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Abstract Booklet

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Establishing an Imaging Assay to identify Potential New Therapeutics that enhance the Neuronal K-Cl Co-transporter

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Inhibitory signalling via ionotropic GABA receptors ($GABA_ARs$) is dependent on a low intracellular chloride concentration ($[Cl^-]_i$), predominantly maintained by the K^+/Cl^- cotransporter type 2 (KCC2). KCC2 is a secondary active transporter that couples K^+ and Cl^- ion efflux, down a K^+ concentration gradient created by the Na^+/K^+/ATPase transporter. A decrease in KCC2 expression and function elevates $[Cl^-]_i$ which reduces the driving force for Cl^- influx through activated $GABA_ARs$ and the resulting hyperpolarizing neuronal inhibition. This loss of KCC2 occurs in several neurological diseases including epilepsy, neuropathic pain, spasticity and trauma, and restoring KCC2 may be a therapy for these disorders. To identify and validate potential KCC2 enhancers we have been developing a BCECF-AM pH-sensitive fluorescent imaging assay in human embryonic kidney (HEK) 293 cells. The application of NH_4Cl induces a transient increase in fluorescence intensity (FI), representing intracellular alkalinisation. KCC2 opposes this increase in FI by transporting NH_4^+ into cells and a resultant acidification. In preliminary control experiments, we have used the H^+/K^+ exchanger nigericin to calibrate the BCECF fluorescence to pH. Application of high K^+ nigericin containing solutions of different pH induces FI changes that reaches a maximum at pH of 8.5-9 with a best fit pK value of 7.778 (n=31 cells across two transfections). Such pH-titration curves allow calibration of FI to pH by a single point nigericin calibration in each experiment. Using NH_4Cl concentration steps between 0 and 20 mM, we could determine the intrinsic buffering capacity of HEK cells (Benjelloun et al. 2005) (55 cells across two transfections). Knowledge of the factors influencing pH and BCECF fluorescence in control HEK cells will enable us to examine the effects of KCC2 on NH_4Cl induced pH transients in HEK293 cells. We hypothesize this assay will have the sensitivity to detect different levels of KCC2 transport activity.

Quantitative Proteomics to Identify Mitochondrial Proteins in Skeletal Muscle Single Fibres of Healthy Humans

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Introduction

Mitochondria are involved in many essential cell functions, including the production of energy and cellular metabolism. Skeletal muscle represent 40-50% of body mass in humans playing a key role in health and performance. The methods for studying mitochondrial protein adaptations in skeletal muscle have mostly been confined to the analysis of whole-muscle samples that contain a mixture of type I and II fibres Recent advances in proteomic techniques and mass spectrometry (MS) allow the quantification of thousands of proteins in small biological samples and enable the analysis of mitochondrial proteins in single muscle fibres. Integrating such techniques may give an increased understanding into mitochondrial characteristics and adaptations to exercise training in single muscle fibres. Moving beyond measurements of just a few proteins may further elucidate the fibre-specific differences in the abundance of mitochondrial proteins, which could provide new information regarding pathways in mitochondria that are divergently regulated in type I & II fibres and could be important in the exercise prescription for health and performance.

Methods & Results

We describe a proteomic method for fibre typing and identification of mitochondrial proteins. The optimised protein processing method allowed for fibre typing with pooling (based on dot blotting) and analysis of proteins by MS. Fibre types were assigned based on the abundance of myosin heavy chain (MYH) isoforms by which the relative amount of each MYH was determined by dividing the intensity-based absolute quantification (IBAQ) of the respective isoform (MYH1, MYH2, MYH4, MYH7) by the sum of the intensities of all four MYH isoforms. Fibres were classified as type I if the relative abundance of MYH7 was >80%, as type-IIa if MYH2 >60%, as type-IIx if MYH1 >60%, and as type-IIb if MYH4 >80%. Corresponding to this method, fibre typing according to proteomic data was consistent ($p>0.05$) with the dot blotting in all experiments. Results will be discussed in the context of adaptations to exercise training.

Discussion

Considering sarcomeric proteins dominate skeletal muscle identification of other cellular proteins is often challenging, we were nonetheless able to quantify the levels of 200-360 mitochondrial proteins, which represented greater than 35% of the total quantified proteins in single muscle fibres. For abundant complexes such as respiratory chain complex I, 39 of the 44 known subunits were quantified in single fibre cells. Our research hopes to further develop proteomic technologies for understanding how mitochondrial proteins function together in pathways and complexes at the resolution of single muscle fibres.

DNA methylation differences between three types of skeletal muscle fibre at rest and in response to high intensity interval training.

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Skeletal muscle is a heterogeneous tissue comprised of diverse cell types. To date, studies investigating DNA methylation (DNAm) in human muscle at rest and after exercise have predominantly focused on the *tissue-level*. However, this approach limits interpretation of results due to 1) the significant role that individual cell identities and the variable cellular composition of tissue play in DNAm, and 2) the specific responses of each fibre type to exercise. Recently, distinct methylation profiles of Type I and Type IIa muscle fibres have been identified, and key fibre-specific genes shown to be differentially methylated (1). Here, we aim to further distinguish DNAm profiles for Type I, Type IIa and *Type IIx* muscle fibres and investigate the *fibre-type-specific* DNAm changes after high intensity interval training (HIIT). Briefly, individual skeletal muscle fibres, previously collected in the Gene SMART (Skeletal Muscle Adaptive Response to Training) study (2), will be isolated, pooled and measured for DNAm using the MethylationEPIC Kit (Infinium). Next, the three major fibre types will be assessed before and after HIIT to identify to what extent exercise differentially alters the methylomes of the three fibre types. This will contribute to a greater understanding of molecular adaptations to exercise in skeletal muscle, with potential to improve treatment strategies for muscle related diseases and maintenance of muscle health.

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Resistance of ryanodine receptor 1 to activation by cytoplasmic Ca^{2+} is essential for thermogenesis in mammalian skeletal muscle

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A major advantage in mammals is the ability to use relaxed muscles for thermogenesis and the maintenance of core body temperature. It has been proposed that the generation of heat in resting mammalian skeletal muscle is largely attributable to ryanodine receptor (RyR) Ca^{2+} leak and the subsequent activity of the SR Ca^{2+} pump. The major difference between mammals and lower vertebrates in their thermoregulatory capacity may lay within in the RyR isoforms present on the SR. Amphibians and most other lower vertebrates have two isoforms, αRyR and βRyR, whereas mammalian skeletal muscle has a single isoform, RyR1. The αRyR isoform is under the direct control of the DHPR, thus activated by voltage; and the βRyR isoform is sensitive to cytoplasmic Ca^{2+}, where increases in cytoplasmic Ca^{2+} cause regenerative release, termed Ca^{2+} induced Ca^{2+} release (CICR). The presence of CICR in mammalian skeletal muscle, however, is controversial, mainly because it has not been possible to establish whether the RyR isoform 1 (RyR1) of mammalian muscle in its closed state can be opened by cytoplasmic Ca^{2+} (Rios, 2018). To test such a critical evolutionary adaptation, measurements of the response of resting RyRs to high cytoplasmic Ca^{2+} were performed in mammalian and amphibian muscle. Additionally, in mammals, Ca^{2+} cycling was altered with RyR1 modulators to highlight the physiological role RyR1 Ca^{2+} leak may have in thermoregulation.

Human muscle biopsies were collected under local anaesthesia from the Vastus Lateralis (VL) muscle. Cane toads (*Bufo Marinus*) were euthanized by double pithing and the Iliofibularis (IL) muscle was extracted. Wistar rats and C57BL/6J mice were euthanized and the EDL muscles were extracted. Single fibres were isolated and mechanically skinned under paraffin oil. Ca^{2+} release properties were observed by rapidly imaging cytoplasmic Ca^{2+} in a solution containing rhod-2 fluorescent dye and 1 mM halothane (RyR agonist) on a Zeiss LSM 5 live confocal microscope. To stimulate a local increase in $[Ca^{2+}]_{cyto}$, two skinned fibres were positioned perpendicularly to each other, in a 'cross' formation. The rationale was that a Ca^{2+} wave passing on an activated fibre would provide an abrupt, local rise in $[Ca^{2+}]_{cyto}$ in the quiescent fibre. In toad muscle, wave propagation onto the quiescent occurred almost instantaneously (0.46 ± 0.02 secs), whereas in the malignant hyperthermia human fibres, there was a delay in the propagation of Ca^{2+} release (3.07 ± 0.16 secs, $P < 0.0001$). No propagation of Ca^{2+} waves occurred in healthy human muscle. This is consistent with significant resistance to cytoplasmic Ca^{2+} activation in mammalian muscle. Heat generation needs to occur in a resting muscle, where mild increases in cytoplasmic Ca^{2+} concentration through a leaky RyR are tolerated. Dantrolene (a RyR inhibitor) has been used to provide indirect evidence that RyR Ca^{2+} leak is involved in the maintenance of core body temperature (Bal *et al*., 2012). Here, we show direct evidence that dantrolene reduces Ca^{2+} leak from the RyR1. In addition, we examined the means in which mammalian skeletal muscle may increase thermogeneration physiologically via activation of the sympathetic nervous system during cold exposure. An increase in RyR channel activity and Ca^{2+} leak was observed when exposed to graded concentrations of cAMP, suggesting the important positive role of β-adrenergic stimulation during skeletal muscle thermogenesis.

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Feasibility of cardiac magnetic resonance (CMR) feature tracking (FT) in the adolescent sheep model of myocardial infarction

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Background: Understanding regional dysfunction resulting from ischemic damage is important for tracking the normal course of dysfunction after an ischemic insult such as a myocardial infarction (MI). This information could also be used to test the efficacy of interventions aimed at improving cardiac function post MI. The current gold standard for assessment of myocardial function is cardiovascular magnetic resonance (CMR). However, recent studies suggest that feature tracking (FT) CMR has advantages as it provides information pertaining to myocardial deformation by measures of myocardial strain (MS) (Schuster *et al.*, 2016). MS is the fractional change in myocardial length in a specific dimension from a resting state (end-diastole) to a contractile state (end-systole). Its implications have been extensively studied in human adults and they have greater prognostic value compared with ejection fraction (EF), particularly in heart failure with preserved EF (Park *et al.*, 2018). Our group has previously reported a significant decrease in left ventricle (LV)-EF in adolescent sheep at 3 days following a MI induced by ligation of a branch of the left anterior descending (LAD) coronary artery (Lock *et al.*, 2019). We used CMR to identify an infarct and reduced EF but quantitative measures of regional ventricular dysfunction are lacking and have not previously been performed in sheep.
Hypothesis: We hypothesized that the use of CMR-FT to measure MS in the adolescent sheep is possible and sufficiently robust to detect diminished cardiac function in response to the MI.
Methods: Six-month old adolescent sheep (n=3) underwent thoracotomy under general anesthesia and the second diagonal branch of the LAD coronary artery was ligated and the jugular vein was catheterized as previously described (Lock *et al.*, 2019). Immediately following the surgery, adolescent sheep underwent CMR (1.5 T) and cine images of the heart were acquired using a standard steady state free precession (SSFP) sequence, and repeated at day 3 post-MI. The CMR cine acquisitions were post-processed by volumetry to measure chamber volumes and EF. MS was measured by using a FT algorithm on the CMR images to measure peak global radial strain (GRS) and global circumferential strain (GCS). The long-axis views were processed to measure peak global longitudinal strain (GLS). A one-tailed Student's paired t-test was used to compare the CMR measurements from day 1 and 3. Data are reported as mean ± SD.
Results: CMR-FT was feasible and measures of MS could be made on SSFP cine images using FT in the adolescent sheep. There was a significant decrease in GRS (26.1 ± 4.2% vs 17.8 ± 5.4%; P=0.042) and GCS (-12.3±3.0% vs -16.4 ± 2.0%; P=0.046; in magnitude, negative value in systole) 3 days post-MI. There was a significant decrease in GLS-3C (-16.4±2.7 vs -8.4±3.5%; P =0.008) at day 3. There was a strong linear relationship between LVEF and GRS (y=0.7154x – 0.1075; R^2= 0.984; P<0.001), LVEF and GCS (y=-0.3636x+0.225; R^2=0.983; P<0.001), LVEF and GLS-3C (y=-0.5224x+0.1144; R^2=0.771; P=0.022).
Conclusion: CMR-FT is feasible in the adolescent sheep and measures of global strain by CMR-FT may be a good surrogate and/or adjunct to EF. This method detects regional changes following a MI by coronary ligation and may be used in future studies for quantification of regional wall motion abnormalities.

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The M1 muscarinic receptor is expressed by myenteric neurons and promotes gastrointestinal smooth muscle contraction

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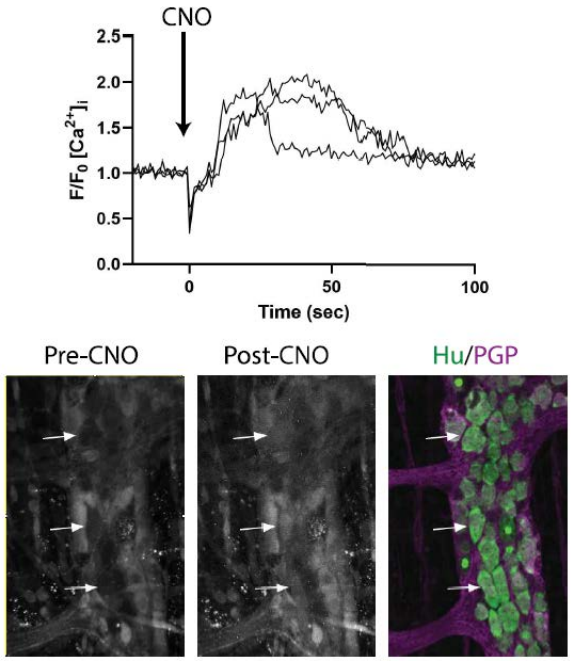
Introduction. ACh is one of the major neurotransmitters essential for gastrointestinal physiology. It is involved in interneuron communication via nicotinic receptors and smooth muscle contraction via muscarinic receptors. Although activation of the M1 muscarinic receptor subtype (M1R) promotes diarrhoea, mechanistic understanding of this process is limited. This is largely due to the lack of subtype-selective pharmacological tools available. Efforts have been made to circumvent this problem including the generation of mice expressing chemogenetically engineered receptors called DREADDs (Designer Receptors Exclusively Activated by Designer Drugs). These receptors are activated by synthetic ligands and are insensitive to ACh.
Aims. To characterise the expression and functional role of M1R in the distal colon using tissues from mice expressing M1R-DREADD tagged with haemagglutinin (HA).
Methods. To determine the expression of M1R in the distal colon, cross-sections were stained with an HA antibody and imaged using fluorescence microscopy. Functional expression of M1R was determined by tissue calcium imaging using myenteric plexus-circular muscle wholemount preparations. To study the effect of M1R-DREADD activation on the smooth muscle, isometric tension recordings of segments of the distal colon were quantified. Clozapine-N-oxide (CNO) was used to activate M1R-DREADD.
Results. HA immunoreactivity was detected in the myenteric plexus of the colon. HA-positive cells were also positive for the pan-neuronal marker PGP9.5. Calcium signalling to CNO were observed in a subset of myenteric neurons. CNO evoked rhythmic contractions of the smooth muscle without changes in the basal tone.
Discussion. M1R-DREADD-HA is localized to myenteric neurons of the distal colon. Functional expression by neurons is supported by calcium responses and the effects of M1R-DREADD activation on smooth muscle contraction. Taken together, these results show that M1R is able to regulate gastrointestinal smooth muscle contraction by a neuronal mechanism.



The effect of sleep restriction, with or without high-intensity interval exercise, on myofibrillar protein synthesis in healthy young men

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We aimed to investigate the effect of sleep restriction, with or without high-intensity interval exercise (HIIE), on the potential mechanisms underpinning previously-reported sleep-loss- induced reductions to muscle mass. Twenty-four healthy, young men underwent a protocol consisting of two nights of controlled baseline sleep and a five-night intervention period. Participants were allocated into one of three parallel groups; a normal sleep (NS) group [8 h time in bed (TIB) each night], a sleep restriction (SR) group (4 h TIB each night), and a sleep restriction and exercise group (SR+EX, 4 h TIB each night, with three sessions of HIIE). Deuterium oxide was ingested prior to commencing the study and muscle biopsies obtained pre- and post- intervention were used to assess myofibrillar protein synthesis (MyoPS) and molecular markers of protein synthesis and degradation signalling pathways. MyoPS was lower in the SR group [fractional synthetic rate (% day –1), mean ± SD, 1.24 ± 0.21] compared to both the NS (1.53 ± 0.09) and SR+EX groups (1.61 ± 0.14) ($P < 0.05$). However, there were no changes in the purported regulators of protein synthesis (i.e. p-AKT ser473 and p-mTOR ser2448) and degradation (i.e. Foxo1/3 mRNA and LC3 protein) in any group. These data suggest that MyoPS is acutely reduced by sleep restriction, although MyoPS can be maintained by performing HIIE. These findings may explain the sleep-loss-induced reductions in muscle mass previously reported and also highlight the potential therapeutic benefit of HIIE to maintain myofibrillar remodelling in this context.

Cardiac maturation across late gestation and the neonatal period in pigs

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Background: Cardiovascular disease (CVD) remains the leading cause of death worldwide, with approximately 18 million deaths attributed to CVD globally[1]. During gestation, cardiomyocytes possess the ability to undergo proliferation, enabling repair of a damaged heart[2]. However, after birth, cardiomyocytes lose their proliferative capacity and enter a non-proliferative, quiescent state as seen in the adult human heart. In postnatal life, injury results in the formation of a fibrotic scar and hypertrophic growth of pre-existing cardiomyocytes to compensate for the loss of contractile units within the myocardium. In some animals such as the adult zebrafish, newborn mice, and fetal sheep, cardiomyocytes can switch from a mature state to an immature state with proliferative capacity, which enables repair and regeneration of the heart after damage[3,4]. Whilst many studies have utilised zebrafish and rodents as a model to study cardiac disease, the use of large animal models such as pigs and sheep are necessary to better reflect human physiology where the switch from proliferative to hypertrophic cardiomyocytes occurs before birth. However, the timing and pattern of major developmental milestones in cardiomyocytes remains ill-characterised in pigs.

Objective: We aimed to characterise and profile cardiomyocyte development across late gestation piglets from 91-112 days (term=115 days) and in neonatal piglets from postnatal day 1 to 20 (n=6 female and n=6 male at each time point). The mRNA expression of genes associated with cardiomyocyte hypertrophy, proliferation and differentiation was determined by qRT-PCR to gain a better understanding of the cardiac profile in during this crucial time period.

Results: Proliferation genes (*CCND2, CCN2, CDK1, PCNA* and *NIFK2)* showed opposing results, with *PCNA* and *CDK1* expression decreasing until birth and then dramatically increasing postnatally, while *CCN2* and *CCND2* showed maximum expression before birth at day 112 gestation, and then decreasing postnatally. Genes involved in cardiac hypertrophy (*ATP2A2* and *NPPA)* demonstrated significantly decreased expression at birth compared to other time points. Gene targets (*HOMER1*, *HOPX* and *CLIC5*) of miR-199a and miR-590, miRNAs previously demonstrated to promote proliferation of cardiomyocytes, showed a significant increase in expression at postnatal day 20 compared to other time points. Targets of miR-133a (*SRF*, *PGAM1*, *GJA1*, *CCND2* and *CCN2*), a miRNA that is lowly expressed during cardiomyocyte proliferation, were also assessed. *SRF* displayed a similar trend to *CCND2* and *CCN2*, with maximum expression at postnatal day 1.

Conclusion: Whilst this preliminary data provides important findings, more research is needed to better characterise cardiac development and the timing of the transition from a proliferative to a quiescent myocardium in pigs. Genes and proteins involved in metabolism and mitochondrial function may provide important findings and uncover novel roles for metabolism and energy utilisation by cardiomyocytes.

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Characterisation of non-nutritive capillaries in skeletal muscle using contrast enhanced ultrasound and fluorescent vascular casting

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Combined, skeletal muscles account for ~40% of total body mass and play a critical role in metabolic homeostasis. A loss of normal vascular responses in muscle leads to development of insulin resistance, prior to any changes in the vascular architecture. Using the isolated, pump-perfused rat hindlimb preparation, we have shown that some vasoconstrictors increase, and others decrease muscle metabolism by redistributing blood flow between nutritive and non-nutritive capillary beds. However, identifying the anatomical location of these discrete capillary beds in muscle has remained elusive. In the present study, we aimed to adapt contrast enhanced ultrasound (CEU) and fluorescent gelatin casting to locate the nutritive and non-nutritive capillaries muscle. Male Sprague Dawley rats (250-280g) were surgically prepared for perfusion of one hindlimb. A modified Krebs-Henseleit buffer was gased with carbogen (95% O2, 5% CO2) and pumped through the hindlimb vasculature at a constant flow rate (0.5 ml/min/g wet wt. muscle). Perfusion pressure, glucose uptake and oxygen uptake were measured for the duration of the experiment. CEU was performed to quantify vascular perfusion at baseline and during infusion of either saline, AngII (15nM) and 5HT (400nM). A buffer containing 1.25% gelatin and 0.1% FITC-albumin was infused to create an *in situ* fluorescent cast of the vasculature under saline, AngII or 5HT mediated conditions. The soleus muscle was dissected, drop fixed in 4% paraformaldehyde, cryosectioned in the transverse plane (20µm) and stained using DyLight 594 Tomato Lectin (1:1000) to quantify the percentage of perfused versus un-perfused capillaries. Compared to saline, infusion of 5HT decreased skeletal muscle vascular perfusion (0.18±0.07AI/sec vs 0.40±0.13AI/sec, $p=0.014$) and decreased the percentage of perfused capillaries in the soleus (7.6±5.4% vs 45.4±10.9%, $p<0.001$). AngII infusion did not change vascular perfusion when measured with CEU, however AngII decreased the percentage of perfused capillaries in the soleus when compared to saline (13.0±9.3% vs 45.4±10.9%, $p<0.001$). These findings suggest that the non-nutritive flow route provides a vascular shunt that enables blood flow to bypass skeletal muscle cells, thus reducing muscle metabolic potential. Whether blood flow is preferentially directed to non-nutritive vessels in insulin resistance to compromise muscle metabolism is not known and warrants investigation.

Agonistic antibody against Fn14 drives positive feedback loop and MyoD expression in acute muscle injury model

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Muscle wasting is a devastating comorbidity associated with an array of chronic and acute conditions. Maintenance of muscle mass and function is essential for improving health outcomes and quality of life in these individuals.
TNFα-like weak inducer of apoptosis (TWEAK) is an important cytokine regulator of muscle homeostasis. TWEAK has been shown to regulate muscle growth, repair, and remodelling via interaction with its receptor, fibroblast growth factor-inducible 14 (Fn14). Regulation of TWEAK-Fn14 and its downstream impact on myogenesis remains poorly characterised.
The current study investigates the effects of two anti-Fn14 antibody variants on degeneration and recovery of mouse *tibialis anterior* (TA) muscle following a Notexin-induced injury. Notexin (venom of common tiger snake, Notechis scutatus scutatus) solution (40 µl, 10 µg/ml) was injected intramuscularly in the right TA and 40 µl saline injected in the left TA. Injuries were performed under isoflurane anaesthesia (2-5% flow rate, recovery within 5-10 minutes) with buprenorphine (0.05mg/kg) injected subcutaneously as analgesic immediately post-injury, and during recovery as prescribed by the supervising veterinarian.
Mice received 20 mg/kg intraperitoneal injections of either anti-Fn14 A, anti-Fn14 B, or no antibody treatment at 6 hours post-injury, and again at 7 days post-injury if applicable. Mice were culled at days 3, 7, and 14 post injury and both TA muscles collected (n=3-4 for each treatment and time point).
Tissue architecture was assessed with H+E staining and key myogenic regulatory factors (myogenin, MRF4, Myf5, and MyoD), catabolic markers (calpain-1, atrogin-1, and MuRF1), and structural proteins (actin, desmin, and myosin) were measured with qPCR or western blotting to establish progression of muscle recovery. Fn14 and TWEAK were also assessed using qPCR.
Compared to untreated, both anti-Fn14 A and anti-Fn14 B treated mice showed significant upregulation of the target, Fn14 at the mRNA and protein level at 14-days post-injury. This was positively correlated with increases in MyoD mRNA, also significantly upregulated at 14-days post-injury in antibody-treated mice relative to untreated controls.
These results indicate a potential positive feedback loop which may positively regulate muscle myogenesis following acute Notexin injury. Notably, structural proteins and tissue architecture (fibre size uniformity, centralised nuclei, non-contractile tissue), as well as infiltration of inflammatory cells, remained pathological in all mice at 14 days post-injury.
While preliminary results indicate that Fn14 may contribute to mouse skeletal muscle remodelling following Notexin injury, further work is needed to clarify the *in vivo* mechanistic actions of the antibody treatments employed and direct future studies.

Sex dependent change in Ca2+ handling properties in response to chronic Ca2+ leak as a result of mutations that affect the RyR

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Ryanodine Receptors are (RyRs) are Ca^{2+} release channels for essential for muscle contraction and heat generation. Furthermore, RyRs passively leak Ca2+ at rest, in theory to generate heat in skeletal muscle (Melzer et al., 1995). Malignant Hyperthermia (MH) is a mutation in the RyR1 that results in an abnormal increase in Ca^{2+} leak (Brini et al., 2005). Subtle changes can be isolated and detected at rest in small compartments sensitive to minute changes, such as the Tubular System (T-system) (MacLennan & Chen, 2009). Therefore, we aimed to examine effect of RyR associated Ca^{2+} leak, directly in RyR1-KI mice, and indirectly through CSQ-KO mice.
The extensor digitorum longus (EDL) muscles of WT, RyR_{Het}, RyR_{Hom} and CSQ-KO mice were excised then rapidly removed. Bundles of muscle fibres were isolated and painted using a membrane impermeable Ca^{2+} sensitive dye rhod-5N (R5N). The fibres are then mechanically skinned to expose the cytoplasm and seal the T-system, trapping R5N. Stained skinned fibres were mounted to a custom built chamber with a coverslip base and bathed in an internal solution with a range of $[Ca^{2+}]_{cyto}$. Ca^{2+} leak was determined using 1mM Tetracaine to completely block the RyR. By applying Tetracaine, we can directly measure the contribution of the RyR to the $[Ca^{2+}]_{T\text{-}sys}$ steady state and the difference in concentration is measured to be RyR "leak." Transients were tracked using confocal microscopy in xyt linescan mode to measure $[Ca^{2+}]_{T\text{-}sys}$ steady states and peak uptake rates (Cully et al. 2018).
It has been shown previously that RyR_{Het} and RyR_{Hom} mice have higher resting $[Ca^{2+}]_{Cyto}$ compared to Wt mice (Yuen et al., 2012). Similarly, we observed a significant gene dose response in the Ca^{2+} handling properties from RyR in the T-system at all $[Ca^{2+}]_{Cyto}$ concentrations. This is shown by a significant increase in steady state $[Ca^{2+}]_{T\text{-}sys}$ scaling from Wt < RyR_{Het} < RyR_{Hom}< CSQ-KO. Furthermore, peak uptake rates of the T-system increases in the same increasing manner corresponding to the genotype. Most notably, the RyR_{Hom} mice presented significant increase in leak and peak uptake capacity compared to both Wt and RyR_{Het} mice, suggesting an adaptation of the muscle reflecting persistent high cytoplasmic calcium. CSQ-KO mice presented a drastically increase in peak uptake capability, however this does not take into account the change in structure of the T-system and therefore the surface area to volume ratio. The increased fluxes of Ca^{2+} through the RyR is a major signalling event in the fibre for adaptation to stress (Ivarsson et al 2019). Furthermore, the increase in capacity to uptake Ca^{2+} suggests an adaptation to compensate the chronically leaky RyR. This chronic leak also causes muscle fibres to produce excess heat due to the upregulated ATP-hydrolysis of SERCA in effort to clear the cytoplasmic Ca^{2+} (Yuen et al., 2012). Furthermore, we were able to identify a sex linked susceptibility to leak, where males presented with increased leak compared to females in the RyR_{Het} and RyR_{Hom} and CSQ-KO, however no difference was found between the WT. Overall, our results provide novel insight into the Ca^{2+} handling properties of muscle with mutations affecting RyR between the sexes.

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Active regulation of red cell mechanics by nitric oxide and calcium

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Classically, the 'passive' physical properties of red blood cells (RBC) were thought to exclusively form the basis of their specific cell mechanics. RBC have the unique capacity to significantly deform when exposed to mechanical shear while traversing the cardiovascular system, and in particular when passing the narrow capillaries of the microcirculation. This exceptional cellular deformability is enabled by the exceeding surface area in comparison to cell volume (facilitated by the bi-concave shape of the RBC), the flexibility of the cell membrane and the intracellular viscosity, which is significantly greater than that of the surrounding plasma. Evidence has accumulated within the past decade, however, supporting the notion that these 'passive' physical properties of RBC may be modulated acutely in response to external stimulation. Mechanical stimulation during traversal of the circulatory system, for example, induces activation of nitric oxide-(NO) and calcium ion-(Ca^{2+}) mediated signalling pathways, respectively. Specifically, RBC express a catalytically active isoform of NO-synthase (NOS), which appears to bear striking similarities to the endothelial type NOS; thus, RBC are capable of independently producing NO by turning over L-Arginine to L-Citrulline. While the molecular activation mechanisms of this novel NOS-isoform are yet to be clarified, convincing evidence suggests that shear stress may activate NOS, potentially via the phosphoinositide 3-kinase-pathway. Finally, NO produced via RBC-NOS is thought to increase flexibility of the cell membrane through post-translational modifications of integral membrane proteins.

Exposure of RBC to shear stress, inducing cellular deformation as it occurs in the microcirculation, has also been shown to raise intracellular calcium-concentration. Influx of Ca^{2+} is known to activate the potassium channel $K_{Ca}3.1$, generally referred to as the Gárdos channel, facilitating efflux of potassium- and chloride-ions causing the cell to lose volume. Although this temporary shrinkage decreases cellular deformability by increasing cytosolic viscosity and decreasing the surface-area-to-volume ratio, it is currently hypothesised that microvascular transit is enhanced due to the smaller cell size. Moreover, given that it is generally assumed RBC-NOS and eNOS share common properties, there is evidence to support that RBC-NOS also requires increased intracellular Ca^{2+} for activation, as does eNOS. It is now tantalising to explore the potential cross-talk between these pathways, especially with increased shear stress and/or deformation of the cell membrane as the potential common activation mechanism.

Synthesis of our efforts to elucidate shear-dependent, acute regulation of RBC physical properties through signalling molecules provides a novel view of these abundant cells, which challenges classic paradigms. Exposure of RBC to physiological-level shear stress (<15 Pa) temporarily improves cell deformability by up to ~20%, an effect lasting for around 300 s. This temporary improvement in cellular deformability was associated with increased NOS-activation, reflected by a ~3-fold increase in ser^{1177}-phosphorylation. When exposing RBC to intracellular oxidative stress, a down-regulation of both RBC-NOS phosphorylation and the up-stream protein kinase B was observed, which occurred concurrent with impaired cell deformability. Mechanical stimulation of these oxidatively damaged RBC significantly ameliorated the impairment in cell deformability via activation of RBC-NOS to levels above-and-beyond those observed in sheared control RBC. These observations demonstrate the complex acute signalling networks that exist in RBC, which both respond to mechanical stimulation and may aid in regulating the physical properties of RBC via mechanisms that are independent of transcription. Interestingly, these investigations were carried out with samples coagulated using ethylenediaminetetraacetic acid, a compound that chelates divalent cations, including Ca^{2+}. Yet, although Ca^{2+} is considered essential for eNOS activation, it appears that the beneficial effects of mechanical stimulation still occurred.

Increased intracellular Ca^{2+} has classically been associated with RBC rigidity, although the effects of Ca^{2+} in dependence of shear stress, and thus cell deformation, have not been explored. Using ionophore A23187, we induced Ca^{2+}-influx into RBC, and observed a dose-independent increase in cell rigidity, accompanied by a differential sensitivity to mechanical stimulation. The rigidity correlated with a decrease in cell volume and was abrogated when the Gárdos channel was inactivated. Moreover, when Ca^{2+} was placed into the shearing medium, time-dependent alterations in RBC deformability were observed, supporting the notion that shear and/or cellular deformation facilitate Ca^{2+}-influx, which then modifies the physical properties of RBC.

Collectively, it appears that RBC mechanics are subject to 'active' regulation through acute changes in concentrations of signalling molecules, supporting the notion of an active cell-type with self-regulated mechanics.

The Influence of Sex on DNA Methylation in Human Skeletal Muscle

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Nearly all human complex traits and diseases exhibit some degree of sex differences. While differences in the sex chromosomes and in genetics contribute to some of the sex-specific phenotypes, the greatest contributor to the observed sex differences is likely epigenetics. Epigenetics, the upstream level of gene regulation, modifies gene expression depending on various stimuli. DNA methylation, a well-studied epiegenetic modification has displayed sex differences in various tissues. Skeletal muscle among one of the tissues with the most sex-biased gene expression, however, it is still unknown whether there are sex differences in DNA methylation in skeletal muscle. We conducted a meta-analysis with our cohort, the Gene Skeletal Muscle Adaptive Response to Training (SMART) study, and two other external datasets. We then overlapped our robust findings with sex-biased skeletal muscle gene expression data from the database Gtex. We found ~60,000 differentially methylated positions (FDR<0.005) between the sexes. Of the 2,866 sex-biased genes from Gtex, 1,014 of those display sex differences at the DNA methylation level. Skeletal muscle displays profound differences at the DNA methylation level. The comparison of male and female DNA methylation profiles uncovers sex-specific mechanisms in muscle metabolism and health. Elucidating sex differences in molecular mechanisms is critical for developing deeper insight into the underlying mechanisms of skeletal muscle health and function, as well as facilitate the use of this information in future research and practice.

Haemodynamics and cerebral oxygenation of neonatal piglets in the immediate *ex utero* period supported by either mechanical ventilation or an *ex utero* oxygenator

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Gestational age at birth is a major predictor of wellbeing; the lower the gestational age, the greater the risk of mortality and morbidity. At the margins of human viability (<24 weeks gestation) immature lungs combined with the need for early ventilatory support means lung injury and respiratory morbidity is common. The abrupt haemodynamic changes consequent on birth may also contribute to preterm-associated brain injury, including intraventricular haemorrhage. Artificial placenta technology aims to support oxygenation, haemodynamic stability and ongoing fetal development *ex utero* until mature enough to safely transition to a true *ex utero* environment. We aimed to characterise the impact of birth transition onto either an oxygenator circuit or positive pressure ventilation on haemodynamic stability and cerebral oxygenation of the neonatal piglet. At 112 days gestation (term=115 days), fetal pigs underwent surgery to implant vascular catheters into the carotid artery and jugular vein. A flow probe was placed around the opposite carotid artery and a neonatal NIRS sensor was placed on the piglet's forehead. Piglets underwent an *in utero* baseline recording of blood pressure (Figure 1), heart rate, carotid artery blood flow and cerebral regional oxygen saturation (rSO_2). Piglets were then transitioned onto either an oxygenator (OXY, n=5) or ventilatory support (VENT, n=8). Blood pressure (BP; Figure 1), carotid blood flow and cerebral oxygenation in VENT piglets rose from *in utero* levels to be significantly higher than OXY piglets post transition. OXY piglet BP, carotid blood flow and carotid oxygen delivery (DO_2) decreased from *in utero* levels post transition; however, cerebral rSO_2 was maintained at fetal-like levels. OXY piglets became hypoxaemic and retained CO_2. Whether OXY piglets are able to maintain cerebral rSO_2 under these conditions for a prolonged period is yet to be determined. Improvements to OXY piglet oxygenation may lie in maintaining piglet BP at *in utero* levels and enhancing oxygenator circuit flow.

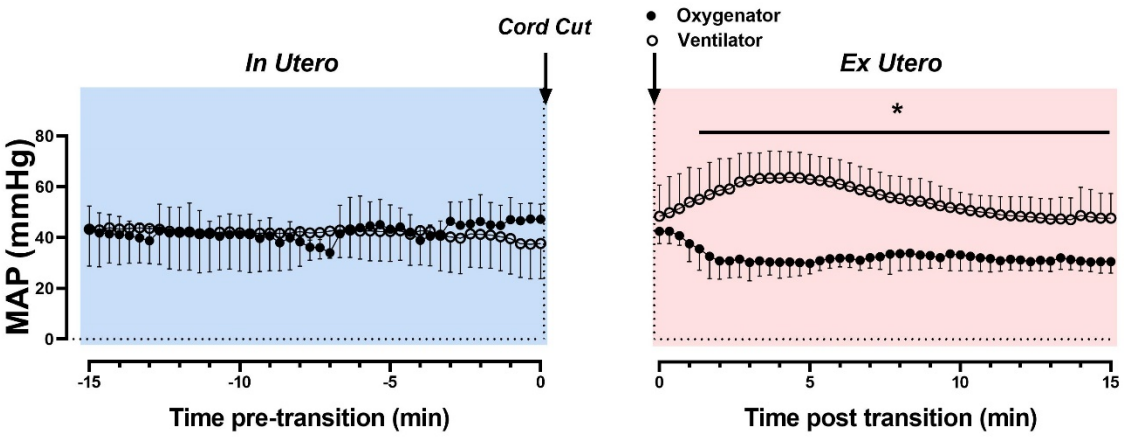

Figure 1. Mean arterial pressure (MAP) *in utero* (shaded blue) and *ex utero* (shaded red) in piglets transitioned onto mechanical ventilation (unfilled circles; VENT) or an oxygenator circuit (filled circles; OXY). Data presented as mean ±SD and analysed by a repeated measures two-way ANOVA with Bonferroni's correction. *, significant difference between VENT and OXY groups. $P<0.05$.

Pericyte and vascular changes are associated with the development of amyloid pathology

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Alzheimer's disease (AD) is the most common form of dementia worldwide and is one of the leading causes of death and disability in Australia. Although the cause of AD is currently unknown, five out of the seven preventable risk factors for AD are cardiovascular related. Therefore, vascular dysfunction in the brain may be an important early event leading to the deposition of amyloid and development of AD. We hypothesise that dysfunction or loss of pericytes, a cell involved in blood flow regulation, blood-brain barrier maintenance and amyloid clearance, may play a role in AD development and onset. To understand the interactions between amyloid deposition, pericytes and the cerebrovasculature, we characterised pericyte and vascular density in 3, 6, 9, 12 and 18-month-old APP/PS1 mice, a transgenic mouse model with overproduction of amyloid. Higher blood vessel density was identified in 3-month-old APP/PS1 mice compared to wild type mice but was not observed in 6, 9, 12 and 18-month-old APP/PS1 mice. Furthermore, a reduction in pericyte number in older animals (6, 9, 12 and 18-month-old) compared to 3-month old mice was found in both APP/PS1 and wild type mice. These results suggest that prior to amyloid plaque formation, there is a strong increase in brain vascularisation to possibly enhance the clearance of increased soluble amyloid. However, a large age-related reduction in pericyte number and vessel density could lead to a prevention of amyloid clearance and facilitate the formation of amyloid plaques, which could explain changes in vascular function during aging. Overall, vascular clearance of amyloid is critical to maintain brain function, and age-dependant reductions in pericyte number and vascular density may put an ageing population at risk of developing AD.

Defining the placental androgen signalling axis in the presence of an inflammatory challenge

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Introduction

Asthma is a highly prevalent disease during pregnancy that is associated with elevated systemic levels of pro-inflammatory cytokines, altered placental function and structure, and an increased risk of adverse outcomes for the developing fetus. Interestingly, these placental dysfunctions and associated risks are sex-specific. Male fetuses continue to grow in the presence of maternal asthma but are more likely to be growth restricted, delivered preterm, or stillborn when maternal asthma becomes uncontrolled. In contrast, females reduce their growth in the presence of maternal asthma which may increase survival when maternal asthma becomes uncontrolled. The mechanisms that contribute to these sex-specific growth and developmental outcomes remain largely unknown, however it is hypothesised that alterations to placental androgen signalling axis may be an important contributor.

Androgens function through the androgen receptor (AR) to transcriptionally regulate genes primarily involved in growth pathways. Recent findings from our group identified sex-specific differences in the expression and subcellular localisation of placental AR isoforms. Unlike females, male placentae from asthmatic pregnancies had decreased nuclear AR-FL and AR-v7, but increased nuclear AR-45 expression. Of the identified nuclear AR isoforms, AR-45 was associated with increased downstream targets involved in growth and angiogenic pathways. These initial findings suggest an important role in a changing placental AR isoform landscape in response to maternal asthma which may ensure continued male growth. In the current study, we have further characterised the function of androgen signalling in the placenta, and how inflammation may impact its response, using an *ex vivo* placenta model.

Methods

Term human placental villi explants collected from normal pregnancies (n=8 male, n=12 female) were cultured in CMRL-1066 media supplemented with 0-10nM dihydrotestosterone (DHT) in the presence or absence of 1μg/ml lipopolysaccharide (LPS) for 24 hours. Tissue was snap-frozen for protein and gene expression analysis.

Results

The expression of cytoplasmic or nuclear AR-45, AR-v7, or AR-FL did not vary between culture conditions in male or female explants. Nuclear AR-45 expression was significantly increased in male placental explants cultured in the presence of 0.1nM DHT, when compared with cytoplasmic AR-45 expression ($p<0.05$). Nuclear AR-FL and AR-v7 were significantly reduced in the presence of LPS, when compared with cytoplasmic AR-FL and A-v7 expression in male placentae only ($p<0.05$). The expression of *IGF-1R* was significantly reduced, and there was a trend towards decreased *VEGFA* expression, in female placental explants cultured in the presence of LPS ($p<0.05$). In contrast, the expression of *IGF-1R* and *VEGFA* was unchanged between culture conditions in male placentae. There were significant, negative associations between nuclear AR-FL and AR-v7 and *IGF-1R* (r^2 = -.975 and -.894, respectively), and cytoplasmic AR-45 and *IGF-1R* (r^2 = -.900) in male placentae cultured in LPS.

Conclusion

In the current study we report distinct, sex-specific differences in the placenta's response to an inflammatory challenge, whereby female placentae, but not male, reduced the gene expression of growth and angiogenic factors. Although there were no significant differences in the expression of AR protein isoforms in the presence and absence of an inflammatory challenge, the subcellular localisation of AR-v7 and AR-FL were altered in male placentae in response to inflammation. These subtle, sex-specific differences in the placental AR isoform landscape may be an important, protective mechanism that prevents the suppression of downstream growth and angiogenic factors in the presence of inflammation in males only. This adaptation may, in part, mediate the continued male growth phenotype often observed in pregnancies associated with elevated systemic inflammation; however, further *in vitro* and *ex vivo* characterisation studies are required to delineate this complex phenomenon.

The Orai1 inhibitor BTP2 impairs the Ryanodine Receptor 1 in skeletal muscle.

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In skeletal muscle, store-operated calcium entry (SOCE) is a retrograde mechanism that leads to the influx of extracellular calcium into the muscle fibres. This process is activated by a decline in free Sarcoplasmic Reticulum (SR) Ca^{2+} concentration, where STIM1 detects this decrease and activates the Store-Operated Ca^{2+} (SOC) channels, located in the sarcolemma invaginations of the fibre, known as t-tubules. In the present work, we used mechanically skinned muscle fibres to assess the effect of the SOC channels inhibitor BTP2 on calcium handling. The highly sensitive system that the sealed t-tubules offer as a Ca^{2+} nanodomain allowed us to detect and quantify a resting Ca^{2+} flux through the SOC channels, sensitive to BTP2. In addition, we found that commonly used concentrations of BTP2 for muscle fibre experimentation impaired the SR Ca^{2+} release channel, Ryanodine Receptor 1 (RyR1). Specifically, we found that BTP2 impaired the basal Ca^{2+} leakage through the RyR1, as well as its Mg^{2+} removal-induced activation or by electrical stimulation to mimic the excitation-contraction coupling mechanism.

In contrast to experimentation with intact muscle fibres, where exposure to BTP2 is extracellular, the usage of mechanically skinned fibres implies a cytoplasmic administration of any drug used; therefore, we tested whether the route of administration was important for the novel effects of BTP2 observed on the RyR1. For that, we designed a novel strategy to deliver drugs to the t-tubules lumen (extracellular space) and skinned the fibre to trap the drug on the extracellular compartment generated by the t-tubules sealing. The delivery of the drug can be tracked by the presence of a fluorophore present on the drug solution and, of importance, can be delivered to a specific population of t-tubules in a single fibre. This leaves a region of the fibre unexposed to the drug, which makes it a suitable in-preparation control to assess any effect of BTP2 on Ca^{2+} handling. From these experiments we concluded that the effects of BTP2 on the RyR1 were not due to the cytoplasmic administration since they were also observed when BTP2 was trapped in the t-system lumen (extracellular administration).

Finally, the RyR1s of saponin-exposed fibres, whose t-tubules membrane were disrupted, were not affected by BTP2. This suggests that BTP2 does not impair the RyR1 as a direct antagonist of this channel, as it depends on an intact t-tubule membrane, required for the proper regulation of protein-protein interactions and Ca^{2+} fluxes that might play a role in this inhibition.

The work presented here shows novel actions of a commonly used drug (BTP2) as well as presents novel approaches to test the effect of molecules on muscle fibre calcium handling.

Exploring the contribution of hypothyroidism-induced placental dysfunction in pregnancy to the development gestational diabetes mellitus

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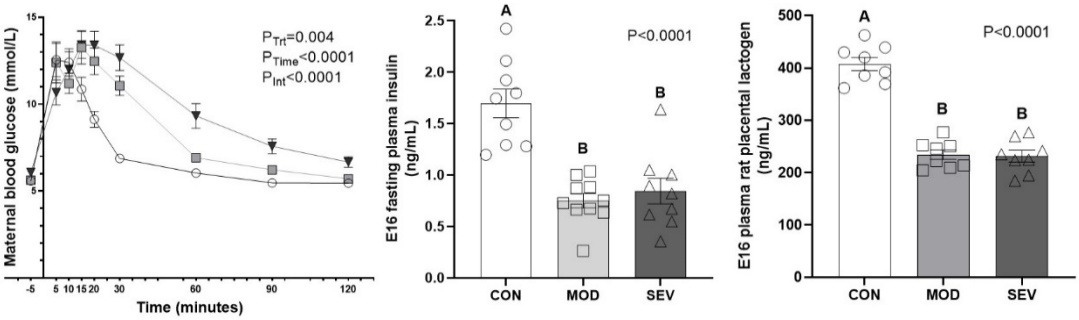
Introduction: Hypothyroidism affects approximately 3% of pregnant women and has been linked to gestational diabetes mellitus (GDM). Few studies have investigated the physiological mechanisms by which thyroid dysfunction in pregnancy may contribute to the development of GDM. This study used a model of hypothyroidism in pregnancy to explore disruption to key pathways that have previously been implicated in GDM.

Methods: Female Sprague-Dawley rats were exposed to either 0.02% (severe hypothyroidism, SEV) or 0.005% (moderate hypothyroidism, MOD) methimazole in their drinking water for seven days prior to mating and throughout pregnancy. On embryonic day (E) 16, pregnant dams were fasted prior to an intraperitoneal glucose tolerance test. This test involves a single injection of a glucose load (1g/kg body weight) and measurement of blood glucose levels over the following two hours (see Figure). On E20, dams were anesthetized by an intraperitoneal injection of a 50:50 mix of ketamine:xylazil (1g/kg body weight) prior to laparotomy and collection of maternal blood via cardiac puncture. Dams were then killed prior to removal of maternal tissues and placentas which were snap frozen for subsequent analysis.



Results: On E16, both MOD and SEV dams were glucose intolerant, and had a significant reduction in both fasting plasma insulin and rat placental lactogen (rPL, see Figure). Furthermore, at E20, SEV dams had significantly higher random blood glucose levels relative to controls. Placental junctional zone expression of *Prl3d1* and *Prl3b1*, two key placental peptides implicated in GDM, were not affected, however maternal plasma rPL remained decreased at E20. Pancreatic expression of *Nkx6-1*, a critical gene required for beta-cell expansion, was significantly reduced in MOD and SEV dams. This suggests that inefficient placental production of rPL may be failing to facilitate appropriate beta-cell expansion during pregnancy in hypothyroid dams, reducing insulin synthesis and secretion. This may explain the glucose intolerance seen on E16. Within maternal skeletal muscle, there was a significant reduction in insulin receptor substrate 1 (*Irs1*) gene expression and glucose transporter type 4 (GLUT4) protein in both MOD and SEV dams. In combination with changes in the pancreas, this suggests that hypothyroidism in pregnancy reduces insulin secretion while also reducing peripheral insulin sensitivity, culminating in a classic GDM-like phenotype. Further investigation into other pathways within the liver and pancreas that may contribute to dysregulated maternal glucose homeostasis in pregnancies affected by hypothyroidism are underway.

Conclusion: This study is the first to provide novel evidence exploring placental contributions in the link between hypothyroidism in pregnancy and pathogenesis of GDM. By further exploration of key metabolic pathways that may be disrupted, this study possesses great potential to improve care, treatment options and long term health outcomes for women at risk of developing GDM in their pregnancy.

The effects of hypoxia on older adults' hormonal responses to resistance exercise

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Older adults commonly experience low muscle mass and strength termed sarcopenia that increase their risk of falls, fractures and physical disability. The proposed mechanisms for sarcopenia include reduced physical activity, altered protein turnover and reduced anabolic hormones such as growth hormone (Ogawa *et al.*, 2016). Although regular resistance training can increase muscle mass and strength in older adults, sarcopenia remains prominent in this population. Therefore, novel exercise interventions are needed to improve muscle health in older adults.

Performing resistance training in normobaric hypoxia is an emerging strategy to improve muscle hypertrophy (Kurobe *et al.*, 2015) and strength (Inness *et al.*, 2016) training outcomes in young adults. Furthermore, in young adults a single bout of resistance exercise in hypoxia, but not normoxia, can transiently increase systemic growth hormone levels in the 30 minutes post-exercise (Kon *et al.*, 2012). Although these findings appear promising for the older adult population, we recently showed that older adults do not experience additional lean muscle mass or strength training benefits from performing moderate-intensity resistance training in hypoxia (Allsopp *et al.*, 2020). To better understand why older adults do not benefit from performing resistance training in hypoxia, this study aimed to characterise the acute and chronic hormonal responses of older adults to resistance training in hypoxia.

Men and women aged 60-75 were recruited into a single-blinded randomised trial, performing resistance training twice weekly in either normobaric hypoxia (14.4% O_2) or normoxia (20.93% O_2) for eight weeks. Participants trained at 70% of their predetermined one repetition maximum (1RM), performing four upper and lower body exercises. Venous blood samples were drawn following the first training session and the last training session of the intervention to characterise the 1) acute responses to the first training session; 2) the acute responses to exercise following eight weeks of training, and; 3) the chronic resting hormonal responses. Growth hormone, cortisol, testosterone and insulin were quantified with commercially available ELISA kits. Results were analysed using repeated measures ANOVA (n=10 normoxia, 10 hypoxia), with Šidák post hoc testing.

The eight week resistance training intervention did not change the resting levels of circulating hormones in either normoxia or hypoxia ($p<0.05$). There were no significant effects of hypoxia on the acute hormonal responses of older adults to resistance exercise at the beginning of the intervention ($p>0.05$). Surprisingly, the participants who trained in hypoxia for eight weeks displayed blunted growth hormone responses up to 15 minutes post-exercise ($p = 0.017$). These finding were unexpected and oppose the findings in young adults following hypoxic resistance exercise. The mechanisms underpinning this response are unknown and require further investigation.

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Altered Mitochondrial Translation Initiation Disrupts Respiratory Complex Assembly and Leads to Cardiomyopathy

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The majority of energy required by our bodies is produced by mitochondria, via oxidative phosphorylation at the respiratory complexes of the electron transport chain. Mitochondrial proteins are encoded by both the nuclear and mitochondrial genomes and the coordinated expression of both genomes is essential for energy production. Impaired energy production leads to mitochondrial dysfunction that causes or contributes significantly to a variety of diseases. Mutations in genes encoding components of the mitochondrial translation machinery are among the most common defects precipitating mitochondrial disease, but little is known about the mechanisms involved in the regulation of mitochondrial translation. We have created a new model of cardiovascular disease caused by loss of mitochondrial translation initiation factor 3 (MTIF3), whose specific role during protein synthesis was unclear. This study was approved by the Animal Ethics Committee of the University of Western Australia and conducted in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes* (NHMRC). Loss of this protein is embryonic lethal, so to characterise its molecular role we developed and investigated a homozygous heart- and skeletal-muscle-specific knockout (KO) mouse model. At 25 weeks, KO mice weighed approximately 25% less than wild-type (wt) counterparts ($n=6$, $p<0.001$) and had developed a dilated cardiomyopathy. Echocardiography was performed under light methoxyflurane anesthesia, and demonstrated that mice had a significant decrease in fractional shortening, posterior wall and intraventricular septum thickness versus wt mice ($n=4$, $p<0.05$). Experiments to characterise the molecular function of this protein were performed using heart and skeletal muscle tissues extracted from mice culled at 10 and 25 week time points. We identify that MTIF3 is vital for regulating translation initiation by proofreading the formation of the preinitiation complex. Knockout mice lack this proofreading capacity, so translation proceeds at an increased rate, at the expense of fidelity and coordination with cytosolic translation. This disruption prevents assembly of specific electron transport chain complexes, leading to compromised energy production, ultimately leading to cardiomyopathy.

Uncovering Robust DNA Methylation Marks for Skeletal Muscle Biological Ageing

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As we age, DNA methylation patterns in skeletal muscle change. Existing studies have reported widespread patterns of age-associated *differential* methylation, and a subset of these methylation sites have been used to predict chronological age with high accuracy. The significance of these sites in biological ageing is, however, limited. This study aims to build on existing knowledge by exploring age-associated patterns of *variable* methylation, which may better capture aspects of biological ageing in muscle. In addition, we will seek to understand the influence of both longevity gene variants and sex on changes in DNA methylation with age. We will follow a large-scale, cross-sectional Epigenome-Wide Association Study (EWAS), utilising more than 900 muscle samples from 10 datasets. Bioinformatics tools will be applied to accurately interpret the dynamic DNA methylation marks that change across the lifespan. This project intends to pave the way for identifying robust epigenetic targets associated with biological ageing in muscle.

Myofibre-type specific differences in disease manifestation of dysferlinopathy

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Dysferlinopathies are a form of limb girdle muscular dystrophy (LGMDR2 dysferlin-related) caused by gene mutations resulting in deficiency of the membrane-associated protein dysferlin. Symptoms manifest post growth in a muscle specific manner, although the pathomechanism is not well understood. One feature is many lipid droplets present in slow-twitch dysferlin-deficient myofibres of humans and mice, and more recently we identified an increased capacity for fatty acid esterification to triglycerides in the predominantly slow-twitch soleus muscle of young adult (3 months) dysferlin-deficient BLAJ, compared with wild type (WT) mice. In older (10 months) BLAJ and WT male mice, our *ex vivo* functional studies showed a greater impact of dysferlin-deficiency on predominantly slow-twitch soleus muscles compared to fast-twitch extensor digitorum longus (EDL) muscles. Muscles were excised from mice anaesthetised via intraperitoneal injection of sodium pentobarbitone (40 mg/kg). The dysferlin-deficient soleus was significantly heavier than WT, with faster contraction and relaxation times and delayed post fatigue recovery (*p*s < 0.05, n = 8). The EDL mass of dysferlin-deficient mice did not differ but had slower relaxation compared with WT mice ($p < 0.05$, n = 8). We also identified differential effects of dysferlin-deficiency, between soleus and EDL muscles, on the levels of some metabolism (glycogen synthase) and calcium handling (calsequestrin 1 and dihydropyridine receptor) related proteins (*p*s < 0.05, n = 6) that likely relate to these differences in metabolism and function. These studies emphasise the importance of carefully considering myofibre-type specific effects to better understand the molecular basis for dysferlinopathies and other muscular dystrophies, especially where pathways related to metabolism and calcium regulation are affected. In particular, pre-clinical studies have the capacity to more carefully investigate disease-specific myofibre type differences. Such insight is critical for the development of targeted future clinical therapies.

Characterising mitochondrial free Ca^{2+} across a spectrum of disease

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The calcium (Ca^{2+}) levels in the mitochondria are an important regulator of mitochondrial ATP production. In skeletal muscle, Ca^{2+} is released from the sarcoplasmic reticulum (SR) via the RyR1 channel, where it binds to contractile filaments before being recycled back into the SR or alternatively taken up by the mitochondria to activate oxidative metabolism[1]. However, mitochondrial Ca^{2+} becomes problematic when supraphysiological levels accumulate as this may activate processes eventuating in cell death. As such, mitochondrial Ca^{2+} overload appears to be a common finding in skeletal muscle affected by heatstroke, dystrophies and ageing[2,3].

One model that can be used to study mitochondrial Ca^{2+} accumulation is a mouse skeletal muscle model of malignant hyperthermia (MH), where the SR ryanodine receptor (RyR) is leaky due to a mutation in the RYR1 gene. MH is a rare but life-threatening clinical condition characterised by a hypermetabolic response to volatile anaesthetics or heatstroke. In this condition, mutations in the RyR1 receptor lead to an excessive amount of Ca^{2+} "leaking" from the SR into the cytosol of the cell[4]. By studying fast and slow twitch muscle of wild type, heterozygous and homozygous RyR1 knock-in mice, we aimed to characterise mitochondrial Ca^{2+} levels across a spectrum of RyR1 Ca^{2+} leak.

All experiments performed were approved by and conducted in accordance with The University of Queensland Human Ethics & Animal Ethics Committees. Wild type (WT) mice (C57BL/6J), as well as mice that were heterozygous (RYR1 KI/WT) or homozygous (RYR1 KI/KI) for a known MH-causative mutation in humans (p.G2435R) were culled at 4 – 6 months of age. The extensor digitorum longus (EDL) and soleus muscles were dissected. Single fibres were isolated and mechanically skinned, exposing the intracellular environment to the bathing solution. Individual fibres were incubated at 4°C in a 67 ηM Ca^{2+} internal solution containing 5 μM Rhod-2/AM for 10 minutes, before being washed for 10 minutes in a 67 ηM Ca^{2+} internal solution to remove any non-specific cytosolic staining. Fibres were imaged with an FV1000 confocal laser with excitation at 543 ηm. They were then exposed 0.25 μM FCCP. Depolarization of mitochondrial membrane potential resulted in the observation of a Rhod-2 transient inside the mitochondria that indicated a release of free Ca^{2+} from its buffer inside the mitochondria, followed by a slow extrusion of Ca^{2+} from the organelle. We used this transient to calibrate the free [Ca^{2+}] inside the mitochondria.

In summary, we have developed an assay to assess the movements on mitochondrial Ca^{2+} by using the membrane potential uncoupler FCCP. This allowed us to quantify mitochondrial free [Ca^{2+}]. We demonstrate that an increase in RyR1 Ca^{2+} leak that is seen in MH affected mice is associated with an increase in mitochondrial free [Ca^{2+}]. Thus implicating the key role of Ca^{2+} in linking the dysfunction of the SR and mitochondria across a spectrum of RyR1 disease.

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Stress: a novel regulator of Duchenne muscular dystrophy pathogenesis?

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Loss of expression or function of the dystrophin protein causes Duchenne muscular dystrophy (DMD). DMD affects boys and causes premature death due to cardiorespiratory insufficiency or acute cardiac failure. Recent evidence indicates behavioural stress in a mouse model of DMD (*mdx*) can trigger acute to sustained hypotension-induced death. Therefore, we tested the hypothesis that stress is a novel regulator of DMD pathogenesis. One hundred male and 100 female *mdx* mice were subjected to two behavioural stressors: scruff-restraint and forced treadmill exercise. Mice were ranked within each sex according to their behavioural response (level of physical activity) to each stressor and classified as either stress-resistant or stress-sensitive. Measures of functional capacity and striated muscle histopathology following death were assessed in both groups (n = 10 – 15/group). Stress-resistant mice are more physically active after stress than stress-sensitive mice. Female *mdx* mice are more physically active after stress than male *mdx* mice. Stress sensitivity was not associated with the ability to conceive or reproduce but it was associated with exercise capacity. Stress-resistant mice completed more distance during a graded exercise test to exhaustion, which was associated with greater myosin heavy chain type IIa expression and capillary density in skeletal muscle. Stress-resistant mice also presented with less cardiac and skeletal muscle fibrosis, fewer central nucleated fibres and greater expression levels of the sarcolemmal associated dystrophin homologue, utrophin. The data indicate stress sensitivity in *mdx* mice is associated with skeletal muscle oxidative capacity and linked to the pathophysiology of DMD.