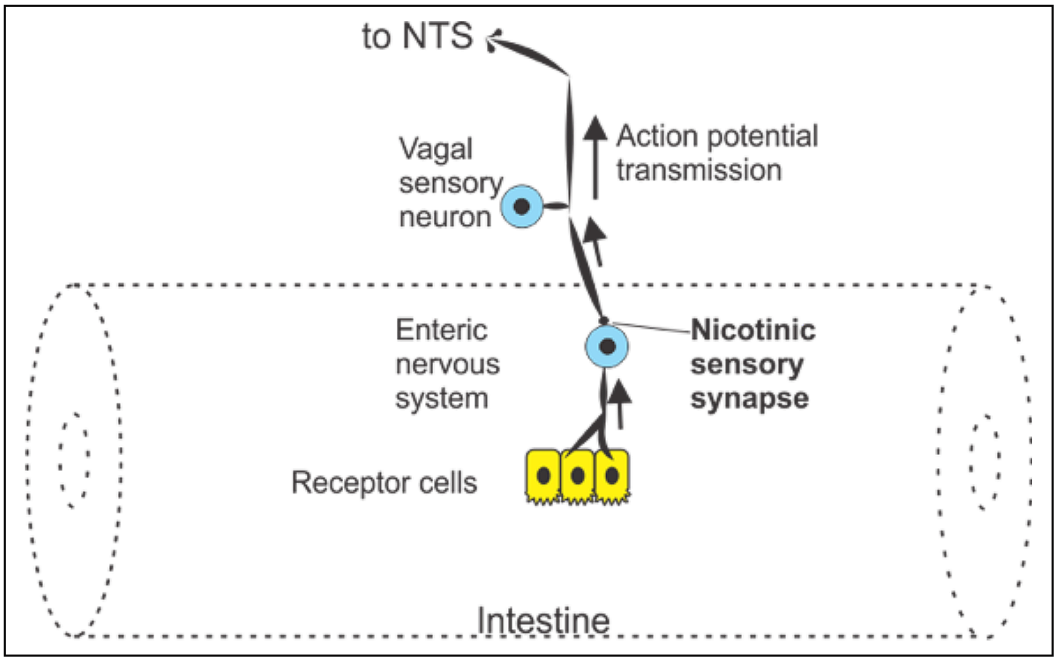

Figure 1: Gut to brain afferent signalling pathway. Sensory neurons within the enteric nervous system respond to luminal stimuli and relay information to the brain via a nicotinic sensory synapse that activates the afferent vagus.

the enteric nervous system. The excited IPANs release acetylcholine and perhaps other neurotransmitters to activate vagal intraganglionic laminar endings (IGLEs) which closely surround and abut the IPANs (*Berthoud* et al., 1997; *Perez-Burgos* et al., 2014). The IPAN to IGLE nicotinic sensory synapse is perfectly positioned to act as a gatekeeper to regulate microbial to brain signalling (Figure 1). Accordingly, the amount of information transmitted to the brain via the vagus would be markedly influenced by whether IPANs are refractory or readily responsive to luminal stimuli, and by the density of IPAN sensory innervation of the epithelium.

Given the important role of enteric IPANs in gut to brain signalling, the vulnerability of the enteric nervous system to old age in terms of numbers and degeneration would have a significant impact on the amount and quality of information reaching the brain from the gut. Thus, even if the number of vagal afferent fibres are not appreciably reduced it can be predicted that there would be decreased constitutive and stimulus evoked vagal afferent responses to luminal microbial stimuli in old age. Exacerbating these potential impediments in gut to brain signalling there is the added problem of decreased microbial diversity (*Nagpal* et al., 2018) demonstrated in old age. In summary, there are several reasons why gut microbes to brain signalling via the vagus is compromised in old age, with a degenerating or dysfunctional enteric nervous system being a major contributor to the reduced signalling.

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STROKE AND MICROBIOTA – CURRENT RESEARCH AND CONCEPTS

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SUMMARY

Stroke is a highly prevalent cerebrovascular disease with limited treatment options. Although therapeutic interventions have evolved over the last 30 years, stroke remains the second most common cause of death and the leading cause for long-term disability worldwide. While primarily a disease caused by acute ischaemia to the brain, stroke pathology is characterized by a strong immune component. The intestinal microbiota and gut immune system have recently been linked to the inflammatory processes observed after stroke. It has been shown that intestinal microbiota composition strongly affects the polarization state of immune cells found in Payer's patches and submucosa of the small intestine. This may result in an imbalance of regulatory and effector type T-cells in the gut. Because gut immune cells traffic to the brain after stroke they influence the local immune response triggered by ischaemic injury by damping or increasing inflammation. In the context of stroke, the communication between brain and gut is bidirectional and stroke can impact the composition of intestinal microbiota. In addition, intestinal microbiota can become a source of opportunistic infectious agents and contribute to post-stroke infections thereby increasing morbidity and mortality. In this review I will summarize current concepts on the role of intestinal microbiota and the intestinal immune system in stroke, review the effects stroke and ageing has on the gut microbiome, and describe studies that identified commensal intestinal microbiota as a source of infectious bacteria participating in post-stroke infections of the respiratory tract.

INTRODUCTION

Ischaemic stroke is a highly prevalent and devastating disease with limited therapeutic options (*Henninger* et al., 2010). Most therapeutic efforts have focused on counteracting the intrinsic brain mechanisms leading to ischaemic injury (*Suwanwela* and *Koroshetz*, 2007). Unfortunately, these efforts have met with limited success in large clinical trials, reflecting, in part, an incomplete understanding of the pathological processes triggered by cerebral ischaemia (*Moskowitz* et al., 2010). Inflammation is a key component in the pathophysiology of ischaemic stroke (*Iadecola* and *Anrather*, 2011). Numerous experimental approaches have concentrated on developing the therapeutic potential of immunomodulation (*Macrez* et al., 2011). However, our understanding of the interaction between resident brain cells and peripheral immune cells infiltrating the post-ischaemic brain, and their role in tissue damage and repair is still incomplete (*Macrez* et al., 2011). The peripheral

immune system plays an essential role in the pathophysiology of stroke, involving both innate and adaptive immune cells (*Iadecola* and *Anrather*, 2011). In turn, neuro-humoral brain signals generated during stroke induce immunosuppression, which contributes to post-stroke morbidity by increasing the risk for infection in stroke patients (*Meisel* et al., 2005). The immune system is forged by the continuous interaction with commensal microbiota that populate the epithelial surfaces and this interaction is essential for immune cell development, maintenance and function (*Mazmanian* et al., 2005; *Hill* and *Artis*, 2010). Specifically, intestinal commensal bacteria, the most abundant symbiotic compartment in the body, have emerged as a potent regulator of the immune system (*Nishio* and *Honda*, 2012). In particular, the microbiota exerts powerful effects on lymphocyte populations, such as regulatory T-cells (Treg) and γδT-cells, which are involved in the mechanisms of cerebral ischaemic injury (*Iadecola* and *Anrather*, 2011). Conversely, stroke also affects the peripheral immune system and alters the composition of the intestinal microbiome. This sets the stage for complex multidirectional interactions within the microbiota-gut-brain axis that is likely to affect stroke outcome.

EFFECTS OF MICROBIOTA ON IMMUNE CELLS INVOLVED IN STROKE PATHOPHYSIOLOGY

Accumulating evidence suggests that commensal microbes influence the outcome of diseases in which the immune system is involved. For example, germ-free (GF) animals are protected from experimental autoimmune encephalomyelitis (*Ochoa-Repáraz* et al., 2009; *Berer* et al., 2011; *Lee* et al., 2011), ankylosing spondylitis (*Sinkorová* et al., 2008), or rheumatoid arthritis (*Wu* et al., 2010; *Chappert* et al., 2013). Increasing evidence implicates systemic immunity in the pathophysiology of ischaemic brain injury (*Iadecola* and *Anrather*, 2011). Thus, cells associated with innate and adaptive immunity play a defining role in the outcome of cerebral ischaemia (*Iadecola* and *Anrather*, 2011). As reviewed below, intestinal microbes are critical for the development, expansion and activity of immune cells implicated in stroke pathophysiology.

Lymphocytes

Th1 and IL-17 secreting T-cells are detrimental in the early phase of cerebral ischaemia and lymphocyte-deficient mice are protected in models of focal ischaemia (*Hurn* et al., 2007; *Kleinschnitz* et al., 2010). The mechanism of T-cell mediated brain injury after ischaemia does not involve classical antigen-mediated T-cell activation and the cytotoxic activity might be related to innate T-cell functions (*Kleinschnitz* et al., 2010). Accordingly, IL-17 secreting γδT-cells, that do not undergo classical antigen-dependent T-cell activation, have been shown to contribute to ischaemic injury (*Shichita* et al., 2009; *Gelderblom* et al., 2012;). Peripheral development of T-cells is greatly affected by microbiota (*Kuhn* and *Stappenbeck*, 2013). Beside Th17 cells (*Ivanov* et al., 2006), intestinal γδT-cells residing in the epithelial layer and submucosa (lamina propria) are also a major source for IL 17 (*Roark* et al., 2008; *Korn* and *Petermann*, 2012; *Hou* et al., 2013). IL-17-producing γδT-cells are responsible for the colitis seen in Treg deficient mice and the disease is abrogated in γδT-

cell-deficient or antibiotic-treated mice, suggesting activation of γδT-cells by commensal bacteria (*Park* et al., 2010). Moreover, intestinal IL-17-producing γδT-cells are reduced in antibiotic-treated mice (*Duan* et al., 2010; *Park* et al., 2010). While effector T-lymphocytes may contribute to focal ischaemic injury (*Yilmaz* et al., 2006; *Hurn* et al., 2007), Treg can have a protective effect by down-regulating post-ischaemic inflammation. Treg appear in the ischaemic tissue after the acute phase and confer neuroprotection by IL-10 secretion, an effect that might be antigen independent (*Liesz* et al., 2009, 2013; *Planas* and *Chamorro*, 2009; *Stubbe* et al., 2013). Intestinal Treg express high levels of IL-10, which is indispensable for maintaining intestinal homeostasis by suppressing Th17 differentiation (*Chaudhry* et al., 2009; *Huber* et al., 2011), γδT-cell proliferation (*Park* et al., 2010), and myeloid cell activation (*Takeda* et al., 1999). The gut microbiota has a role also in the development of intestinal and extra-intestinal Treg. For example, colonic FoxP3$^+$ Treg cells are reduced in GF mice (*Round* and *Mazmanian*, 2010; *Atarashi* et al., 2013). Furthermore, in a mouse model of EAE, the generation of Treg from splenic and lymph node lymphocytes was increased in GF animals (*Lee* et al., 2011). Therefore, mucosa-microbial interactions influence Treg lymphocytes in the gut as in lymphoid organs.

Myeloid cells

In experimental stroke, as in human stroke, monocytes/macrophages infiltrate the brain early after ischaemia and persist for several weeks (*Schilling* et al., 2003; *Gelderblom* et al., 2009). Inhibition of post-ischaemic monocyte recruitment by interfering with CCR2 and CX3CR1 chemokine receptor utilization is beneficial in experimental stroke (*Hughes* et al., 2002; *Soriano* et al., 2002; *Denes* et al., 2008; *Schilling* et al., 2009). Monocyte/macrophage development is influenced by signals from intestinal bacteria. Systemic monocyte counts are normal in GF mice, but they are functionally impaired and do not mount an IFNγ response after bacterial stimulation (*Ganal* et al., 2012). Dendritic cells (DC) have been implicated in stroke pathology (*Felger* et al., 2010; *Gelderblom* et al., 2017). Intestinal DC and macrophages are essential to maintain a tolerogenic environment in the gut and are strong inducers of Treg (*Coombes* et al., 2007; *Sun* et al., 2007). Neutrophils play a part in post-ischaemic inflammation by limiting tissue perfusion due to intravascular clogging (*del Zoppo* et al., 1991; *Dawson* et al., 1996), destabilizing the BBB by releasing MMP9 (*Rosell* et al., 2008; *Ludewig* et al., 2013), and by generating ROS and NO (*Garcia-Bonilla* et al., 2014). Neutrophils isolated from the bone marrow of GF or antibiotic-treated mice exhibit impaired *ex vivo* bacterial killing (*Clarke* et al., 2010). All together, there is ample evidence that the intestinal environment shapes the activation state of immune cells involved in the immune response to ischaemic brain injury.

ALTERED INTESTINAL IMMUNE STATUS AND ITS EFFECTS ON ISCHAEMIC STROKE

The role of altered intestinal microbial composition on stroke outcome has been investigated in mice undergoing transient focal ischaemia by intraluminal obstruction of the middle cerebral artery (MCA) (*Benakis* et al.,

2016). In this study mice were treated with amoxicillin (amoxicillin/clavulanic acid; AC) to alter the microbiome resulting in elimination of *Clostridia* and *Bacteroidetes* with concomitant expansion of *Proteobacteria*. The authors observed remarkable neuroprotection both on the anatomical and functional level. Because protection was not observed after one week of AC treatment, a time point when microbial alterations were already present, let the authors to hypothesize that changes in the immune system leading to an altered inflammatory response after stroke might be at the basis of the observed neuroprotection. Analysis of the intestinal immune system revealed an increase in Treg and a decrease in $IL17^+$ γδT-cells, whereas Th17 cells were not affected. In addition, the authors could show that DC isolated from AC treated animals showed higher expression of the "tolerogenic" marker protein CD103 and were more potent in inducing Treg when co-cultured with naïve $CD4^+$ T-cells. After MCA occlusion $IL17^+$ γδT-cells were increased in the meninges of control but not AC-treated mice indicating that the deleterious IL17 response was blocked in mice with altered microbiota. Accordingly, the protective effect of AC treatment was absent in IL17 deficient mice. Similar alterations in T-cell homeostasis resulting in reduced Treg and increased Th1 and Th17 cells were observed after ischaemic stroke in mice and post-stroke immune changes could be prevented by faecal matter transplants from healthy donors (*Singh* et al., 2016). It was also shown that T-cells trafficked from the intestine to the brain after stroke indicating that the polarization state of intestinal T-cells might determine the course of post-ischaemic inflammation in the brain (*Benakis* et al., 2016; *Singh* et al., 2016). A recent study by Singh and coworkers (*Singh* et al., 2018) compared GF animals with GF animals recolonized with conventional SPF flora in a permanent focal ischaemia model in mice. Interestingly, GF animals showed increased infarct volume and decreased inflammatory response to cerebral ischaemia. Using $Rag1^{-/-}$ mice that lack T and B cells, the authors found that ischaemic lesions in GF $Rag1^{-/-}$ mice were not different from lesions of recolonized $Rag^{-/-}$ mice, albeit the lesions were smaller than in wild-type mice. These studies identified disturbances of the intestinal immune homeostasis as an important contributor to ischaemic brain injury.

EFFECTS OF STROKE ON THE INTESTINAL MICROBIOME

The relationship between microbiota and stroke is complex, given that ischaemic brain injury has been found to alter gut microbiota composition. After stroke, up to 50% of patients experience gastrointestinal symptoms, including dysphagia, gastrointestinal bleeding, and constipation. Stroke is likely to alter the intestinal microbial environment by altering intestinal epithelial permeability, motility, mucus biosynthesis and the immune system. Large ischaemic infarcts in mice after transient MCA occlusion altered the gut microbiome partially by inducing intestinal paralysis leading to reduced microbial diversity and overgrowth of *Bacteroidetes* species (*Singh* et al., 2016). Another study found that after stroke, relative abundance of *Peptococcaceae* increased in the mouse caecum, whereas the proportion of *Prevotellacea* decreased (*Houlden* et al., 2016). This alteration was paralleled by aug-

mented release of noradrenaline and the reduction of mucoprotein-producing goblet cells. Investigating the mucosa-associated microbiome it was found that microbial communities within the mucosa were significantly different between sham-operated and post-stroke mice at 24 hours following surgery (*Stanley* et al., 2018). Microbiota composition was substantially different in all sections of the gastrointestinal mucosa. The main changes in mucosal microbiota composition were due to increased abundance of *Akkermansia muciniphila* and clostridial species while operational taxonomic units (OTUs) potentially belonging to the *Barnesiella* genus were reduced.

ROLE OF INTESTINAL MICROBIOTA IN POST-STROKE INFECTIONS

Secondary infections, including pneumonia and urinary tract infections, are major complications in stroke patients. Pneumonia occurring in up to 20% of all stroke patients is the most common serious medical complication in stroke care resulting in a 2.5-fold increased mortality rate (*Meisel* et al., 2005). Post-stroke immunodepression has been recognized as a key factor in facilitating infections. Immunodepression in stroke patients manifests itself in reduced peripheral blood lymphocyte counts and impaired T- and natural killer (NK)-cell activity (*Meisel* et al., 2005). Experimental stroke in rodents induced a rapid and extensive apoptotic loss of lymphocytes in lymphoid organs and peripheral blood (*Prass* et al., 2003). The effects were linked to increased noradrenalin and glucocorticoid secretion through overactivation of the hypothalamic-pituitary-adrenal axis. Moreover, stroke also reduced intestinal lymphocyte counts likely resulting in suppression of the intestinal immune system and possibly facilitating bacterial invasion (*Schulte-Herbrüggen* et al., 2009). Further it has been shown that the majority of bacteria detected in stroke patients who developed infections were common commensal bacteria that normally reside in the intestinal tract including *Escherichia coli*, *Enterococcus spp.*, and *Morganella morganii* (*Stanley* et al., 2016). Using the transient MCA occlusion model in mice, the same study found that loss of intestinal epithelial barrier function, which was observed as early as 3 hours after stroke, facilitated the translocation of facultative pathogenic bacteria into the blood stream from where they may have colonized the respiratory tract. Using bioinformatic algorithms it was predicted that the small intestine and liver were the most likely origins of microbial communities present in the lung of post-stroke mice.

STROKE, MICROBIOME AND AGEING

Age has a strong effect on stroke outcome and the composition of the intestinal microbiome. Aged (18-20 month) mice show increased *Firmicutes* and reduced *Bacteroidetes* (*Spychala* et al., 2018). The ratio of *Firmicutes* to *Bacteroidetes* (F:B) increased 9-fold compared to young (8-12 week) mice. Interestingly, stroke increased the F:B ratio in aged as well young mice. When young mice were transplanted with faecal microbiota harvested from aged

mice, stroke outcome was worse. On the other hand, aged mice transplanted with a "young microbiome" performed better than control mice. It was also found that short chain fatty acids, potential mediators of neuroprotective effects afforded by commensal microbiota, were decreased in faecal matter of aged mice and levels could be recovered after faecal transplantation. Aged mice are also more likely to develop sepsis after cerebral ischaemia than young animals (*Crapser* et al., 2016). While high bacterial burden was detected in the mesenteric lymph nodes and spleens of both young and aged mice after stroke, substantial bacterial colonization of liver and lung was found only in aged stroke mice and not in those from young. Increased bacterial dissemination in aged mice was associated with increased gut permeability, lymphopenia, and increased plasma levels of inflammatory markers as compared to young mice.

CONCLUSIONS

Increasing evidence points to a role of intestinal microbiota in stroke pathophysiology and outcome. Recent studies have identified a gut-brain immune axis as an essential component of the inflammatory response to stroke. The microbiota-gut-brain axis is complex and shows a strong bi-directional interaction. Stroke disturbs intestinal motility and epithelial barrier function and alters the composition of intestinal microbiota. Therefore, it is likely that stroke itself induced changes of the microbial environment contribute to stroke pathology in the subacute and chronic stages of the disease. The current research also evokes many questions. Is the presence of certain microbiota a risk factor for stroke? Could one predict stroke outcome based on gut microbial composition? Besides the immune axis, are there humoral or neural pathways engaged in microbiota-brain communications that will affect stroke? Future studies are likely to address some of these questions.

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THE MICROBIOME, IMMUNE DEVELOPMENT, CHRONIC INFLAMMATION, AND CANCER

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INTRODUCTION

Our microbiota is an integral part of our mammalian selves. Indeed, all multi-cellular eukaryotic hosts across the tree of life have an essential and characteristic microbiota that influences host development and resistance to disease. Within complex organisms such as vertebrates, we harbour numerous microbial communities whose composition and function are relevant to their habitat at different body sites, such as the intestines ("gut"), skin, and oral cavity. Our gut microbiota is perhaps the best studied, most abundant, and arguably, the most influential microbiota that impacts host phenotypes. In the recent decades, the development of several scientific tools has exponentially increased our understanding of the microbiota and interactions with its human host. These include model organisms, most notably "gnotobiotic" laboratory mice that are born and raised germ-free (GF) and then colonized with individual strains or groups of microbes. Through the use of GF and gnotobiotic mice, we have been able to demonstrate causality of specific microbes and microbial groups with distinct processes of immune development and non-infectious diseases like chronic inflammation and cancer, among others. To validate the physiologic relevance of observations made in model organisms with human disease, we can now survey the human microbiota at an unprecedented depth using culture-independent molecular methods (i.e. targeted 16S "microbiome" sequencing, metagenomics, metatranscriptomics, and metabolomics) coupled with sophisticated bioinformatics pipelines. An important finding from population studies of the microbiome has revealed that the compositional fluctuations in an individual's microbiome over time are less substantial than inter-individual differences at a particular stage in development. However, the developmental changes that occur during early life and over an individual's lifespan certainly shape the composition and function of the microbiota. Indeed, the composition and functional capabilities of the microbiota shape host development. The focus of the 2018 Old Herborn University Seminar Series 32 is "Ageing and the Microbiome". Accordingly, in this chapter we discuss the current state of knowledge regarding the influence of our mammalian microbiota on the immune system, chronic inflammation, and a prominent disease of the ageing – cancer.

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THE IMMUNE SYSTEM AND MICROBIOME IN AGEING

The immune system is our major host defence system that is educated early in life to distinguish harmful stimuli, including bacteria. It is broadly subdivided into two branches, the innate and adaptive immune systems. The innate immune response is the first line of defence towards invading pathogens. For example, this branch of the immune system is involved when host cell pattern recognition receptors are triggered by conserved pathogen specific molecules which promote an immediate and broad-spectrum protective response to pathogens. Conversely, the adaptive immune response relies on antigen presenting cells (APCs) to present bacterial products to effector T and B lymphocytes and create a pathogen-specific immunological memory and response. Through precise coordination, these systems provide host defence against foreign invaders. However, to efficiently mediate its response, the immune system must accurately distinguish between normal host-associated organisms and those that are potentially deleterious (*Chaplin*, 2010). Over the course of our lifetime, our immune system encounters a diverse range of stimuli in a variety of contexts, which challenges the ability of the immune system to differentiate between self and non-self (*Ponnappan* and *Ponnappan*, 2011). Alterations in this response can result in the development of a variety of diseases that include inflammatory bowel disease (IBD) and cancer (*Narendra* et al., 2013; *Gálvez*, 2014). As these immune responses are shaped over a lifetime, it indicates that age impacts the recognition of stimuli, which could transfer towards inappropriately reacting to residential host microbes. Inappropriate reactions to normal commensal bacteria may underlie a variety of pathogenic processes.

Microbial influences on immune development

Establishment of the microbiota begins from birth and continues until post-weaning (*Bergström* et al., 2014). During establishment, the microbiota is highly diverse and prone to fluctuations based on environmental and dietary changes (*Penders* et al., 2006). After 2-3 years of age, this complex community stabilizes with the majority of bacterial community members remaining unchanged throughout the lifespan of an individual (*Rajilić-Stojanović* et al., 2013). After stabilization, the core human microbiota mainly comprises the following phyla: *Bacteroidetes* and *Firmicutes*, with a smaller abundance of *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (*Arumugam* et al., 2011). The ageing process strongly impacts the composition of the microbiota as individuals with increased frailty, an assessment of biological age based on current health status and life expectancy, lose bacterial diversity and form a *Bacteroidetes* dominant population (*Claesson* et al., 2011). While not directly correlated to chronological ageing, this loss of diversity most often begins between 75-80 years of age and is a form of dysbiosis that potentiates disease development (*Biagi* et al., 2010). These results are a generality from a diverse population, and only consider numerical age (lifespan) rather than the individual's ageing-related health status (health span). Despite this correlation, it is important to remember that the microbiota play a largely protective role in disease development by initiating and educating the host immune system (*Pickard* et al., 2017).

Early observations in GF mice demonstrated that the host microbiota is essential for the maturation of the immune system (*Clavel* et al., 2017; *Pickard* et al., 2017). In the absence of

a microbiota, GF mice have several immunological defects, including reduced lymphoid cell numbers and function (*Fiebiger* et al., 2016). For example, GF mice have less T helper type 1 (Th1) cells compared to their conventionalized counterparts (*Wu* and *Wu*, 2012). Th1 cells promote cell-mediated immune responses and phagocyte-dependent inflammation to target intracellular pathogens (*Damsker* et al., 2010). Th1 responses in GF mice can be restored through host colonization with a variety of microbes, including *Listeria monocytogenes* which promotes Th1 development through macrophage production of the T-cell-stimulating factor, interleukin 12 (IL-12) (*Hsieh* et al., 1993). In the gut, Th1 responses are driven by intracellular bacteria like *L. monocytogenes* (*Atarashi* et al., 2015). Additionally, GF mice have a reduced number of T helper type 17 (Th17) cells. Th17 cells are like Th1 cells in that they are pro-inflammatory; however, they drive the production of IL-17, a cytokine which mediates defence against extracellular pathogens and auto-immune disease (*Damsker* et al., 2010; *Wu* and *Wu*, 2012). Colonization of GF mice with intestinally adherent pathogens, such as Clostridia-related segmented filamentous bacteria (SFB), induces the development of Th17 cells in the small intestine by driving the release of serum amyloid A from intestinal epithelial cells (IECs). The release of serum amyloid A results in the production of innate lymphoid cell group 3 activating cytokines which upregulate the Th17 response (*Ivanov* et al., 2009). Fine-tuning of Th1 and Th17 responses is essential for immune tolerance towards the host microbiota, as seen in the case of IBD where aberrant populations of Th1 and Th17 cells lead to enhanced pathology (*Gálvez*, 2014). Therefore, underdevelopment of these responses may underlie the pathogenesis of other diseases associated with chronic inflammation, such as cancer (*Bailey* et al., 2014; *Vinay* et al., 2015).

The absence of a microbiota impacts most, if not all, aspects of the immune system (*Round* and *Mazmanian*, 2009). However, it is not fully understood where the window of opportunity lies for correcting many of these immune deficiencies. One study examining colonic invariant natural killer T (iNKT) cell populations revealed that this opportunity for modulation likely occurs in early life (*An* et al., 2014). At birth, GF mice have an enriched population of colonic lamina propria iNKT cells compared to specific-pathogen-free (SPF) mice (*Olszak* et al., 2012). iNKT cells are pro-inflammatory and mediate cell tolerance to commensal microbes (*Chandra* and *Kronenberg*, 2015). Colonization of adult (>5 weeks of age) GF mice with a complex microbiota does not influence the number and activity of iNKT cells (*An* et al., 2014). However, if the colonization occurs when GF mice are neonates, the number of iNKT cells is reduced and their later activation is well-controlled (*An* et al., 2014). This early education of the colonic iNKT cell population is important for limiting morbidity associated with IBD (*An* et al., 2014). This supports the idea that exposure to specific microbes and microbial products is needed within a certain developmental time for the host to appropriately educate their target immune population and prevent disease.

The gastrointestinal tract houses one of the largest and most diverse collection of microbes in the human body (*Thursby* and *Juge*, 2017). In the intestine, a single layer of epithelial cells separates the underlying mucosal immune system from the microbiota. Due to this proximity, much work has focused upon the protective role of the microbiota on inflammatory immune

responses that can lead to intestinal inflammation or colitis. For example, GF mice colonized with a beneficial non-toxin-producing strain of *Bacteroides fragilis* exhibit a marked expansion of regulatory T-cells (Tregs) in the intestinal mucosa (*Round* and *Mazmanian*, 2010). This expansion of Tregs limits inappropriate inflammation and is important for preventing autoimmune disease through immunotolerance towards self-antigens (*Round* and *Mazmanian*, 2010; *Rothstein* and *Camirand*, 2015). This protection is mediated by *Bacteroides spp.* through the production of polysaccharide-A (PSA), a commensal microbial surface molecule presented by APCs to modulate Treg specific immune responses. Indeed, colonization with PSA + *B. fragilis* protects mice from chemically TNBS-induced and *Helicobacter hepaticus*-induced colitis (*Round* and *Mazmanian*, 2010). Furthermore, colonization of conventional mice with *Clostridium butyricum* protects against experimentally-induced colitis by promoting the expansion of immunosuppressive interleukin-10 (IL-10) secreting macrophages and mature Tregs (*Hayashi* et al., 2013). *B. fragilis* and *C. butyricum* belong to the most highly represented bacterial phyla within the murine and human microbiota (*Bacteroidetes* and *Firmicutes*, respectively) and mechanistically represent some of the many ways the commensal microbiota can modulate host immune responses to prevent the onset of disease (*Belkaid* and *Harrison*, 2017; *Clavel* et al., 2017; *Pickard* et al., 2017).

The immune system and ageing

The presence of a microbiota in early life is essential for immune system maturation. However, education of the immune response is a lifelong process. Alterations to the innate and adaptive immune systems which occur with increased frailty are linked to a complex biological process known as immunosenescence (*Fülöp* et al., 2016). Specific changes associated with immunosenescence can best be understood through functional differences within the unique cell types of the innate and adaptive immune systems. For cells of the innate immune system, there are reported functional differences for every major cell type (*Castelo-Branco* and *Soveral*, 2014). However, the most distinct differences are within neutrophil and macrophage populations. Neutrophils isolated from the blood of individuals, aged 62-83, displayed reduced phagocytic capabilities and decreased production of reactive oxygen species (ROS) when infected with *Staphylococcus aureus* (*Wenisch* et al., 2000). These neutrophils, versus cells isolated from younger adult patients, also had impaired bactericidal activity (*Wenisch* et al., 2000). Neutrophils are the first line of defence towards invading pathogens. Therefore, immunosenescence related changes to this cell type suggest an age-related decline in pathogen tolerance (*Chaplin*, 2010). Similarly, primary macrophages isolated from aged mice (18-24 months old) have impaired phagocytosis and reduced ROS production in response to infection when compared to macrophages isolated from young mice (2-3 months old) (*Davila* et al., 1990; *Swift* et al., 2001). Additionally, macrophages from aged mice display altered antigen presentation and reduced production of pro-inflammatory cytokines (*Herrero* et al., 2001; *Renshaw* et al., 2002). Alterations in macrophage antigen presentation and cytokine release may lead to altered immune signalling between the innate and adaptive immune systems, resulting in a weakened immune response (*Chaplin*, 2010). Overall, age-related changes to the innate immune system strongly reduce the host's initial re-

sponse to pathogens.

While changes in the innate immune system have been noted with ageing, alterations to the adaptive immune system may be more pronounced (*Linton* and *Dorshkind*, 2004; *Castelo-Branco* and *Soveral*, 2014). The adaptive immune system is used for long-term protection from environmental insult and invading pathogens. Therefore, long-term education of this subsystem could have additive effects on immunosenescence. B-cells are one of the major cell types of the adaptive immune system. The population of B-cells is divided into plasma cells or memory B-cells. Plasma cells produce pathogen specific antibodies, while memory B-cells provide long-term recognition of antigens (*Eibel* et al., 2014). Peripheral blood isolated from elderly individuals (aged 86-94 years) showed a reduction in B-cell population diversity, likely due to a decrease in memory B-cells (*Gibson* et al., 2009). This decline in B-cell diversity was linked to increased frailty and may be used as a predictor for general health status (*Gibson* et al., 2009). A reduction in memory B-cells may cause an inappropriate immune response towards the microbiota, as B-cells are important for establishing the distinction between pathogenic and commensal bacteria (*Eibel* et al., 2014).

T-cells are the second major cell type of the adaptive immune system. Under normal immunological conditions, T-cells differentiate into either T Helper (Th) cells or natural killer T (NKT) cells. This differentiation is based on antigen presentation by APCs and is necessary to form a pathogen-specific immune response (*Chandra* and *Kronenberg*, 2015). Populations of T-cells isolated from the peripheral blood of elderly humans (aged 72-89 years) showed a reduction in the proportion of NKT-cells versus cells isolated from young patients (aged 25-30 years) (*DelaRosa* et al., 2002). This finding was matched by a study which showed a reduction in the proportion of NKT-cells isolated from the peripheral blood of adults aged 61 years and over (*Jing* et al., 2007). Additionally, NKT-cells isolated from the liver of aged mice (aged >20 months) demonstrated a decline in cytotoxic function, and reduced cytokine release versus T-cells isolated from young mice (aged 2 months) (*Mocchegiani* et al., 2004). Under non-immunosenescence conditions, NKT-cells respond to antigenic activation by robust proliferation and directed cytotoxicity (*Chandra* and *Kronenberg*, 2015). Therefore, the reduced proportion of NKT-cells and cytotoxic ability in elderly organisms may exacerbate disease development by weakening the host's response to pathogens.

The host microbiota initiates immune system maturation in early life. However, to keep up with a lifelong antigenic load, the immune response must be fine-tuned and properly educated across the lifespan. Changes to the immune system that occur with age are noted within immunosenescence whereby functional differences develop within innate and adaptive immune cell populations. Despite these observations, it remains unclear how these immunological changes impact cellular crosstalk and overall immunocompetence. On top of this, how the microbiota impacts the immune system during immunosenescence remains to be elucidated. It is likely that changes to the immune system result in an inappropriate response towards commensal microbes, as indicated by diseases like IBD (*Sun* et al., 2015). Therefore, these inappropriate reactions to the native microbiota may contribute to the development of chronic inflammation and the onset of age-related diseases, such as cancer (*Tilg* et al., 2018).

MICROBIOME AND CANCER – A DISEASE OF AGEING

Cancer is considered a disease of old age. As life expectancy increases, the estimated rate of cancer is predicted to increase by 45% from 2011 to 2030 in the United States (*Smith* et al., 2009). It is also estimated that by 2030, individuals 65 years and older will contribute to 70% of all cancers in the United States (*White* et al., 2014). The risk of developing cancer increases dramatically with age as the duration of time in which an individual is exposed to carcinogens increases (*Harding* et al., 2012), the proliferative capacity of ageing cells decreases (*Pompei* et al., 2001), and immunological competence decreases (*Bonafè* et al., 2002). Cancer typically results from a series of genetic mutations or epigenetic modifications that develop sequentially overtime (*Loeb* et al., 2003). The colon, which harbours the largest and most diverse microbiota of all organs, has the highest incidence rate of all reported cancers in the 85+ population (*Thakkar* et al., 2014). Over the past decade, we have become increasingly aware of the roles that the microbiota play in the development of cancer and modulation of cancer therapies. We have also elucidated several mechanisms underlying the microbial influences on cancer. However, these roles are diverse and seem to influence many aspects of immune and cancer development (*Fulbright* et al., 2017). Microbes can contribute to the onset and progression of cancer through direct means, such as by producing genotoxins, and indirect means through the modulation of immune responses to tumours and immunotherapy (*Rhee* et al., 2009; *Arthur* et al., 2012; *Kostic* et al., 2013; *Boleij* et al., 2015; *Gopalakrishnan* et al., 2018). Additionally, several members of the native microbiota can alter chemotherapeutic drugs, resulting in unpleasant side effects for the host or even rendering them clinically inert (*Al-Dasooqi* et al., 2011; *Geller* et al., 2017). It is therefore important to divulge the significance of microbial interactions on age-related diseases, such as cancer, in order to fully understand disease progression and design suitable therapies.

The ability to manipulate the microbiota using GF and gnotobiotic mice has demonstrated the importance of the microbiota on immune system development. The microbiota assist in training the immune system to recognize harmful and non-harmful stimuli, both from the environment and within the self. Here we will discuss known mechanisms by which the microbiota can influence anti-cancer chemotherapeutic activities and anti-tumour immunomodulatory responses. For example, *Clostridia spp.* are capable of suppressing the body's anti-tumour immune responses. Host-derived primary bile acids are converted into secondary bile acids by the gut microbiota, primarily members of the genus *Clostridia,* and circulated systemically throughout the body via hepatic circulation (Ridlon et al., 2006). Previous work illustrated that secondary bile acids can increase the risk of obesity-associated hepatocellular carcinoma in susceptible mice (*Yoshimoto* et al., 2013). Recent data suggests that antibiotic elimination of the gut microbiota in mice decreases both primary and metastatic tumours within the liver by facilitating the build-up of primary bile acids, which trigger liver-specific NKT-cell recruitment to target cancer cells (*Ma* et al., 2018). The profound effects bacteria illicit on cytotoxic immune cells provide key insights on how the native microbiota influence host anti-tumour responses.

Another example, *Fusobacterium nucleatum*, a Gram-negative oral commensal overrepresented in colorectal carcinoma, can promote tumourigenesis through the modulation of the innate immune system (*Castellarin* et al., 2012; *Kostic* et al., 2013). A known target is the natural killer (NK) cell, which kills compromised host cells, such as infected or cancerous cells. *F. nucleatum* inhibits the cytotoxicity of NK-cells via the Fusobacterium protein Fap2 which binds the NK-cell inhibitor receptor TIGIT (T-cell immunoglobulin and ITIM or immunoreceptor tyrosine-based inhibition motif domain) (*Gur* et al., 2015). In addition to targeting the immune system, it should be mentioned that *F. nucleatum* exerts pro-carcinogenic activities directly on epithelial cells through β-catenin signalling, altering cell fate (*Rubinstein* et al., 2013). *F. nucleatum* can also alter the efficacy of chemotherapeutic drugs by inhibiting host cell apoptotic pathways (*Yu* et al., 2017). *F. nucleatum* is a prime example of one species of the microbiota that exhibits a variety of different effects on the host to mediate tumourigenesis and hinder cancer therapy. In the next few sections, we will discuss a variety of known bacterial mechanisms that act upon cancer development and treatment.

Cancer immunotherapy and the microbiota

Several independent groups have recently demonstrated that some members of the microbiota play critical roles in determining patient responsiveness to cancer immunotherapy. The exact mechanisms by which individual species of bacteria exhibit these effects are not fully understood. However, current data suggest that bacterial modulation of the immune system may be one critical mode of altering host response to cancer therapy. Recent data regarding anti-PD1 therapy supports this notion. Anti-PD1 treatment is a type of immune checkpoint inhibitor that enhances anti-tumour immune responses by maintaining T-cell activation via blocking the immune inhibitory receptors programmed death ligand-1 and 2 (PDL-1 and PDL-2) (*Shields* et al., 2017). Anti-PD1 therapy is often prescribed to patients with lung cancer and advanced melanoma. However, the efficacy of anti-PD1 immunotherapy ranges from only 19 to 43% for both cancer types (*Jiang* et al., 2015; *Larkin* et al., 2015). Several members of the microbiota are enriched in PD-1 responders, including *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* (*Matson* et al., 2018). *Faecalibacterium,* an abundant Gram-positive genus of commensals in the human gut (*Shabbir* et al., 2016), was also enriched in PD1-responders (*Gopalakrishnan* et al., 2018). Tumour-bearing mice that were given faecal microbiota transplants (FMTs) from PD1-responders exhibited decreased tumour burden and tumour size when receiving anti-PD1 therapy. *Faecalibacterium* promoted cytotoxic (CD8+) T-cell recruitment to tumours, which may be an important mechanism underlying the ability of this bacterial group to enhance anti-PD1 responses and reduce tumour burden (*Gopalakrishnan* et al., 2018). Similarly, another group demonstrated that FMTs from PD-1 responders enhanced PD-1 treatment in recipient mice, which was further augmented with oral supplementation of the commensal *Akkermansia muciniphila* (*Routy* et al., 2018). Antibiotic treatment reduced the efficacy of PD-1 immunotherapy in mice, consistent with clinical reports of reduced PD-1 efficacy in patients simultaneously taking antibiotics (*Routy* et al., 2018). These studies demonstrate that multiple species of bacteria have the capability

of altering immunotherapeutic responses in patients. Moving forward, it may be critical to consider the contributions of these microbial communities when developing anti-tumour immunotherapies.

Chemotherapy and the microbiota

Multiple members of the microbiota can differentially influence cancer chemotherapy, with some enhancing and some inhibiting the clinical effects of chemotherapeutic drugs. An important early observation was that genotoxic platinum chemotherapies, including oxaliplatin and cisplatin, were ineffective in tumour-bearing GF mice (*Iida* et al., 2013). Thus, the presence of a complex microbiota is essential for these chemotherapies (*Iida* et al., 2013). Platinum chemotherapies utilize ROS to induce cytotoxicity. The DNA damage induced by cisplatin is augmented via the production of mitochondrial ROS within cancer cells themselves (*Marullo* et al., 2013). Data also indicates that the ROS production from tumour-associated inflammatory cells are critical for the efficacy of these platinum chemotherapeutic drugs (*Iida* et al., 2013). It may be that in the absence of a native microbiota, inflammatory cells are not effectively primed to produce ROS during development, leading to shortcomings in ROS production later in life. This data highlights the influential capacity of the microbiota on the host and how in their absence the immune system may have substantial deficits in anti-tumour responses. Conversely, the microbiota can have negative effects on chemotherapeutic efficacy. Deep sequencing for microbes within pancreatic tumour biopsies revealed that 57.5% of pancreatic tumour tissues tested (65 of 113 samples) were positive for bacterial reads, with *Gammaproteobacteria* being the most abundant (51.7% of reads) (*Geller* et al., 2017). Interestingly, 98.4% of *Gammaproteobacteria* contain genes that encode a specific isoform of the enzyme cytidine deaminase (CDD_L), which has the ability to breakdown gemcitabine and confer chemotherapeutic resistance in tumour tissues (*Geller* et al., 2017). Accordingly, bacterial migration from the gastrointestinal tract into the pancreatic ducts and tumour tissue may be a significant source of drug failure in clinical pancreatic cancer cases. Gut bacteria are also responsible for re-activating chemotherapeutic drugs in the distal intestine. Irinotecan, a chemotherapeutic drug used to treat colorectal cancer (CRC), is inactivated by the liver, but reactivated into the activate drug by *Clostridia spp.* through β-glucuronidases in the gut (*Stringer* et al., 2009). This re-activation contributes to the typical unpleasant gastrointestinal side effects of irinotecan therapy, including mucositis and diarrhoea (*Stringer* et al., 2009; *Al-Dasooqi* et al., 2011). This evidence illustrates the profound impact the native microbiota can have on the response to cancer therapies. Therefore, future treatment plans should account for the influence of these patient-specific microbial factors to ensure successful outcomes.

Direct effects of the microbiota on tumourigenesis

Specific members of the microbiota have the capacity to directly contribute to tumourigenesis (*Schwabe* and *Jobin*, 2013; *Fulbright* et al., 2017). Commensal *Enterobacteriaceae*, including several strains of *Escherichia coli,* are capable of inducing DNA damage in mammalian cells by producing a genotoxin termed colibactin (*Cuevas-Ramos* et al., 2010; *Arthur* et al., 2012). The bacterial polyketide synthase (*pks*) pathogenicity island encoding colibactin is up-regulated in CRC models and

the presence of these gene products promotes tumourigenesis by inducing double-stranded DNA breaks (*Nougayrede* et al., 2006; *Arthur* et al., 2014; *Tomkovich* et al., 2017). Colibactin can also induce premature cellular senescence in cells that initially survive the DNA damage (*Secher* et al., 2013; *Cougnoux* et al., 2014) Furthermore, the *pks* pathogenicity island is overrepresented in the microbiota of CRC and IBD patients (*Arthur* et al., 2012; *Buc* et al., 2013; *Prorok-Hamon* et al., 2014). This suggests that bacterial genotoxins likely contribute substantially in the development of human cancer and chronic inflammatory diseases.

Bacteria also have the capacity to induce a pro-tumourigenic environment through chronic inflammation. Enterotoxigenic *B. fragilis,* a member of the most abundantly represented genus in the gut, produces its own flavour of toxin called *B. fragilis*-derived toxin (BFT) (*Wu* et al., 2002). BFT is a zinc-dependent metalloprotease that can induce colitis and promote tumourigenesis through the generation of ROS and subsequent initiation of DNA damage in epithelial cells (*Goodwin* et al., 2011). Enterotoxigenic *B. fragilis* robustly activates Th17 immune responses, which involves the inflammatory cytokine interleukin-17 (IL-17), and may lower host anti-tumour immune responses, encouraging unhindered tumour growth (*Wu* et al., 2009; *Geis* et al., 2015). Enterotoxigenic *B. fragilis* is overrepresented in patients with CRC when compared to healthy individuals (*Boleij* et al., 2015) and exacerbates tumourigenesis in susceptible mice (*Wu* et al. 2009). Interestingly, the tumourigenic effects of *pks+ E. coli* and enterotoxigenic *B. fragilis* act synergistically *in vivo* to quicken tumour onset and increase mortality in susceptible mice beyond the capability that either species has individually (*Dejea* et al., 2018). Given that the native microbial community is quite complex, the cumulative effects of microbial products on the host may significantly contribute to the onset of cancer.

One of the earliest identified microbial suspects of inflammation-mediated cancer development is *Helicobacter pylori*. While widely considered a pathogen, *H. pylori* is estimated to be present in the gastrointestinal tract of over half of the human population worldwide and a major risk factor for gastric adenocarcinoma (*Arthur* and *Jobin*, 2011; *Hooi* et al., 2017). It is estimated that *H. pylori* infection increases the attributable risk of gastric cancer by 73% (*Herrera* and *Parsonnet*, 2009). Chronic *H. pylori* infection results in inflammation and tissue damage by the bacterial virulence factor CagA (cytotoxin-associated gene A), which initiates the development of the hallmark precursory lesions of gastric cancer, including intestinal metaplasia and dysplasia (*Díaz* et al., 2018). It remains unclear why *H. pylori* infection only progresses to malignancy in a subset of infected individuals; however, it is postulated that host immune responses and the genetics of both host and bacteria contribute to neoplastic development (*Polk* and *Peek*, 2010). The evidence presented here illustrates the diverse microbial mechanisms that contribute to tumourigenesis, whether that be by directly targeting the DNA for damage through a toxin or by providing an augmented environment for unrestricted cellular proliferation.

In summary, the mechanisms by which the native microbiota influences cancer development and therapy are numerous and diverse. As more data surfaces, it will be imperative to synthesize and apply knowledge on microbial contributions towards cancer

development and treatment. By doing so, we can more effectively assess cancer risk and ultimately design more potent therapies.

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AGEING AND THE MICROBIOME: SUMMARY OF THE 32ND OLD HERBORN UNIVERSITY SEMINAR AND THE DISCUSSIONS

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Following a warm introduction, *Volker Rusch* opened the Seminar. The focus of this year's meeting was ageing, with an emphasis on whether the gut microbiome played any role in the process.

Michael Zasloff presented a brief introductory lecture ("*Some Insights about Ageing from a Shark and an Oyster*"). He pointed out that the two documented longest-lived animals were the "Ming Clam" (400 years) and the Greenland shark (500 years). Their survival obviously required non-senescent stem cells, and an immune system that could protect against all potential pathogens. He pointed out that the details of the animal "ageing clock" is unknown. Zasloff finished his presentation with a discussion of recently published studies in mice that suggested that hypothalamic inflammation leading to the reduced expression of certain hypothalamic hormones (i.e. GnRH) drives peripheral ageing. These observations suggest that an "elixir of youth" might be more real than we believe.

Robert Pignolo presented a general overview of the physiology of human ageing from the perspective of a research gerontologist ("*Physiology of Human Ageing*"). He made the point that it becomes difficult to separate ageing per se from the onset of disease processes that are associated with ageing. If we could slow down ageing, how would we document the response? One approach is to focus on the loss of function of a system, such as the GI tract, the immune system, the endocrine system and the nervous system cataloguing the known progressive changes that occur in the otherwise healthy person. He highlighted the known progressive changes that occur with respect to specific tissues, such as muscle, bone, skin and adipose tissue. He stressed that a deeper analysis of these processes could be extended mechanistically to dissect the specific pathways leading to the age-related changes. Ageing in humans is frequently associated with a loss of resilience and those factors that increase an individual's fragility, and reduce their probability of recovery. He stressed that much current interest in human ageing is focused on the mechanistic basis of reduced stem cell survival, cellular senescence and the underlying mechanisms responsible for chronic inflammation. In particular, considerable research in human ageing is now focused on drugs that deplete senescent cells.

Brian Kennedy posed the provocative question of whether we could intervene to delay human ageing ("*The Age of Ageing: Can We Intervene*"). He reviewed the known conditions that have been shown in various systems to extend lifespan, such as caloric restriction. Of particular interest were small molecules that had already been developed and were currently in human use for various medical conditions. In particular he highlighted metformin and rapamycin. While the mechanism

underlying the benefit of metformin was still unclear, rapamycin's effects on ageing appear to be attributable to mTOR inhibition. mTOR regulates protein synthesis in response to nutrient and energy availability. Recent studies suggest that mTOR inhibition extends stem cell survival. He presented remarkable studies that supported the effect of mTOR inhibition on age related stem cell loss in the murine trachea.

He then tackled the persistent theme of how best to measure the impact of an intervention that delayed ageing in humans. One promising potential biomarker is the accumulation of methylation across the 450K CpG site assessable in the human genome. As an individual age the number of methylated CpG sites increases providing an "epigenetic clock" that might be of use. Kennedy described another approach his colleagues have explored, that being facial recognition. Facial recognition technology has advanced to such an extent that chronological age can be reliably estimated through this modality, possibly providing a tool to assess the impact of anti-ageing interventions.

Dario Riccardo Valenzano presented his work with the turquoise killifish as a new model animal to study the ageing process ("*Life Span Regulation by the Gut Microbiota in the Naturally Short Lived African Turquoise Killifish*"). The killifish has a lifespan of between11-26 weeks, bridging the brief lifespan of *C. elegans* (3 weeks) and *Drosophila* (9-13 weeks) and the longer lifespan of the mouse (130 weeks). This species is indigenous to Africa. The genomic sequence was published in 2015 as well as techniques to genetically modify the animals. Ageing in killifish is characterized by dramatic skeletal disturbances including a form of scoliosis, reduced capacity to learn, decreased fertility, slower overall movement, sarcopenia, and gut fibrosis. Valenzano noted that the microbiome of the killifish changes dramatically as the animal ages, and then posed the question of whether the gut microbiome influenced ageing. The most abundant taxa in its microbiome are shared with mammals, and common microbial taxa are shared between animals caught in the wild and those maintained in captivity. Can the course of ageing be changed by altering the microbiome? Microbial transplants from young or old fish were introduced into middle aged fish that had been pretreated with antibiotics. The most dramatic outcome of this study was with young microbial transplants: survival was extended, swimming activity was increased to a level normal for young animals, changes were seen at the transcriptional level in the intestinal tissues. He hypothesized that the effects observed could in part be due to the differences in serum metabolites associated with the young or old gut microbial communities and the interaction with host physiology.

Christoph Kaleta presented a broad overview of ageing ("*Evaluating Potential Contributions of the Microbiome to Ageing Pathologies*"). His overriding thesis was that a major factor in the ageing process is due to the accumulation of dead or damaged cells, which, in turn, provokes chronic inflammation. Might the ageing microbiome impact on the clearance of senescent cells? Perhaps metabolites of microbiome origin play a role. And if so, we might be able to intervene.

Thomas C. G. Bosch presented an expansive perspective on ageing focusing on his studies of *Hydra* ("*Ageing and the Microbiome: Lessons from Non-Senescent Models*"). He stressed that both genetics and environment play a role in longevity. He highlighted the remarkable role of FoxO in the biology of *Hydra*, FoxO being one of the

two genes that have been associated with longevity in humans. A three classes of stem cells in *Hydra* express FoxO, and genetic inhibition of FoxO expression slows down the population growth rate in this animal. Remarkably, FoxO also plays a role in managing the microbiome of *Hydra*. It turns out that FoxO is required for expression of antimicrobial peptides, which in turn, regulate the composition and density of the microbiome. So, by virtue of FoxO's integration into the design of *Hydra*, it controls cross-talk between the stem cell and the microbiome. This relationship is dramatically altered in the setting of a *Hydra* strain in which a tumour has arisen. The presence of the tumour, which is of an epithelial nature, dramatically alters the microbiome, increasing the proportion of spirochetes. These spirochetes are not passive bystanders, since their elimination with antibiotics causes regression of the tumour. Re-inoculation with these bacteria causes a recurrence of the tumour. Careful analysis of the microbial interactions involved, demonstrate that a *Pseudomonas* strain is also required for tumorigenesis, implicating the two species in this process. Bosch stressed that this story underscores the principle that the physiology of an organism cannot be understood without including the microbial elements with which coexists, without viewing the entirety of the holobiont.

Wolfgang Kunze focused on the ageing of the enteric nervous system (ENS) and how gut microbes might influence the activity of the ENS ("*Ageing and Gut to Brain Signalling*"). He reminded us that gut microbiota change with ageing and that feeding *Bifidobacteria* to mice has been shown to increase lifespan. He suggested that gut microbes could contribute to ageing through neural communication to the brain, for example, via the vagus. When the *Lactobacillus* strain, JB-1 is introduced into the mouse gut, 90% of the signals stimulated by JB-1 are transmitted through vagal afferents. He highlighted that as we age the ENS progressively deteriorates. Peristalsis is reduced. Afferent activity decreases. Ion channel activity, such as the Ik potassium channel, diminishes. The consequences of this deterioration can be observed in the brain. For one, stimulation of the vagus has been shown to activate growth hormone releasing factor in the hypothalamus, demonstrating a clear neural linkage between the hypothalamus and the gut. Introduction of JB-1 or squalamine into the intestine (studied *ex vivo*) leads to increased vagal firing in aged mice, suggesting that the diminished activity of the aged ENS can be corrected therapeutically.

Josef Anrather introduced the concept that inflammation within the brain surrounding stroke could be modulated by the gut microbiome ("*From the Gut to the Brain: The Roles of Microbiota in Stroke*"). He described several models of stroke as studied in rodents. The basic premise of his hypothesis is that IL-17 T cells are major players in the inflammatory events that follow a stroke, that the gut is the major reservoir of IL-17 T cells, and that the microbiome plays a role in the differentiation of IL-17 T cell population. Following a stroke intestinal IL-17 T cells traffic to sites such as the meninges, and then into the brain. By altering the population of intestinal microbes, it is possible to influence the dendritic cells of the intestinal lamina propria to shift the T cell population towards Tregs. He has begun studies that suggest that a factor extractable from the "good" flora can recapitulate the beneficial effects on the intestinal T cell population of the gut. These studies suggest an entirely novel understanding of the pathophysiology of the events that follow a

stroke, and treatments that might be developed.

Janelle C. Arthur focused on the role of the microbiome in colorectal cancer, a disease of ageing ("*The Microbiome and Cancer*"). She reminded us that the current explanation for the occurrence of colorectal cancer is chronic inflammation, caused either by microbes or by organic compounds of dietary origin present in faeces. From studies with IL-10 knock out mice, an animal that develops colorectal cancer, it is clear that the microbiome plays a role, since germ-free animals of this strain do not exhibit this phenotype. Arthur asked whether specific microbes might be involved. Since inflammation precedes the appearance of cancer in these animals, might inflammation produce a change in the microbiome that encourages the appearance of carcinogenic microbes? To answer these questions, she used germ-free IL-10 knock out mice. The animals were inoculated with SPF microbiota, then treated with azoxymethane, a chemical that provokes both intestinal inflammation and colorectal cancer. The microbiome was then examined and compared with the initial inoculum with respect to its species. Treatment with Azoxymethane led to a 100-fold increase in *E. coli*. If the same experiment was performed with either a pure culture inoculum of *E. coli* or *E. faecalis* (rather than the SPF inoculum) only the *E. coli* inoculated mice developed significant colorectal cancers. It appears that strains of *E. coli* that harbour the polyketide synthase (*pks)* pathogenicity island appear to be the culprits. A specific substance produced by these strains of *E. coli* can induce cancer independently of inflammation. The product of the *pks* gene cluster is a poorly characterized molecule called Colibactin which has been shown to cause chromosomal damage by an unknown mechanism and requires contact between the bacterial cell and the epithelium. These studies suggest that age related changes in the microbiome could be a cause of colorectal cancer, and clearly amenable to intervention.

The formal Academic ceremony followed the completion of the Seminar, with the granting of Honorary Professorships at the Old Herborn University to the speakers.