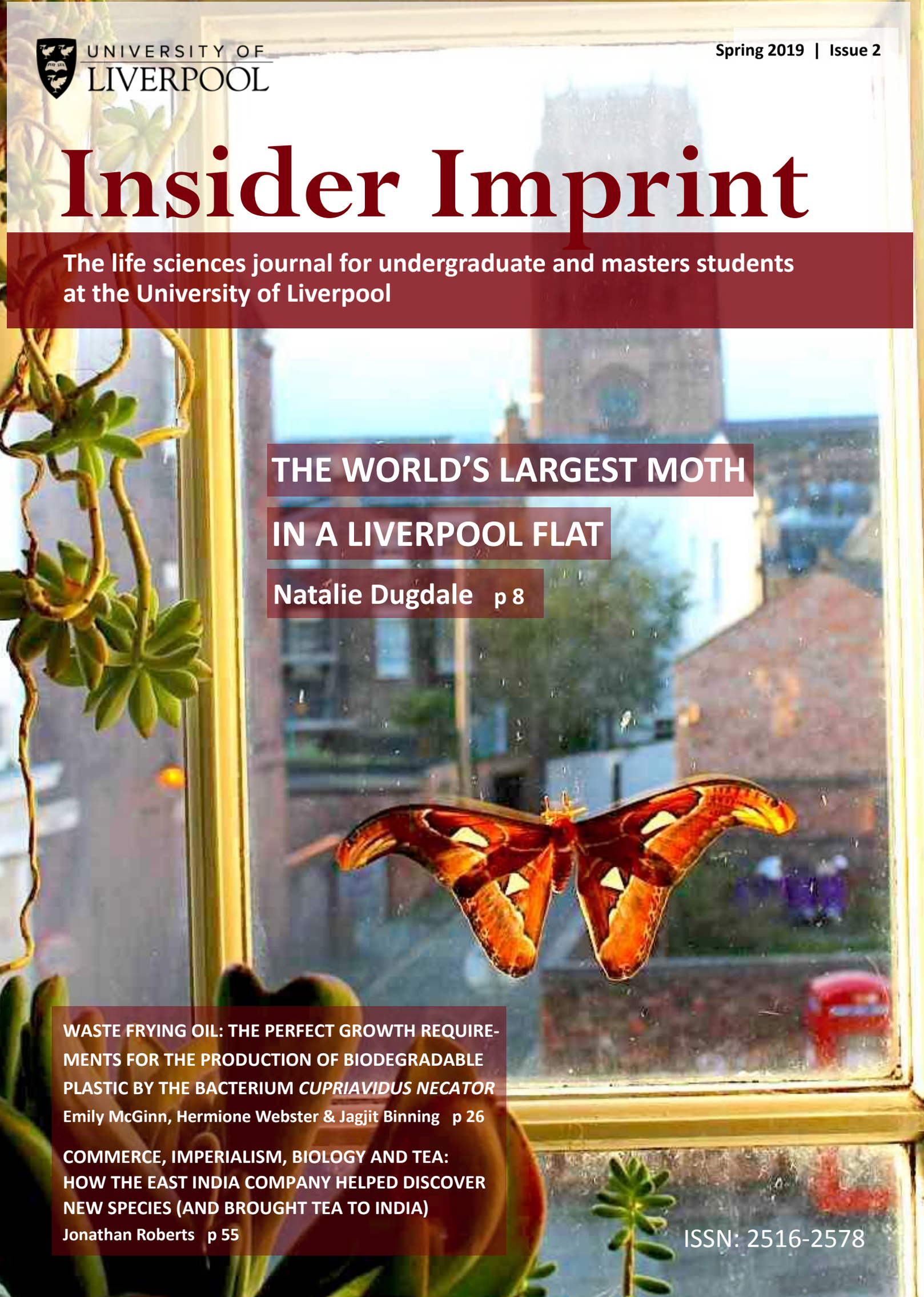
UNIVERSITY OF LIVERPOOL

Spring 2019 | Issue 2

Insider Imprint

The life sciences journal for undergraduate and masters students at the University of Liverpool



THE WORLD'S LARGEST MOTH IN A LIVERPOOL FLAT

Natalie Dugdale p 8

ISSN: 2516-2578

INSIDER IMPRINT

Managing Editor: Dr Fabia Allen

Editorial Board: Juhi Gupta, Dr Kerry Hanna, Alistair Jones, Declan Manning, Shahjahan Shigdar

Peer Review Manager: Shahjahan Shigdar

Public Relations Manager: Juhi Gupta

Social Media Manager: Alistair Jones

Artistic Director: Declan Manning

Peer Reviewers: Aleksandra Beliavskaia, Elizabeth Lomas, Amy Manson, Lorenzo Ramos, Ian Richards

The author(s) retain copyright to their work, and grant Insider Imprint a non-exclusive right to publish the article with a Creative Commons - Attribution licence.

Insider Imprint (Print) ISSN 2516-256X

Insider Imprint (Online) ISSN 2516-2578

Published Spring 2019

Image Accreditations

Page 6 Background image: Pixabay License free for commercial use https://pixabay.com/photos/lake-water-bank-chair-li%C3%A8ge-4026593/

Page 11: Image by Michael Ruiz/CC BY 2.0 https://www.flickr.com/photos/simax/3598360283

Page 18 Background image: Pixabay License free for commercial use https://pixabay.com/illustrations/virus-microscope-infection-disease-4030721/

Page 48 Background image: Pixabay License free for commercial use https://pixabay.com/photos/light-bulb-lightbulb-light-bulb-1246043/

Page 57 Bee image: Pixabay License free for commercial use https://pixabay.com/en/insects-hymenoptera-apis-mellifera-696191/

Page 57 Tea farming image: Pixabay License free for commercial use https://pixabay.com/en/tea-green-planting-the-tradition-3039559/

Page 63 Background image: Pixabay License free for commercial use https://pixabay.com/photos/desk-ink-education-paper-table-1869579/

Page 65 Meerkat image: Pixabay License free for commercial use https://pixabay.com/photos/meerkat-animal-nature-zoo-3520068/

Contents

Insider Imprint | Issue 2 Spring 2019

Foreword

Welcome to the second issue of Insider Imprint, the first journal of its kind in the University - run by students for students. It is wonderful to see such a diverse range of inspiring content from across Health and Life Sciences in this, even bigger, second edition. The University works hard to give students opportunities to build up skills and experience and Insider Imprint is a great example of this. I would like to thank everyone involved in its production – its success is due to the hard work and dedication of the editorial team and the enthusiastic response from contributors.

I do hope that you enjoy reading this issue and encourage you to contact the team with your comments and ideas for future publications. This is, after all, an opportunity for you to showcase and share your many activities and successes.

Professor Dame Janet Beer

Vice-Chancellor

Meet the Team

Meet the team behind *Insider Imprint*! We are a dedicated team from across the Faculty coming together with a common goal of creating a space for undergraduate and masters students to showcase their achievements, to inspire the upcoming generation of students and to support students in becoming confident and competent presenting their work, ideas and experiences.

DR FABIA ALLEN

Managing Editor

Lecturer in the School of Life Sciences

DECLAN MANNING

Editor & Artistic Director

PhD student in Cellular Physiology

DR KERRY HANNA

Editor

Lecturer in Orthoptics

JUHI GUPTA

Editor & Public Relations Manager

PhD student in Pharmacology

ALISTAIR JONES

Editor & Social Media Manager

PhD student in Translational Medicine

SHAHJAHAN SHIGDAR

Editor & Peer Review Manager

PhD student in Musculoskeletal Biology

One year on from our inaugural issue of Insider Imprint, we are proud to present issue 2! Even more jam-packed than issue 1, we are so happy with the variety of submissions and diversity of students getting involved. Find out about what some of your peers have been up to, from study abroad to raising giant moths in their bedroom! Read the current research students are working on and about topics your peers find interesting.

If you are thinking about publishing your work, there has never been a better time! Insider Imprint is open to any topic related to the Life Sciences, and will take submissions from all students in the Faculty of Health and Life Sciences. We will work with you to get your work to publication standard, and once published your work will be available to show off on our beautiful website insiderimprint.com, as well as in print.

Enjoy issue 2, we hope it will inspire and inform you, and we look forward to working with some of you soon!

Your Insider Imprint Team

Reflections

What are your fellow students up to?

Find out about what your fellow students have been getting up to from starting something new, studying abroad and extracurricular activities.

Learn about how your peers have grasped opportunities and their advice to you.

Adjusting to life at Masters level

Advice from a current MSc student

April Hayes, MSc Advanced Biological Sciences (Microbiology)

I remember coming home after the first introduction lecture in welcome week with one sentence rebounding around my head – 'You will be given more work than at undergrad'. This prediction has most definitely come true. At times, it has felt a lot, mainly when we have had several deadlines due for the same day which was tricky to balance. I would say that the workload is doable once you have adjusted to it. The assignments start early – the first was due in the third week of term – and do keep coming, but there's never been a time when it hasn't been possible to get them all written and submitted on time.

Organisation is a big deal at masters level. Mainly due to the increased work load that you are given immediately. I know this is something that everyone knows they should do, but often, don't really do it well. However you like to do it, whether it is with Google calendar or a physical diary, getting organised is the biggest thing I would recommend. There are a lot of things that you will have to juggle. In addition to your modules, there are also meetings with your supervisor, time in the lab, and any part-time work or extracurricular activities. The deadlines are published online but it is your responsibility to keep track of them. I like to use a whiteboard to cross them off when I hand them in, but whatever you do, make sure that you are making note of what is due and when.

The biggest change I have noticed from being a third year is the amount of responsibility we are given by the lecturers and our supervisors. Though it is refreshing to have so much responsibility and freedom at times, if you don't handle flexibility well it can be difficult. You are expected to take control of your project and arrange to meet with your supervisor, and you are expected to remember the course deadlines. You won't be reminded of them! However, since everyone is in the same boat someone is likely to remind you if you've forgotten.

One of my favourite changes is the research project. As an MSc student I don't start in the lab until the summer (unlike the MRes and MBiolSci students) but it has been nice to really get to grips with my research area in semester one by reading the literature and talking to my supervisor. I have been able to spend some time in the lab with some of the PhD students, shadowing them to learn the skills that I'll need in May. It's been one of my favourite parts of the degree so far. I've really come to get some ownership over my project, especially since I was able to direct where it went a little more than at undergrad. I've even got my family and friends interested in biology, which hasn't always happened before! As a master's student you are expected to drive the project more than you might be used to at undergrad, this is one example where you are expected to take more responsibility for your work. This can be a little awkward in the beginning, but as time progresses it gets easier to talk to your supervisor and the PhD students and post-docs in the lab.

As a masters student you are encouraged to attend seminars given by visiting academics hosted by the different research institutes. The seminars are given across a range of topics. I have sat in on seminars about avian influenza, cyanobacterial photosynthesis and DNA replication. I've had the opportunity to listen to a lot of interesting academics, and it is interesting to hear what other people are working on, and to see them so interested in their work. Attending these seminars is a great way to get a flavour of the research going on in areas of life sciences that you may not have thought about before. As a microbiologist, I hadn't really thought much about replication forks, but the seminar was really interesting!

To anyone considering a masters degree I would say to make sure you are willing to put the work in. My MSc so far has been really rewarding, but if I was even the slightest bit apathetic about it all I don't think that I would have got as much out of it as I have done. The learning curve can be steep at times, especially if you're tackling topics and techniques that you have never dealt with before. Before this year I had never used any form of bioinformatics techniques, so the first few weeks of starting the Informatics module were tricky but I have learnt so much, and it's been one of my favourite modules thus far. I have heard a masters degree described as 'third year on steroids' and at times, it can be. However, it is completely manageable, you just need to be aware that it's probably not easily cruised through. It can be very fulfilling, especially if you are willing to put the work in, and there are a lot of opportunities to get involved with, like writing for Insider Imprint! It's so rewarding, and I have learned so much in my first two semesters; it has absolutely flown by. If you are considering a masters degree, I would seriously consider it, especially if you are excited by science, and want to get involved at a deeper level than at undergraduate.

The world's largest moth in a Liverpool flat

A commentary on the experience of raising one of the world's largest moths, *Attacus atlas.*

Natalie Dugdale, 3rd Year Genetics BSc

The internet is a wonderful place; from same-day delivery of groceries and clothing to purchasing a hot tub or a light aircraft, you can get hold of practically anything in no time at all. In my case, I decided to purchase some newly hatched moth larvae from the world's largest moth. I have kept many different animals in my lifetime but have never delved into insect keeping before now. The Atlas moth (*Attacus atlas)* is the largest species of moth by wing surface area (1) and the second largest by wingspan, second only to the White Witch (*Thysania agrippina)*. Atlas moths are a member of the *Saturniid* family and along with several other subspecies, they inhabit the forests of India and South-East Asia, and recently, a one-bed flat in Liverpool.

At the end of July, I received a tiny cardboard box in the post, inside of which was a petri dish containing a few leaves of privet and ten tiny caterpillars. Barely a centimetre long, prickly in appearance and pale green, it was merely days before they had their first moult. Interestingly, the caterpillars changed substantially in appearance with almost every moult following the first. Unlike the vast majority of moth species, which shed their skin four times, the Atlas moth grows so huge that it requires a fifth shed, meaning it goes through six growth phases known as "instars" throughout its life as a larva. With the caterpillars growing as large as 11.5 cm in eight weeks (1), they quickly started a serious regime of eating. The green spiky larvae soon became white and powdery-looking and had doubled in size in less than a week. In the space of five weeks, they had grown to the length of my index finger (Figure 1). Once they reached their fourth instar, a size difference was becoming apparent between the caterpillars and the sexes became distinguishable, with females now growing larger than males. By seven weeks, I could barely keep up with replacing the number of leaves they were eating. The caterpillars were massive and eating privet at a rate at which I had to replace branches every few hours and empty their tank of droppings twice a day. Eventually, after a total of eight weeks of gorging themselves (Figure 2), the caterpillars abruptly stopped eating and their behaviour changed drastically. From energy saving, eating machines they transformed into wanderers, carefully evaluating every stem and leaf they could reach, before picking a place to weave a cocoon. Using the silk glands beneath their mouth, they spent the next two days carefully constructing intricately latticed silk cocoons where they could safely pupate inside. These structures

"From energy saving, eating machines they transformed into wanderers, carefully evaluating every stem and leaf they could reach, before picking a place to weave a cocoon"

Figure 1. Caterpillar in its 4th instar.

Figure 2. Caterpillar in its 6th instar, hours before building her cocoon.

Figure 3. Female pupa removed from her silk cocoon for sexing.

Figure 4. Adult male Atlas moth resting on a window.

Figure 5. Adult male Atlas moth resting on the author's hands.

were wrapped in leaves, well camouflaged and protected by the silk which solidified into a hard shell around them. By the ninth week, my enormous caterpillars had transformed into pupa within enormous silk cocoons. Females have visibly smaller antennae, even as pupa, allowing for easy sexing (Figure 3).

Aside from various butterfly and moth websites and a few blogs, I struggled to find any solid information on timings referring to complete metamorphosis from caterpillar to imago (adult moth). From the information that I had gathered, I initially expected my pupae would eclose as moths within four weeks. As six weeks had passed and there were still no moths, I researched a little further and discovered that Atlas moth pupa are able to lie dormant and overwinter until conditions became more suitable (3). All I knew was that they were still alive and still responding to a touch stimulus, so they went into a well ventilated and insulated box kept at a pleasant 18^{o}C to wait out the winter. With a thermometer, a hot water bottle filled twice daily and regular misting of the cocoons, it was apparent that I had created an ideal environment. On the sixth of December, I opened the box and was shocked to discover my first moth. Within 24 hours of the first emergence, a second male eclosed to join him (Figure 4). With the largest of the two having a wingspan of around 21.5 cm, these magnificent moths enjoyed their final week of life fluttering around a flat in Liverpool (Figure 5). Interestingly, the first two moths to eclose quickly became tame and amicable. They climbed onto my finger voluntarily, of their own accord, and ceased all their defensive tactics such as vibrating, flapping and spraying, all of which were commonplace in the first days following their emergence. Having found that caterpillars are able to retain memories from their final instars (2), I would like to think they were quick to warm to me as they may have had memories of my handling of them in the past with no ill consequence. This theory is probably a leap, of course, with their tame behaviour likely being learned (albeit quickly), or perhaps is simply typical of this species.

After months of eating and growing followed by months of pupating, the adult moths live for a week or less. They do next to no flying because the effort is so great, and instead they conserve their energy in hope of coming across a mate during their short adulthood. I am incredibly grateful for being able to experience the amazing life cycle of these wonderful creatures and will definitely be raising more butterflies and moths in the future.

References

1 Association of Zoos and Aquariums. Atlas Moth (*Attacus atlas*). Available from: www.waza.org/en/zoo/choose-a-species/invertebrates/insects-and-millipedes/attacus-atlas [Accessed 13th December 2018]

2. Blackiston D J, Silva C E and Weiss M R. Retention of memory through metamorphosis: can a moth remember what it learned as a caterpillar? PLoS ONE 2008. 3(3): e1736

3. Worldwide Butterflies. Giant Atlas Moth (*Attacus atlas*) Cocoons. Available from: www.wwb.co.uk/giant-atlas-moth-attacus-atlas-cocoons%22 [Accessed 18th December 2018]

My PhD experience

A journey of independence and investigation

Alistair Jones, PhD Student, Institute of Translational Medicine

As a wide-eyed undergraduate fresh out of Leeds University, with a BSc (Hons) in Neuroscience, I was still unsure if academia was for me. What I did know was that I really enjoyed my dissertation research project and putting my energy into making discoveries of my own.

So, I came over to Liverpool in 2015 to complete the MRes in Translational Medicine, a course that encompassed everything I loved about my dissertation. I found here was the opportunity to work in a lab full-time and it was the first chance I had to become a semi-independent researcher in the lab. It was in this year that I found out that EVERYONE is always learning and it's ok to make mistakes! Even your most confident looking post-doc has to do some techniques for the first-time. This made the jump to a PhD a lot less intimidating for me and I learned that I loved lab-work (even the more tedious experiments). So I hit the website *findaPhD,* and found all the projects that peaked my interest.

Currently, I am on the MRC DiMeN PhD programme in my final year. I was fortunate to be able to find a project that was really interesting to me without having to even change lab benches. My project investigates the mechanisms by which epilepsy develops and I am working on creating new treatment options for patients who currently can't control their seizures. As neurological diseases and molecular biology have always fascinated me, I find this a rewarding way of combining my interests and helping patients.

Working in a lab, learning new techniques and the ups and downs of scientific discovery can be stressful but also incredibly fulfilling. From my first smeary looking western blot to my most recent endeavours into visualising neurons in *C. elegans* (nematode worm), I've really enjoyed learning something new and having different challenges each day. Unlike undergraduate study, I am now completely in charge of my own learning, and that space to learn exactly what I'm interested in is one of the best elements of my PhD.

I was told "You will get exactly as much out of your PhD as you put in" which sounds obvious but, there really are so many projects and training programmes you can take advantage of as a graduate student. I am working with collaborators all over the UK, attending research conferences and I have taken as many training initiatives as possible including making it as far as the USA. Here, I got the fantastic chance to work in Seattle for a month with a unique opportunity to learn and train with some of the great minds in epilepsy pharmacology (as well as trying out Seattle's expansive selections of coffee) through winning an MRC training grant.

I'm also actively involved in brilliant parts of science that don't take place at the lab bench. I've been able to challenge myself in several other areas such as editing for Insider Imprint, organising conferences and being a student representative for my PhD programme. Each of these opportunities, at home and aboard, has given me the chance to meet some incredible people from all sorts of scientific arenas.

If you're thinking "what if I want to leave academia after my PhD" that's ok too! It's not *all* about academia, and most supervisors support interests in alternative career paths during your PhD. I've worked alongside students who have moved on to biomedical industry, medical writing, policy-making and NHS scientist training schemes following their projects. All these options are helped through a PhD as you develop a range of skills that are valuable assets to many jobs outside academia. So don't feel that by taking the PhD step you're in academia forever as it is unlocking doors to career paths I hadn't expected.

A PhD is a passion project, a challenge that you embrace because you can bring your ideas to the table and make your research area better. With some hard work and a little luck, I've had the opportunity to meet some outstanding people and had some fantastic opportunities. I am of course incredibly biased, but if you're thinking of a PhD go find what makes you curious and make your contribution because my PhD has been an incredible experience which I would recommend to anyone.

" I am completely in charge of my own learning, and that space to learn exactly what I'm interested in is one of the best elements of my PhD"

Become a published author

Write for *Insider Imprint* – the life sciences journal for undergraduate and masters students at the University of Liverpool

Currently accepting: research articles, reviews, commentaries, essays, artwork and much more

Submit your work and queries by email to: insiderimprint@liv.ac.uk

We would love to hear from you

www.facebook.com/insiderimprint

Insider Imprint

www.insiderimprint.com

From PhD to Masters to trainee Patent Attorney!

My adventures over the last year, discovering my new career path

Megan Booth, Liverpool Graduate, BSc Molecular Biology and Biotechnology

Over a year ago I wrote an article about studying for a PhD at the University of Cambridge, which was published in the first edition of Insider Imprint in May 2018. However, since then I have 'quit' my PhD, written up my results for a Masters and I am now a Trainee Patent Attorney at Appleyard Lees in Cambridge. In the following article I describe my adventures over the last year, including my realisation that 'quitting' when things really are not right, does not make you a failure.

My lifelong love of science led to me pursuing a degree in molecular biology at the University of Liverpool and I was fascinated by all aspects of my degree. It was sitting in a virology lecture and learning about bacteriophages that inspired me to embark upon a PhD. In September 2016 I started my PhD and my first year passed with what I assumed were the "normal" feelings for a green PhD student, finding their feet in a new lab, in a new city over 150 miles away from home. From feeling like an imposter and dreading repeating the same experiment *again* that week, to longing for some structured learning, my first year passed in a flurry of anxiety and lack of fulfilment. These feelings came as a shock to someone who had always succeeded in everything so far, and they did not go away over time.

In January 2017, I started to attend careers talks and workshops to explore possible career options as I had decided that post-PhD I did not want to continue in academia. One talk was given by a Patent Attorney and I was immediately captivated by what sounded like the perfect career for me in. It would allow me to combine my technical background in molecular biology with my other interests in science communication, writing and marketing. I decided then that this would be the perfect career for after my PhD, as I was under the impression that to become a trainee you required a PhD. Although not all firms demand a PhD, I also thought that no one would employ a Cambridge 'drop-out'. I decided that I would continue with my PhD and just fill my spare time with extra-curricular activities (such as writing blogs, editing articles and science communication) which would allow me to develop the other skills a Patent Attorney requires so that after my PhD I could apply for trainee positions. I rapidly approached the end of my first year with no real improvement to my mood and I realised something had to change.

My PhD came with the opportunity to take three months away from the lab and to complete a work experience placement in a career outside of academia. I embraced this opportunity and managed to obtain a three month placement with an Intellectual Property (IP) firm in Cambridge. Many PhD programmes come with this option to take a three month break to explore careers outside of academia. A well researched three months away from the lab can help students take a step back and do something different. It is worth taking the time to fully research potential placements and write targeted CVs and cover letters in the same way as you would for a job. This can seem like quite a daunting, near impossible task when you're trying to juggle a research project, but it is very useful.

> **"*Quitting* when things are not right does not make you a failure"**

I started my placement in January 2018 and I thought taking some time away from research would help to re-inspire me and provide that motivational push for the remaining two years. However, my three months away had the opposite effect in that I enjoyed it so much that all I wanted to do was become a trainee Patent Attorney. During the placement I learnt a lot about the career and I got to experience the role first hand as I was given real cases to work on from day 1. It confirmed that this was the perfect career for me and despite feeling very nervous about being viewed a 'Cambridge drop-out', I decided to apply for trainee

positions. I was also lucky that I had managed to generate enough data for a masters and so it was agreed that I could write up and get something out of my time at the university.

I applied to three firms, but the job at Appleyard Lees was the one I wanted most. I had read a lot about them on their website and reading so many positive interviews with current trainees online made me want to work for Appleyard Lees even more. Everyone I met at both stages of the interview process was incredibly friendly and I felt that the office had a very welcoming, supportive atmosphere. I was delighted to be offered the job and I started with Appleyard Lees in May 2018.

My first eight months have been incredible. Every day presents new challenges and the opportunity to learn something new and solve problems in interesting ways. Whether this is new scientific or technical knowledge, a new skill or a new rule or article, I find that every day my brain is in constant use from 9am until I turn off my computer around 5pm. I absolutely love being a Trainee Patent Attorney and I love all aspects of the job. There is a lot of reading to do, exams to pass and it is a steep learning curve as with most scientists making the transition, I have never studied law before. I have also had to juggle writing up a masters thesis with working full-time, which I am pleased to say has now come to an end as I submitted my thesis in January 2019!

I always preferred reading and writing to carrying out lab work and this job allows me to still use my science and keep in touch with new developments, but in a much broader area than studying one specific protein or gene in one specific bacteria or cell! It is very varied and I work on a variety of cases, which is one of the many things I love about the career. As well as learning new science and law, there is also a lot of application and problem solving to do, such as thinking of ways you can overcome an examiner's objection to get a case through to grant. Patent law is incredibly rewarding and ticks all of my boxes. There are frustrating days, but there will be in any career and the imposter syndrome still finds a way of creeping in.

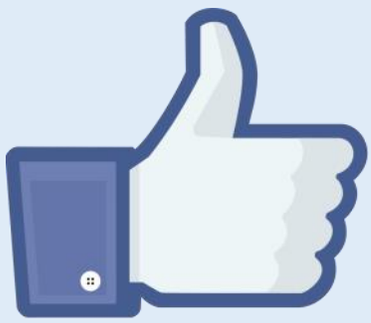
PhDs are not all bad and I think it is normal to be frustrated during the process, providing it does not become unhealthy. A lot of people do really love doing their PhD, becoming experts in a niche area of research, making new discoveries and having responsibility over planning their own time and experiment plans. However, it is important to recognise when negative thoughts and feelings are unhealthy and where to seek help for these. In my case, it was an obvious decision to make as I didn't enjoy the main bulk of the work. The way I thought about it was if I was doing a job that I hated, rather than a qualification, I would actively seek out alternative employment, so why should my PhD be any different? There is a huge stigma surrounding 'dropping out' of a qualification and for so long I continued on a path because I was worried about what other people might think.

If something isn't working out, don't be afraid to challenge the 'norm'. Never just put up with a situation for fear of the unknown or what others might think. I made the best decision for me and I do not regret it. I now work for an incredibly friendly and supportive law firm, doing something that I love.

If any readers would like more information about being a trainee or has any questions about the career or my journey, please do not hesitate to contact me –

meg.booth@appleyardlees.com

https://www.appleyardlees.com/people/meg-booth/



LIKE US ON FACEBOOK

Like our page to see our latest news and receive updates about writing and art opportunities

Insider Imprint

facebook.com/insiderimprint/

Studying and volunteering abroad in Australia

From Macquarie University to volunteering with marine turtles on the Great Barrier Reef!

Megan Johnson, 2nd Year Bioveterinary BSc

The University of Liverpool offers a wide range of opportunities to study abroad, including a summer programme, a semester abroad, or a full year in China. I can't recommend study abroad highly enough after having spent my second semester last year at Macquarie University, Sydney, Australia. Studying abroad is a once in a lifetime experience. Apart from having a fab time, it will look great on your CV as it is such a unique opportunity. Furthermore, you can gain many soft skills from it; independence, organisation, and adaptability. Your host university and country will offer you the chance to develop your existing skills or find new ones. For me this was learning to surf on the famous Bondi beach, and I had some amazing experiences while travelling.

I took every opportunity that I could to see as much of Australia as possible. I went hiking in the Blue Mountains and I spent a weekend in Melbourne where I was lucky enough to spot a few wild koalas! However, my favourite Australian spots had to be Bruny Island in Tasmania with their white wallabies along with the Great Barrier Reef, Queensland.

Study abroad is a fantastic opportunity on its own but I wanted to make my experience a little bit more unique. As I am studying Bioveterinary Science and hoping to work in conservation biology, I thought it would be a good idea to do some work experience while in Australia. I spent a week volunteering at Cairns Turtle Rehabilitation Centre, based on the stunning Fitzroy Island, a 40 minute ferry ride from Cairns Marina.

In the morning I would scrub the tanks and filter bags, then tempt turtles with fish or squid. It was dirty and smelly work, but ultimately very rewarding! While I was there the centre was looking after six turtles. The main species found around the island is the green sea turtle (*Chelonia mydas*) but the centre also had two olive ridley turtles (*Lepidochelys olivacea*). There was Margaret, estimated to be around 100 years old and found starving near the island. Lou was badly injured by a ghost fishing net and lost his front right flipper and his left rear flipper was later amputated by a vet. Lou's amputations were on opposite sides, so he will manage in the wild once rehabilitated. Francis was injured in a possible boat collision and suffered muscle damage to a front flipper, but will make a full recovery. Then there were the two 'babies', Alex and Layla, they were around 5 months old when they were found by fishermen washed up on the beach. They were examined by a vet and found to be full of micro plastics, which is why they couldn't swim. They will be released into the wild when they grow a little bigger, to give them the best chance of survival. Lastly, there was Angie, who was the only permanent resident. Angie probably suffered from a boat collision or crocodile attack, causing damage to her shell, this led to her developing a condition similar to emphysema in humans. As you can see in the picture, Angie's shell is much more domed than it should be, the excess air in her lungs prevents her from diving to the sea floor to feed and is why she wouldn't survive in the wild. I was also surprised to learn that the turtle shell or carapace is live tissue, which means they have nerve endings and blood vessels here. Angie of all the turtles especially enjoyed having her back scratched, as I am doing in the photo, and this would feel similar to a massage to you or me.

"It was dirty and smelly work, but ultimately very rewarding!"

While volunteering at the centre I found that marine sea turtles don't reach sexual maturity until they are 35 years old and continue to grow throughout their lives, though at a much slower rate as they become older. Their gender is determined by the temperature the egg is incubated at, female turtles above 30˚C and males below 28˚C. This is why climate change will be devastating for marine turtle populations, the num-

ber of female sea turtles is estimated to be as high as 87% of the total global turtle population and this is increasing. Once they reach 35, male turtles grow a longer tail but it is very hard to tell the difference otherwise. At the centre all the turtles were considered female unless we knew otherwise, therefore Lou was the only known male and Angie and Margaret were of such a large size, so older than 35, and therefore definitely female.

Fitzroy Island is surrounded by coral reefs, which form part of the Great Barrier Reef, I spent some time snorkelling here after volunteering with the turtles. It was an incredible sight, with so many beautiful fish species. Unfortunately, a lot of the coral has suffered bleaching due to human activities. If you are lucky enough to be travelling to a coral reef, do make sure you're using phosphate-free sun protection. It is very important to keep protected from the sun but most sun lotions contain phosphorus which contributes to coral bleaching. There are organic alternatives on the market which protect us, as well as the environment. If you are spending time outdoors in nature please make sure you take away all your litter with you and dispose of it correctly. Discarded waste plastics really do cause injuries to animals, as shown by the turtles.

My time spent in Australia, both at Macquarie University and Cairns Turtle Rehabilitation Centre, was absolutely fantastic and I would eagerly encourage everybody to consider study abroad or volunteering in another country. I found the project through *volunteerworld.com,* they are an organisation that connects volunteers to projects and oversees the whole process so you have nothing to worry about. They have projects all over the world from conservation, to providing healthcare and teaching and so there really is something for everybody.

You might also be interested in the article by McGinn et al. on the production of biodegradable plastic:
See Research, page 26

Study abroad: Year in China

Experience and advice on studying abroad at Xi'an Jiaotong-Liverpool University (XJTLU) in China

Adewuni Sorungbe, 4th Year Biological Sciences with Year in China BSc

Between my second and third year at university I undertook the Year in China. As a biological sciences student, the Year in China was the perfect opportunity that allowed me to learn about subjects that weren't related to my degree all whilst living in a new country, experiencing a different culture and meeting people from all over the world.

I knew I wanted to study abroad whilst at university; it's an incredible opportunity and the study abroad opportunities at the University of Liverpool are one of the reasons I wanted to study here. Before I saw the Year in China advertised I'd never considered studying in China before, but I'm so glad I took the opportunity to apply for it.

What is the year in China?

The Year in China is a study abroad opportunity offered to study at Xi'an Jiaotong-Liverpool University (XJTLU) in Shanghai, China. XJTLU is an international university where lectures are taught in English. On the Year in China you get the opportunity to take Chinese language modules but you don't need to be proficient in Chinese language to take part in the year abroad!

What is the application process like?

The application process was very straightforward, I had to submit an online application form through the study abroad section of the university website after semester 1 exams. It included basic information about myself and the degree program that I'm registered on. The application process involves writing a short essay about yourself; I included information about myself, why I wanted to study abroad and why I thought I would be suited to studying in China. Unlike with a semester abroad and due to the nature of the relationship between Liverpool and XJTLU there aren't a very limited number of places for students to compete for. My acceptance onto the Year in China was dependent on me maintaining a minimum of 40% in all of my second year modules without any resits and obtaining my visa. The study abroad team in Liverpool and the XJTLU global team are really supportive after the application process, helping you to apply for accommodation and giving advice about all of the preparations you need to make before you leave.

What it was like arriving?

Arriving in China was surreal, I was very jetlagged and it was so hot! I definitely wasn't prepared for the heat. Although I had prepared for the year abroad well in terms of vaccinations, travel money, insurance etc, I was less prepared in terms of my expectations and what I thought it would be like. I think in some ways this was probably beneficial to how easily I adjusted to life in China, I had no prior expectations so everything was just new and exciting. Saying that, although there were very few things I was worried about, the main two were definitely the language barrier and making friends.

Language barrier

XJTLU is an English speaking university so everyone you interact with in terms of lecturers, administration staff and the students will be proficient in English meaning that you

"Having been on the Year in China, I realise that I'm capable of a lot more than I thought"

shouldn't have any problems communicating on campus. That being said it is very helpful to learn some Mandarin to help in day to day life such as buying food, asking for directions and asking for help. All of this is taught to you in a module called Practical Chinese for Everyday Life; I can honestly say this module was incredibly helpful in everyday life and helped to make me feel like I really was living in China rather than just being like a tourist.

Making friends

Making friends at XJTLU wasn't as challenging as I thought. As well as the approximately 9,000 local students there are other Liverpool students doing different programmes at XJTLU as well as international students studying abroad. I made a lot of great friends within my accommodation, it was a very social year. When you study abroad you don't want to stay in every evening and not go out and travel. Living with lots of international students we travelled around China and Suzhou, organised weekend trips and nights out. Even if you didn't want to go out, there would always be someone to get dinner with or go for a drink. The university put on some events as did student societies which really let you get to meet more people and make friends outside of lectures and your accommodation. While I was there I went to some yoga classes and joined the boxing society, this really helped me to get involved in life at the university and meet more people.

What I studied

For me the subjects offered at XJTLU were a huge incentive to want to study there on a year abroad. You don't get many opportunities to learn about Chinese history, culture and society at an amazing university all whilst living in China! In my first semester I took the modules: International China, China: Social Stratification and Change, Practical Chinese and Genome Expression and Maintenance. Although I wasn't required to take a module related to my degree I wanted to and found it really interesting to see the differences and similarities between teaching styles in China and the UK. In my second semester I was also able to take a public health module - for me this was an amazing opportunity as it is an area of study that I'm really interested in.

I found it quite hard adapting to the teaching and learning styles in China, going from the Biological Sciences programme to a China studies course I felt that the lectures were really interactive and there was a lot more reading and discussion to be done. I also found that whilst in Liverpool my degree is mainly exam based, the Chinese studies programme is majority coursework. I found it hard to get used to consistently doing work throughout the semester instead of working hard for a few weeks before a final exam. It really made me assess how I manage my time and luckily that has translated back to my final year in Liverpool.

Living in Suzhou

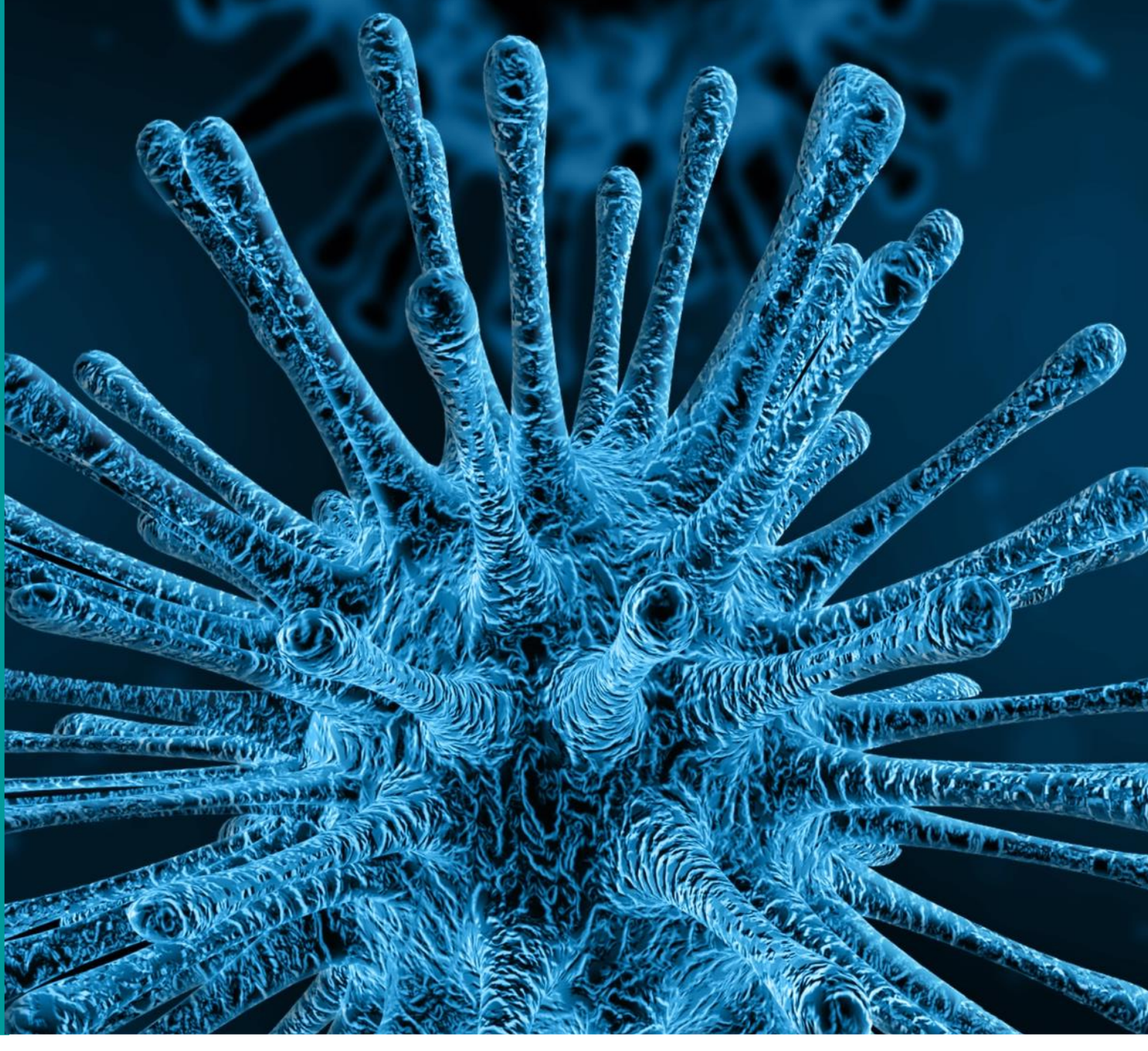
Suzhou is an amazing city to live in, it has a very well developed city centre but there are areas which still embody the traditional aspects of Suzhou life. It doesn't sound like these completely different environments would blend together well but they really do; I've never been to anywhere quite like Suzhou before. The opportunity to travel was a huge incentive for wanting to study abroad and the Year in China offered many opportunities to travel. There's so much to do within Suzhou itself but because of the extensive rail transport system it's also easy to visit places around Suzhou on the weekends.

Overall

The Year in China was an incredible year that I'm grateful I had the opportunity to go on. While it is a scary thought moving to a country that's completely different to what you're used to for a whole year it is an experience that you'll probably never get again. It has equipped me with many skills that are useful in aspects of my life today and that I am sure will help me in future. Having been on the Year in China I realise that I'm capable of a lot more than I thought.

Research at Liverpool

Read about research from current students



The correlation between retinal haemorrhage and the clinical outcome of children with malarial retinopathy

Melissa Leak[1]

Cerebral malaria (CM) is a form of severe malaria that affects children in sub-Saharan Africa. It is transmitted by mosquitos that have previously fed on infected humans. The parasite causing CM leads to red blood cells collecting together in brain vessels causing oxygen deprivation so patients often present with a coma. However, as other diseases also induce comas, it can be difficult to distinguish CM from these. Early patient diagnosis is key to effective treatment and can influence patient survival. One diagnostic method is to examine the back of the eye for any irregularities in blood vessels found in the retina. These 'irregularities' are termed 'malarial retinopathies' (MR). This study investigated the significance of one feature of MR called a haemorrhage, which is an escape of blood from a ruptured blood vessel. We aimed to determine the relationship between the presence of bleeds (haemorrhage) in the retina and the probability of survival for a child suffering with CM. Computer software was used to highlight any haemorrhages in 259 retinal photographs from CM patients in Malawi. The results were then tested for any statistically significant relationship between presence of haemorrhage and patients who died or survived. Our statistical tests indicated that although there was a difference between the two groups, it was not statistically significant. It was concluded that, although this was a unique attempt to analyse the relationship between CM patient clinical outcome and haemorrhages, future studies should be conducted with a larger sample size in order to demonstrate a statistically significant difference.

Abstract

Cerebral malaria (CM) is a critical form of severe malaria, which is commonly misdiagnosed in sub-Saharan Africa. Misdiagnosis is responsible for patient fatality and inappropriate treatment of those infected with other diseases. Establishing the presence of malarial retinopathy (MR) in a patient, opens a new window for finding accurate methods of early diagnosis as this is an abnormality unique to CM. This project aimed to ascertain the relationship between haemorrhages in the retina and the prognosis for children suffering with CM. Analysis of 259 montaged haemorrhage retinopathy fundus data sets (259 eyes of 259 subjects, one eye per subject) using a software programme, consisted of determining the haemorrhage to retinal ratio and calculating the difference between children who either died or survived. Out of the 259 patients, 38 died and 221 survived. Of the 38 that died, the mean haemorrhage:retina ratio was 0.129 with a standard deviation (SD) of 0.246. From the remaining sample who survived, the mean ratio was 0.0219, SD=0.0660. The Shapiro-Wilk normality test presented a significance value of <0.050, so a following non-parametric Mann-Whitney U test was performed. This produced a result of 0.090 implying that the difference between the two groups was not significant. The results showed a difference between the two groups, however this difference was not statistically significant suggesting that future research with a larger sample size is warranted. This may be the first step towards accurately predicting prognosis in severe malaria by utilising an innovative method for quantitatively analysing the relationship between clinical outcome with CM and haemorrhage in the retina.

Introduction

Severe malaria is defined by clinical/lab evidence of vital organ dysfunction in a patient who may also harbour high levels of parasitaemia, the most critical form being cerebral malaria (CM). CM is a neurological complication with a high mortality, primarily characterized by coma and parasitic observations on blood smears (1), a key indication of the disease being the sequestration of parasites in the vessels of the central nervous system (CNS) (2).

The causal *Plasmodium falciparum* parasite is vectored by female *Anopheles* mosquitos which pick up the disease from previously infected humans and pass the parasite on to the uninfected during subsequent feeding (3). Geographical dependency and poverty constrain the disease prevalence and transmission of CM to subtropical regions with a significantly lower average income (4), notably affecting children in sub-Saharan Africa. Those infected

[1]Third Year Biological & Medical Sciences Student, School of Life Sciences, Liverpool, UK, L69 7ZB

with CM suffer coma, severe disability or death. As a major ongoing disease that poses risk to over 3.4 billion people, early diagnosis of severe malaria remains one of the most effective methods of improving survival of patients. Paediatric severe malaria commonly involves one or more of three syndromes: CM, malarial anaemia and metabolic acidosis.

Retinopathy is defined as any abnormality associated with the human retina, and is a pathology identified as an important clinical marker in the prognosis and diagnosis of CM. As a direct extension of cerebral vasculature, the retina remains the only region of the body with a vascular bed capable of examination using non-invasive techniques, providing a unique opportunity to observe vascular pathology (5). Malarial retinopathy (MR) is represented by retinal whitening, papilledema, capillary non-perfusion (CNP) and varying haemorrhage presence in the ocular fundus, all of which may be studied through validated retinal image analysis (2). The isolation of haemorrhage presence in this study was necessary due to significantly limited research of this retinopathy proportional to other features of CM. The similarity of eye and brain embryonic origins has led to shared features of the relevant microvascular systems. Both the brain and the retina are embryologically derived from a mutual neuroectoderm (5); as demonstrated from past research (6), the number of retinal haemorrhages present is consequently comparable with the degree of cerebral haemorrhages.

Isolating MR hallmarks within malarial histopathology has provided an alternate means of distinguishing malarial coma from comas from other causes and has served to strengthen the accuracy of treatment (7). It can be assumed that MR represents similar manifestation mechanisms that act on different parts of the CNS to cause the neurocognitive sequelae - as a consequence of parasitized red blood cells (8). Accordingly, early detection of retinopathy in sufferers of severe CM would serve as a strategical vantage point with regards to understanding the pathophysiological mechanisms of the disease enabling more efficient treatment of the patient. It is imperative to note, that in a field where there is predominately qualitative research regarding CM haemorrhages, quantitative analysis of haemorrhage incidence may provide a more appropriate method of preparing data for dissemination to others. For this reason, the novel objective of this research was the quantitative interpretation of haemorrhage incidence with regards to the clinical outcome of a patient suffering with CM.

A 2004 study of paediatric CM in Malawi indicated that 24% of patients fulfilling the criteria for CM before death presented post-mortem evidence of an alternative cause for coma (17); thus indicating the need for more accurate diagnosis. It is apparent that detection of retinopathy may be the only clinical feature able to distinguish patients presenting with CM from those with alternate causes. Hence, it is likely that diagnosis of this association may become a routine component of specifying encephalopathy of malarial aetiology (18).

A combination of retinal examination, imaging analysis and statistical techniques were used to investigate whether retinal haemorrhage does in fact share a causal relationship with the severity of CM or is a non-causal correlation and its potential uses in predicting the pathophysiological processes present in the CNS. As haemorrhage prominence is apparent in most fatal cases of CM, quantifying the relationship contributes to an understanding of the various pathophysiological processes that occur in the disease. Early diagnostic methods may contribute to treatment efficacy and further reduction of mortality caused by the disease.

Methods

Prior ocular fundus image collection

Samples of 259 montaged MR fundus datasets were collected from children suffering with CM who had been admitted to the Paediatric Research Ward, Queen Elizabeth Central Hospital, Malawi. A few patients, with other malarial/non-malarial diagnoses were also included in the study. Haemorrhages occur in a variety of shape and colour formats so being accustomed to detecting different features of haemorrhages present in cases of MR was essential (9). This was ensured by repeated analysis of ocular fundus retinopathies and confirmation of the collected Grader's annotations with the project supervisor. Children meeting the WHO criteria for CM were sampled from an exposure-control study for haemorrhage analysis over a 3-year period, after medical consent was obtained from parents/guardians and following enrolment procedures. The original fundus images were captured using a Nikon-E1 digital camera (Nikon, Tokyo, Japan) after pupil dilation with Tropicamide 1% and Phenylephrine 2.5% (8). These images, with a field view of 50°, were derived from two groups of patients labelled died vs survived, and consisted of one image per patient (9). After approval from local and collaborating ethics committees at the Universities of Malawi (College of Medicine) and Liverpool, a collection of representative images at different phases was obtained for analysis. These images consisted of low compression JPEG files and montages were formed by stitching together multiple original images of the same eye to visualise a larger area of the retina. Montages composed of several images from similar time points gave a summary of the haemorrhage features in the ocular fundus. The subsequent methodology consisted of three major phases. The first was image analysis using an in-house programme, the second was data generation using alternative software and in the third phase statistical tests were conducted to determine significance. As a potential new method of diagnosis, precautions were taken to ensure the data collected was as dependable as possible. Whilst executing phases 1 and 2, no information of patient clinical outcome in relation to the fundus images being analysed was provided. This

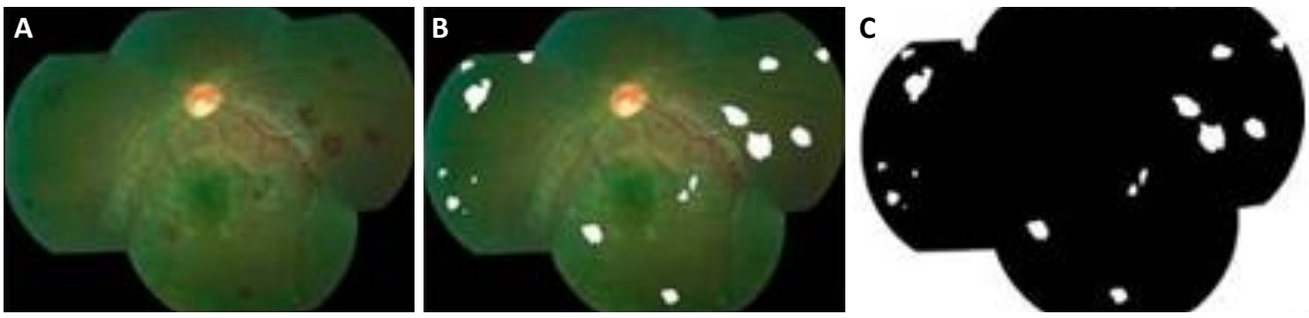


Figure 1. Use of an annotation tool on ocular fundus photograph IM048684 (A), produced a highlighted haemorrhage region over the image which was indicated by a white filling (B). After completion, the Matlab software generated a Grader's annotation (C) which would then be used to calculate the following haemorrhage:retinal area ratios. Photographs were collected by Ian MacCormick (2) and Nick Beare at different phases, for analysis at the University of Liverpool.

aimed to prevent potential researcher bias during the study.

Production and analysis of Grader's annotations

The first step was to appropriately annotate the haemorrhage regions within the retinopathy fundus images, for which an in-house Matlab programme was used (R2017a, Mathworks, Natick, MA). This programme was used to delineate regions of haemorrhage by hand in an interactive manner. Use of the interface involved telling the software which MR feature was being analysed, selecting an outline colour and tracing haemorrhages by hand, essentially outlining the affected area with a stop-point marker (Figure 1). The graphic user interface of the Matlab programme then automated this task for subsequent analysis. Haemorrhages were defined by regions of pink/red that stood out against the green ocular fundus image (green due to patient skin pigmentation). These were outlined with 50% confidence and any uncertainties were corrected for by exclusion from the study.

Step two generated a black and white Grader's annotation image, highlighting only the boundaries of the haemorrhagic areas within the retina (Figure 1C). The ratio between the area of haemorrhage and the area of the visible retina was derived using the software programme for each image. The clinical outcome for each patient was then provided after annotation was complete, to form the data required for statistical analysis.

Statistical analysis

The final step utilised SPSS (version 24, SPSS; IBM, Chicago, IL, USA), for statistical analysis of the haemorrhage ratio data previously derived. Normality was tested to decide whether a parametric/non-parametric test would be used to test for a ratio difference between the samples of died vs survived. A subsequent P value of 0.05 was deemed as significant, as standard to scientific hypothesis testing.

A frequency histogram was generated as part of normality testing, with a Shapiro-Wilk significance value indicative of deviation from normal distribution. Subsequently, a Mann-Whitney U test was the non-parametric alternative used to compare the sample means from the 'died' vs 'survived' groups and test for a significant difference in the haemorrhage:retina ratio.

Results

Of the 259 patients that were admitted with CM, 38 died and 221 survived. SPSS descriptive analysis concluded that from the sample who died of CM, a mean haemorrhage:retina ratio of 0.129 was derived with a 0.246 standard deviation. In contrast, from the 221 patients who survived, the mean haemorrhage:retina ratio was 0.0219 with a standard deviation of 0.0660 (Table 1).

Patients who died from CM had a larger mean ratio of haemorrhage:retina however there was also a larger standard deviation from this mean. The sample of patients who survived had a much smaller mean ratio, indicating an observable difference in haemorrhage incidence between the two groups.

Figure 2 provides sample ocular fundus images from pa-

Descriptive Statistics						
A						
	N Statistic	Minimum	Maximum	Mean	Standard Error	Standard Deviation
Survived	221	0	0.652	0.0219	0.004	0.066
Valid N (listwise)	221					
B						
Died	38	0	0.869	0.129	0.0398	0.246
Valid N (listwise)	38					

Table 1. Descriptive statistics from a data set depicting the ratio of haemorrhage incidence to visible retina region. Derived from a sample of 259 patients of which 38 'died' (A) and 221 'survived' (B). A mean statistic of 0.129 was derived from the sample who died, SD=0.246. Of those who survived a smaller mean ratio of 0.0219 was derived, SD=0.0660.

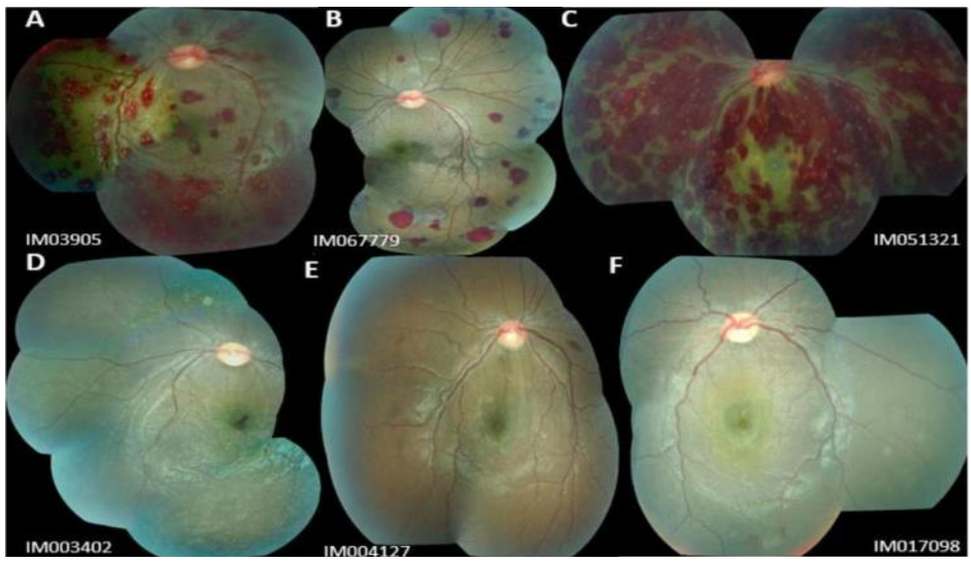


Figure 2. Example fundus ocular image montages from a sample of 'died' (A, B, C) and 'survived' (D, E, F) CM patients. This figure highlights some observed differences in the retinal regions occupied by haemorrhagic features. Photographs were collected by Nick Beare and Ian MacCormick (2).

tients who either died or survived. These photographs have observable haemorrhages which can be seen as a pink/red pigmentation that stands out against the visible fundus. In this figure, the top row (patients who died), has a larger proportion of haemorrhage incidence in the retina compared to the bottom row (patients who survived CM).

The Matlab programme generated a Grader's annotation as demonstrated by Figure 3B, automating the regions highlighted as haemorrhagic for the following ratio calculation.

The frequency histogram and plot showed a skewed curve with significant deviation from a normal distribution, this was supported by the Shapiro-Wilk test significance statistic of 1.96×10^{-29}. This step was completed using SPSS software.

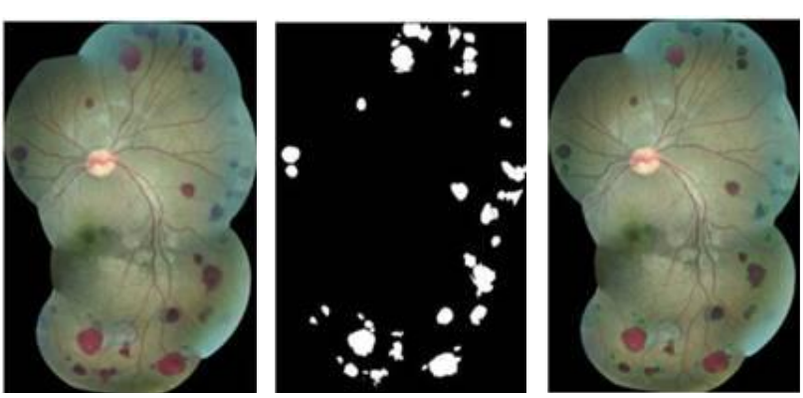
A Mann-Whitney U test was conducted generating a value of 0.090 (Table 2). This indicates an observable but statistically insignificant difference. The hypothesis test summary concluded that the distribution of ratios of haemorrhage:retina was the same across patients who survived and died, however the slight difference detected by the Mann-Whitney U test implied that the project hypothesis still holds.

Discussion

CM neuropathological manifestations burden children in sub-Saharan Africa, signature characteristics being the sequestration of parasites in CNS vasculature (10). This project focused on determining a relationship between retinal haemorrhages (as a feature of MR) and the clinical outcome for patients suffering with CM. Statistical analysis of data from patients who either survived or died showed a measurable but not statistically significant difference between the two outcome groups, using a confidence interval of 95%.

Limitations

The lack of significance may be due to the small sample size of ocular fundus images being analysed, as the results provided some evidence of difference despite this being insufficient for statistical significance. This assumption is supported by previous research demonstrating the relevance of haemorrhages in CM infection (11). This Indian study investigated the results of opthalmoscopic examination of 214 adult patients who had been admitted to hospital with CM and concluded that 14.6% of patients had retinal haemorrhage with fewer other ocular abnormalities found. The significance of haemorrhage



A) Fundus Image **B)** Grader's Annotation **C)** Annotation Contours

Figure 3. Example result using IM03905. (A) The original fundus image that was uploaded into the initial Matlab software. With the use of an outlining tool, retinal haemorrhage highlighted in white and a preview Grader's annotation image shown in (B) was subsequently produced. This was later quantified for statistical analysis, calculating haemorrhage ratio and region. Contours of the previous annotation have been overlaid onto the original (produced by Dr Yalin Zheng) for visualisation purposes (C). Images were collected by MacCormick (2).

involvement in CM should therefore be researched and elucidated further, as the study in India is just one example in which haemorrhages remained a sole distinguishing retinopathic feature in patients suffering severe malaria. These results affirm that it would be unjustified to discredit the conclusion that monitoring a particular feature within MR could help predict patient prognosis/ clinical outcome.

The limitations of this research were mainly due to time constraints. Due to workload we were unable to establish the intended intra-observer agreement (IOA) analysis. This would have been conducted by taking a small sample (20-30) of montaged haemorrhage retinopathies for repeated analysis, enhancing the reliability of the results. Thus, incorporation of IOA analysis is considered to be a critical element missing from this project. If the experiment were repeated, IOA would be computed by reliability analysis in SPSS i.e. Intraclass Correlation (ICC). Nevertheless, the innovative methodology and objective distinguished this research from previous studies as it provided quantitative analysis of haemorrhage occurrence in MR. For this reason, this research may contribute a novel route for future CM research.

An additional limitation was the compromised quality of a few select ocular fundus images. Distorted pigmentation of the photograph rather than the retina itself made it difficult to distinguish haemorrhages. This variation in image quality could have resulted from factors such as eye movement, media opacity, pupil size or camera misalignment/focus and may have restricted the accuracy of the Grader's annotation (12).

Although this research uses survival as the benchmark for good prognosis, most patients who suffer CM are subject to multiple neurological deficits. A follow up study of 62 CM survivors recorded that over a 16-month period, 17% of patients had manifestations consisting of cortical blindness, mono-paresis and speech deficits (13). Thus, the benefit of a prognosis predicting a poor quality of life for the patient, especially in regions already burdened with socio-economic stress and poverty is questionable. Nonetheless, any decrease in mortality for a prominently life-threatening disease is beneficial.

Table 2. Results from a non-parametric Mann-Whitney U test performed on sample ocular fundus images from patients suffering CM. A significance value of 0.09 was obtained; the null hypothesis was retained.

Mann-Whitney U Test				
Rank				
	Outcome 2	N	Mean rank	Sum of ranks
Ratio HM:Retina	1.00	221	126.80	28022.00
	2.00	38	1148.63	5648.00
	Total	259		
Hypothesis Test Summary				
	Null Hypothesis	Test	Sig.	Decision
1	*The distribution of Ratio "HM:Retina" is the same across categories of "Outcome"*	Independent samples Mann-Whitney U test	0.090	Retain the null hypothesis
Asymptotic significances are displayed. The significance level is 0.05				

Researching MR as a clinical marker of CM

MR is a characteristic result of CM pathogenesis, occurring in 60% of infected children (19). Survivors are not only rendered vulnerable to high mortality rates but are prone to long-term neurocognitive impairments as elucidated by a past study on severe malaria in Kenyan children (20). Evidence supports the importance and value of using MR detection as a prognostic tool regarding cerebral vasculature and related research has demonstrated strong similarities between sequestration existing in the brain and the retina (21). This supports use of MR as a method of clinically assuming the neurovascular pathophysiology in paediatric CM. Research into MR has enabled doctors to isolate significant hallmarks of malarial histopathology and has offered a method of distinguishing malarial coma, strengthening therapeutic approaches.

Existence of retinal haemorrhages can be observed in 6-8% admissions of children suffering CM (22). Studies of the mechanics behind haemorrhage occurrence have suggested that erythrocyte 'rosetting' is responsible for not only the pathogenesis of CM but also that it is subsequent occlusion of the retinal vessels that leads to haemorrhage development (23). In surviving patients, retinal haemorrhages are commonly absorbed spontaneously (24). It can be presumed that any failed visual recovery is a result of the photoreceptor being vulnerable to disrupted tissue oxygenation. Previous autopsy research evidenced petechial haemorrhages in the brain, emphasising the correlation between haemorrhage presence and patient clinical outcome (17).

In previous research, data were analysed qualitatively and only later analysed quantitatively, providing evidence of a relationship without the direct exploitation of quantitative data. The few studies which conducted quantitative evaluation of retinal haemorrhage were performed in conjunction with results of other retinopathy features (25), but never on a large scale with analysis focused solely on haemorrhage as a feature of MR. Thus our research highlights a unique approach utilising an innovative method of predicting CM prognosis.

Relevance to future research

Observing changes in ocular vessels and more specifically homing in on characteristic retinopathies, provides a unique opportunity to understand the disease mechanisms and how they relate to patient prognosis. Current

diagnostic methods rely on detection of parasitic proteins and fail to give information on infection density and have a low sensitivity to lower parasitic densities (30). Although this work did not identify a significant difference, future integration of quantitative analysis with a large enough sample of known patient outcomes may allow the production of a diagnostic tool, sufficient to distinguish malarial coma in malaria burdened regions. The combination of retinopathies correlated with severe malaria is unique, *P. falciparum* malaria is responsible for around 250,000 cases of paediatric retinal haemorrhage annually, thus justifying further work. More importantly, this project may have been the first to outline a direct quantitative relationship between the clinical outcome of patients with CM and haemorrhage incidence in the retina.

Conclusions

Although various limitations restricted the significance of our project analysis, there remains sufficient evidence to support the notion that there is a recordable association between haemorrhage incidence and patient clinical outcome in paediatric CM. Microscopic diagnosis is not always reliable when parasitaemia is low, so this research project serves as a new approach towards more accurate patient diagnosis.

Acknowledgements

I would like to acknowledge the University of Liverpool for the opportunity to complete this research project. I would also like to acknowledge Ian MacCormick and Nick Beare for collecting retinal images for the analyses carried out in this study.

Most importantly, I would like to express my gratitude to my supervisor Dr Yalin Zheng for his continued support and encouragement.

References

1. Idro R, Marsh K, John C C and Newton C R J. Cerebral malaria; Mechanisms of brain injury and strategies for improved neuro-cognitive outcome. Pediatric Research 2010. 68 (4):267-274

2. MacCormick I J C, Maude R J and Beare N A V *et al.* Grading fluorescein angiograms in malarial retinopathy. Malaria Journal 2015. 14 (1):367

3. Lehane M J. Biology of Blood-sucking Insects. London: Harper Collins Academic; 1991

4. Sachs J and Malaney P. The economic and social burden of malaria. Nature 2002. 415 (6872):680-685

5. Maude R J, Dondorp A M and Sayeed A A *et al.* The eye in cerebral malaria: What can it teach us? Transactions of the Royal Society of Tropical Medicine and Hygiene 2009. 103(7):661-664

6. White V A, Lewallen S and Beare N *et al.* Correlation of retinal haemorrhages with brain haemorrhages in children dying of cerebral malaria in Malawi. Transactions of the Royal Society of Tropical Medicine and Hygiene 2001. 95:618–621

7. White V A, Lewallen S and Beare, N A V *et al.* Retinal pathology of paediatric cerebral malaria in Malawi. PLOS ONE 2009. 4(1):e4317

8. Boivin M J, Vokhiwa M and Sikorskii A *et al*. Cerebral malaria retinopathy predictors of persisting neurocognitive outcomes in Malawian children. The Pediatric Infectious Disease Journal 2014. 33(8):821-824

9. Zhao Y, MacCormick I J C and Parry D G *et al.* Automated detection of leakage in fluorescein angiography images with application to malarial retinopathy. Scientific reports 2015. 5:10425

10. Joshi V S, Maude R J and Reinhardt, J M *et al.* Automated detection of malarial retinopathy-associated retinal haemorrhages. Investigative Ophthalmology & Visual Science 2012. 53(10):6582-6588

11. MacCormick I J C, Beare N A V and Taylor T E *et al.* Cerebral malaria in children: Using the retina to study the brain. Brain 2014. 137(8):2119-2142

12. Kochar D K, Shubhakaran, Kumawat B L and Vyas S P. Prognostic significance of eye changes in cerebral malaria. Journal of the Association of Physicians India 2000. 48 (5):473-7

13. Zheng Y, Kwong M T and MacCormick I J C *et al.* A comprehensive texture segmentation framework for segmentation of capillary non-perfusion regions in fundus fluorescein angiograms. PLOS ONE 2014. 9(4):e93624

14. Ponsford M J, Medana I M and Prapansilp P *et al.* Sequestration and microvascular congestion are associated with coma in human cerebral malaria. The Journal of Infectious Diseases 2012. 205(4):663-671

15. Clark I A and Rockett K A. The cytokine theory of human cerebral malaria. Parasitology Today 1994. 10(10):410-412

16. Moxon C A, Heyderman R S and Wassmer S C. Dysregulation of coagulation in cerebral malaria. Molecular and Biochemical Parasitology 2009. 166(2-3):99-108

17. Taylor T E, Fu W J and Carr R A *et al.* Differentiating the pathologies of cerebral malaria by post-mortem parasite counts. Nature Medicine 2004. 10(2):143-5

18. Beare N A V, Lewallen S, Taylor T E and Molyneux M E. Redefining cerebral malaria by including malaria retinopathy. Future Microbiology 2011. 6(3):349-355

19. Lewallen S, Bronzan R N and Beare N A *et al.* Using malarial retinopathy to improve the classification of children with cerebral malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene 2008. 102(11):1089-1094

20. Carter J, Mung'ala-Odera V and Neville B *et al*. Persistent neurocognitive impairments associated with severe *falciparum* malaria in Kenyan children. Journal of Neurology, Neurosurgery, and Psychiatry 2005. 76(4):476-481

21. Barrera V, Hiscott P S and Craig A G *et al*. Severity of retinopathy parallels the degree of parasite sequestration in the eyes and brains of Malawian children with fatal cerebral malaria. The Journal of Infectious Diseases 2015. 211(12):1977-1986

22. Newton C R, Kirkham F J and Winstanley P A *et al*. Intracranial pressure in African children with cerebral malaria. The Lancet 1991. 9;337(8741):573-576

23. Carlson J, Helmby H and Hill, A V *et al.* Human cerebral malaria: Association with erythrocyte rosetting and lack of anti-rosetting antibodies. The Lancet 1990. 15;336 (8729):1457-1460

24. Choi H J, Lee S Y, Yang H and Bang J K. Retinal haemorrhage in *vivax* malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene 2004. 98(6):387-389

25. Dorovini-Zis K, Schmidt K and Huynh H *et al.* The neuropathology of fatal cerebral malaria in Malawian children. The American Journal of Pathology 2011. 178(5):2146-2158

26. World Health Organisation. Severe Malaria. 2014. Tropical Medicine and International Health, 19 Suppl 1: 7-131

27. Chanda E, Hemingway J and Kleinschmidt I *et al.* Coetzer Affiliation Ministry of Health, National Malaria Control Centre, Lusaka, Zambia

28. Coleman M. Insecticide resistance and the future of malaria control in Zambia. PLOS ONE 2011. 6(9):e24336

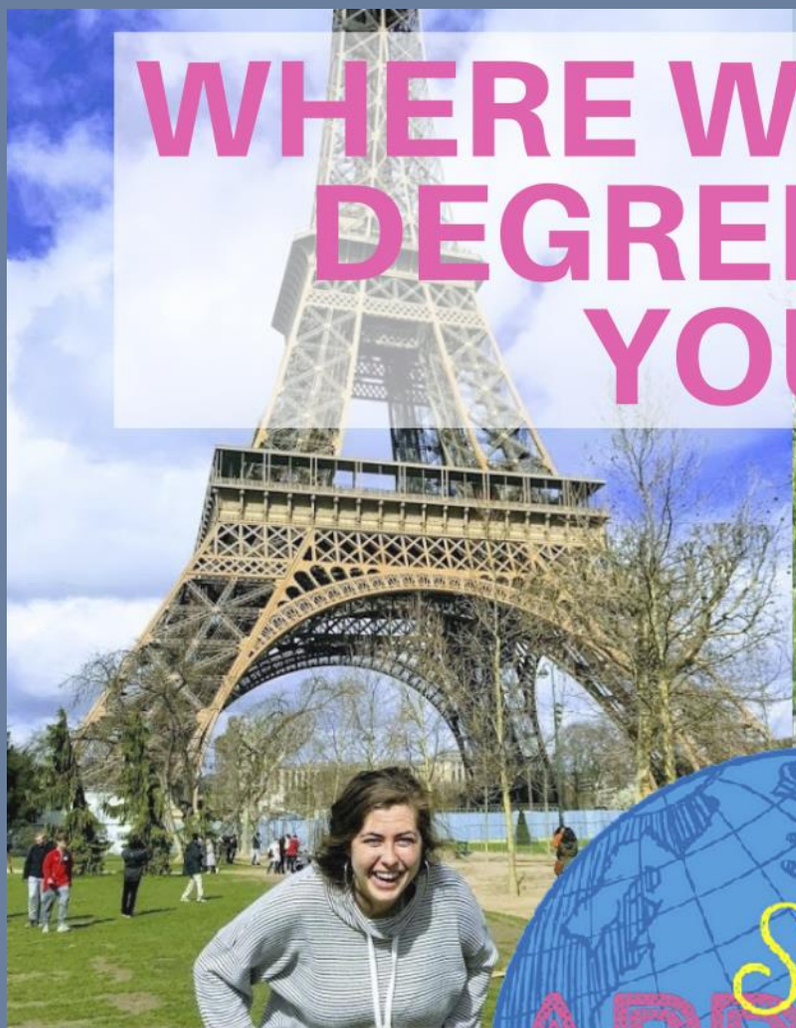
29. Bastawrous A, Giardini M and Bolster N M *et al.* Clinical validation of a smartphone-based adapter for optic disc imaging in Kenya. JAMA Ophthalmology 2016. 134(2):151-158

30. Gaca-Wysocka M and Grzybowski A. New technologies in diabetic retinopathy. In: Grzybowski A ed. Ophthalmic care for the diabetic patient. Foundation of ophthalmology development and support. Poznan: 2016. p. 88-96

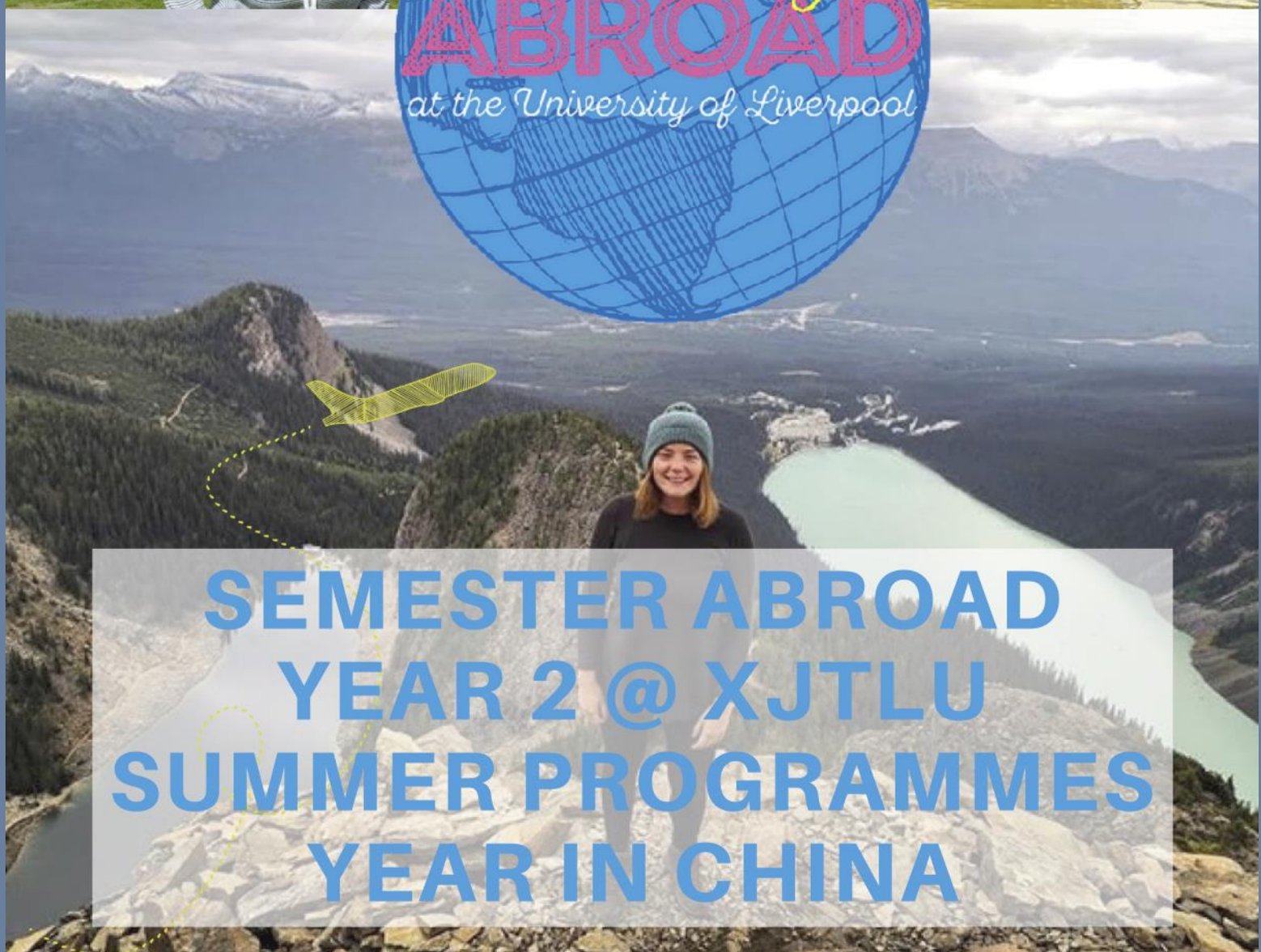
31. Hänscheid, T. Current strategies to avoid misdiagnosis of malaria. Clinical Microbiology and Infection 2003. 9(6):497-504

You might also be interested in Emma Khoury's article on screening for diabetic retinopathy: see Insights, page 68

WHERE WILL YOUR DEGREE TAKE YOU?



Study ABROAD
at the University of Liverpool



SEMESTER ABROAD
YEAR 2 @ XJTLU
SUMMER PROGRAMMES
YEAR IN CHINA

Waste frying oil: The perfect growth requirements for the production of biodegradable plastic by the bacterium *Cupriavidus necator*

Emily McGinn[1], Hermione Webster [2], Jagjit Binning[1]*

Plastic is increasingly becoming a global challenge, with millions of tons of non-degradable plastic produced a day, filling up natural habitats and polluting the earth. Here we show how the bacterium *Cupriavidus necator* can not only produce a biodegradable plastic (PHB) but further produces the highest PHB yield when grown on a waste oil substrate. Investigating different oils including rapeseed, soy and peanut oil under three different conditions, new, heated and 'used' showed that used peanut oil, a common waste product of many food industries, produced the highest PHB yield in *Cupriavidus necator*. This could give new innovative solutions to decreasing non-degradable plastic pollution whilst also profiting from a waste product.

Abstract

The Gram-negative soil bacterium *Cupriavidus necator* is capable of producing poly-3-hydroxybutyrate (PHB), a polymer contained within intracellular granules. The bacterium utilizes these polymers as an energy source during periods of environmental stress. When extracted, the polymer may also be exploited by humans for industrial plastic manufacturing as an eco-friendly alternative to petrochemical plastic production. However, this is an expensive process as it harbors little production over cost. By using recycled materials e.g. media supplemented with waste oil (oil already used in the food industry), as an energy source for PHB producing bacteria, a cheaper approach to this form of plastic production could be provided. The aim of this study is to investigate PHB production in *C. necator* when supplemented with a range of widely used cooking oils, and further to investigate whether the use of 'used' oil resulted in a higher yield of PHB. Absorbance readings were used to calculate the amount of PHB produced. On comparing maximum PHB content, our observations suggest peanut oil produces the greatest yield (16.657 % weight). With waste oil at large within the food industry, and peanut oil a popular cooking oil used worldwide, exploiting waste peanut oil would be a cheap and eco-friendly alternative approach to polymer production.

Introduction

Cupriavidus necator is a Gram-negative soil bacterium capable of producing a bio-degradable polymer; poly-3-hydroxybutyrate (PHB) which when extracted, may be exploited for industrial purposes (1). *C. necator* produces the fatty acid compound PHB which is stored within intracellular granules called granzymes when nutrients are in excess. The PHB storage compound can then be utilised by the host bacterium as a carbon source during periods of environmental stress (2). The metabolic production of PHB is by the enzymatic pathway conversion from acetyl-CoA to poly-3-hydroxybutyrate via the utilisation of glucose (3).

Upon extraction, PHB can be used as a resource for plastic production in replacing the more commonly used petro-chemically produced plastics, presenting an environmentally friendly alternative (1). Microbes can utilize the plastic as an energy source by secreting enzymes to degrade the PHB, hence the plastics have biodegradable properties (4). With the pollution of our oceans dominated by plastic waste - over 250,000 tons of plastic afloat our seas (5) - it is more important than ever to explore resources which have less environmental consequences.

Investigation into possible applications of PHB and other polyhydroxyalkanoates (PHAs) have been ongoing since the late fifties (6) when they were initially commercialized, however the economic complexities involved were also identified and so interest in the idea declined rapidly. Hence, a cheaper alternative to the existing methods of PHB extraction and processing may overcome some of these economic boundaries.

Previous research by Verlinden *et al.* (1) showed that the use of waste frying oil as a source of nutrient intake by *C. necator* resulted in an improved yield of PHB production than that of pure, unused oil. However, only one type of oil; rapeseed oil, was under investigation in this previous study. Our investigation sought out to investigate a variety of well-known industrial frying oils, including rapeseed, soy bean, and peanut oils as substrates for PHB production.

Materials and Methods

Chemicals

All chemicals used were derived from the School of Life Sciences Teaching Labs, University of Liverpool.

[1]Third Year Biological Sciences Student; [2]Third Year Microbiology Student; School of Life Sciences, University of Liverpool, Liverpool, UK, L69 7ZB
*Corresponding author

Oils

The oils used were 1) rapeseed (Crisp 'n Dry), 2) soybean, and 3) peanut. Each oil condition was exposed to 3 treatments as follows; unused 'new' oil, oil heated for one hour at 180 °C (50 mL), and 'used' oil (fried with 100 g of raw potato and 50 g of raw chicken for one hour at 180 °C), plus a negative control (no oil added). Oils were sterilized separately in a steam autoclave before use.

Growth media

Liquid growth medium tryptone broth consisted of 17 g/L tryptone, 3 g/L peptone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, 2.5 g/L D-glucose, and distilled water. Tryptone agar consisted of 15 g/L tryptone, 5 g/L peptone, 5 g/L NaCl, 12 g/L agar powder, and distilled water. These measurements were followed as stated by Verlinden *et al.* (1).

Culture conditions

A single colony of *C. necator* was inoculated with 25 mL tryptone broth and grown at 28 °C for approximately 24 hours producing a starter culture. Following growth, 2 mL starter culture inoculated with 400 mL tryptone broth was incubated at 30 °C for approximately 24 hours. The following standard protocol was followed as suggested by Verlinden *et al.* (1). Small scale batch fermentations were performed in 500 mL flasks, containing 225 mL tryptone medium and 20 g/L oil. Succeeding this, the sterile medium was sonicated for 10 minutes to ensure a homologous mixture. Of the starter culture inoculum, 25 mL was added to each of the flasks containing the different oils and treatments, giving a final volume of 250 mL (10% (v/v)). All flasks were incubated in a rotary incubator (150 rpm) at 30 °C for 60 hours. All experiments were conducted in triplicate giving a total of 12 samples, including the negative control (broth without oil).

PHB extraction

All centrifugation steps were carried out at room temperature and set to 4565 x g unless stated otherwise. PHB dry cell weight was assayed in 250 ml of tryptone broth bacterial culture with the differential oils and treatments. Recovery of bacterial cells by centrifugation was at 4 °C for 25 minutes. These were re-suspended in sodium hypochlorite (250 ml, 5%), and incubated at 37 °C for 1 hour, followed by further centrifugation for 25 minutes, and resulted in PHB granules (further suspended in distilled water (250 ml)). Repeated centrifugation took place, with suspension in acetone (5 ml), vortexed and centrifuged for a further 20 minutes. The process was repeated with ethanol (5 ml). Pellets were incubated with chloroform (3 ml) in a boiling water bath for 2 minutes. This was repeated to achieve a final volume of 9 ml, followed by centrifugation for 15 minutes. PHB chloroform solutions were placed into a boiling water bath to dissolve solvent, leaving only PHB. Concentrated 5% sulphuric acid (10 ml) was added and placed in a water bath for a further 10 minutes. The concentration of PHB was measured at 235 nm and correlated to a standard curve giving the dry net weight of PHB per ml of colony.

Statistical analysis

Statistical analyses of the different oils and treatments were achieved by a One-Way ANOVA, followed by a post-hoc Bonferroni's Multiple Comparisons test to analyse differences within oil treatments. The statistical software used was GraphPad.

Results

The results of PHB production weight percentage (% weight) from small scale fermentations with *C. necator*, and 20 g/L oils in tryptone broth are shown in Figure 1. Figure 1a-c shows the PHB production of all three oil conditions tested within their different treatments. 'Control' refers to the negative control (all treatments were tested against this control). A significant difference in PHB production was shown within the 'none' vs. 'used' oil conditions for all oils investigated, hence all 'used' oils i.e. fried with 100 g of raw potato and 50 g of raw chicken for one hour at 180 °C, represented an increased PHB production (rapeseed, P<0.01; soybean, P<0.01; peanut, P<0.0001).

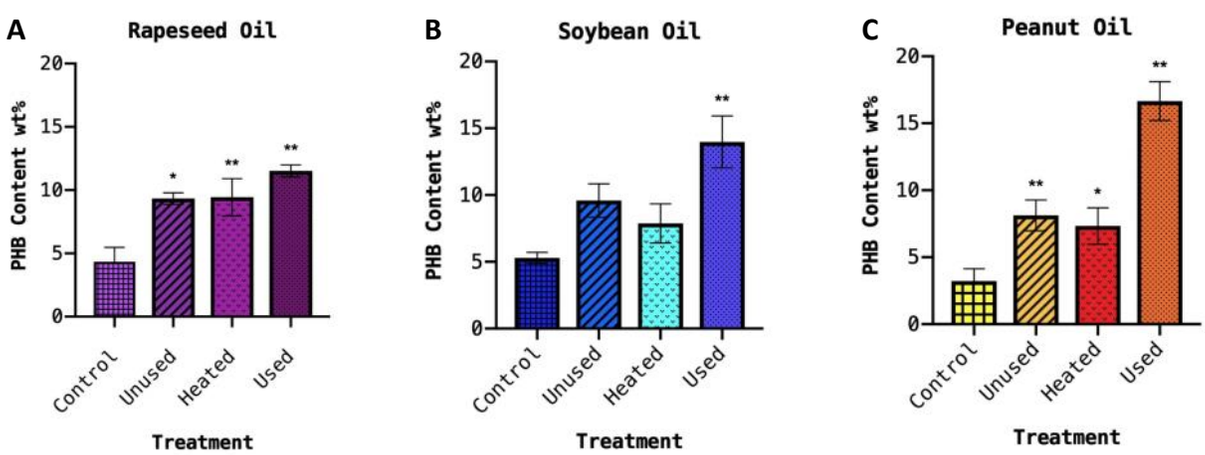


Figure 1. PHB content (% weight) extracted from *C. necator* and inoculated with three separate oil conditions in tryptone broth within four treatments; no oil (control), uncooked 'new' oil, heated oil, and used oil (a-c). (* = P<0.05; ** = P<0.01; *** = P<0.0001)

Within the 'used' oil treatment, peanut oil is the most significantly different (P<0.05) from rapeseed oil with respect to PHB production (% weight), and so presented the highest yield when compared to the other oils. With a mean PHB content of 16.657 % weight, compared with rapeseed at 11.517 % weight. Furthermore, peanut oil produced the lowest PHB when subjected to the uncooked oil treatment compared to that of rapeseed and soybean, and so represented the highest difference between uncooked oil and 'used' oil. However, within the uncooked oil treatment, this was not significant when tested between the three separate oil conditions. Only soybean oil showed no significant difference between none vs. uncooked oil treatments, with rapeseed showing significance; P<0.05, as well as peanut oil; P<0.01 showing an increase in PHB production from the bacterium when subjected to oil compared to the control. Furthermore, this was consistent when comparing none vs. heated (rapeseed; P<0.01, peanut; P<0.05).

When looking at the heated oil treatment results are similar, although no significant difference for any of the oils were found between the treatment; uncooked vs. heated, showing little relevance of heating oil upon PHB production. Significant differences were found between heated vs. used treatments for oils, soybean (P<0.05) and peanut (P<0.0001), but not rapeseed oil (P>0.05).

Discussion

The results of PHB production (% weight) from small scale fermentations with *C. necator*, with 20 g/L oils in tryptone broth showed the PHB production of all three oils tested was highest in the 'used' oil treatment. Hence, the addition of oils which contained additional nutrients i.e. fried with 100 g of raw potato and 50 g of raw chicken for one hour at 180 °C enabled an increased utilization of energy sources for *C. necator* to produce intracellular granules containing PHB. As these findings of used oil producing the most PHB in *C. necator* is consistent across the oils investigated, we can assume that waste oil use is a clear opposition to petrochemical derivatives.

These results reiterated the findings of Verlinden *et al.* (1), in that utilization of waste oil allows an increase in PHB production due to 1) increased metabolism of *C. necator* from excess nutrients in food residue, and 2) the change in chemical composition of PHB due to prolonged heating and frying, in that it decreases the relative amount of unsaturated fatty acids. Through fatty acid analysis, they found that saturated fatty acids tend to lead to an increase in PHB within bacteria. The natural chemical pathway of PHB production within bacteria involves the β-oxidation of fatty acids to acetyl-CoA and so forth (3), and so it may be that saturation increases the conversion rate within this cycle. More research should be conducted into the significance of the by-products of waste oil i.e. food residues and the additional chemical pathway alterations introduced during frying, with relevance to PHA production.

Due to the chemical composition of PHB, extremely high melting temperatures result in a physically stiff structure (7) making it particularly limited in its applications. Various further investigations conducted, have involved the enhancement of performance and efficiency of PHB extraction and use, for example, the practice of physical blending with co-polymers such as hydroxyvalerate (HV) (8). By altering, or adding to the chemical properties in this way (e.g. creating lower melting temperatures), PHB can also be useful within industrial food packaging, which is the most widely produced execution of plastic manufacturing (9). Following from this, co-polymers containing PHB can also be produced with the use of waste oil as well as pure oil, and glucose as carbon sources by *C. necator* (10). As this is noted, the use of waste oil can contribute to a wide spectrum of industrial plastic productions.

Within the 'used' oil treatment, peanut oil is found to be significant compared with rapeseed oil with respect to PHB production (% weight), with a mean PHB content of 16.657 % weight. Peanut oil is one of the most widely used oils in the food industry worldwide, with over half of this production market dominated by China and India according to Transparency Market Research (11) due to its extensive use in Asian cuisine. China is also responsible for 24.8% of the world's plastic production (9) and so has a strong audience for an alternative plastic source considering ocean pollution in the North Pacific. This presents possibilities of introducing plastic innovations such as those in this study to combat two problems; a) plastic waste, and b) the route of the problem, by using more environmentally friendly sources.

In the peanut industry there are many different by-products or waste products, including peanut meal, with an average of 5.78 million metric tones produced between 2000 and 2010 (12). Although this is a significant amount of a by-product produced to achieve the high value edible peanut oil, peanut butter and more, it has been shown that fermented peanut meal may also be an efficient substrate for biodegradable plastic production, in particular poly lactic acid (PLA) biodegradable plastics. The limitations to PLA include a high melting point, however modifications such increasing the relative amount of D-lactic acid can resolve this problem, yet current methods to increase D-lactic acid production are high cost low productivity. Recent research has shown that peanut meal fermentation by *Sporolactobacillus sp.* results in the highest yield of D-lactic acid from peanut meal (13), and therefore further showing how peanut by-products can be utilised in the production of biodegradable plastics.

Peanut oil in its natural state contains 79% unsaturated fatty acids, and 16% saturated (14). This saturation is shown to be higher than that of our lowest contributor of PHB production in this study, rapeseed at 6% saturated fatty acids. Additionally, the most prominent unsaturated fatty acid in peanut oil is linoleic acid (15); it may be that this is more easily converted to saturation during heating. However, research by Verlinden *et al.* (1) found an excess of unsaturated fatty acids, regardless of oil treatments, and so it was expected that other major factors are involved in PHB production, and that unsaturat-

ed fatty acid composition may play more of a role in the general bacterial cell metabolism. However, the saturation figures do seem to contribute to the notion that saturation may have increased acetyl-CoA conversion in this study and previous. If this correlation stands, it may be beneficial in future experimentation to test bacterial utilization of waste oils with a higher level of saturated fatty acids, such as coconut oil at 80% saturated fatty acids (14), which is also frequently used within the cooking industry.

Considering experimental results and previous research, it is clear that the use of waste oil has the capabilities of producing a high yield of PHB from *C. necator*, and potentially other PHB producing bacteria. As waste oil is an abundant resource, wide-scale applications of its use as a practical replacement of contemporary approaches to PHB production and extraction, has the potential to replace the current market of petro-chemical plastic production as a cheaper, eco-friendly alternative. Peanut oil is a potential candidate in this approach, as is shown in this study to produce the highest PHB yield from those tested in this experiment, as well as being widely used in the food industry world-wide. The PHB production from waste oils of several other widely used cooking oils should be investigated, including that of coconut oil.

Acknowledgements

Thanks to our mentors Mr. Richard Heaton, and Dr. Ewan Young, as well as module coordinator Dr. Heather Allison, and the University of Liverpool Life Science Lab Technicians.

References

1. Verlinden R A, Hill, D J and Kenward M A *et al.* Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. AMB express 2011. 1(1):11

2. Cavalheiro JM, de Almeida M C M, Grandfils C and Da Fonseca M M R. Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Process Biochemistry 2009. 44(5):509-515

3. Rehm B H, Krüger N and Steinbüchel A. A New Metabolic Link between Fatty Acid de Novo Synthesis and Polyhydroxyalkanoic Acid Synthesis. Journal of Biological Chemistry 1998. 273(37):24044-24051

4. Luzier W D. Materials derived from biomass/biodegradable materials. Proceedings of the National Academy of Sciences 1992. 89(3):839-842

5. Eriksen M, Lebreton L C and Carson H S *et al.* Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLOS ONE Science One 2014. 10;9(12):e111913

6. Holmes P A. Applications of PHB-a microbially produced biodegradable thermoplastic. Physics in Technology 1985. 16(1):32

7. Cox M K. Properties and applications of polyhydroxyalkanoates. In: Doi Y and Fukuda K. Studies in Polymer Science. Elsevier: 1994. Vol. 12 p. 120-135

8. Modi S, Koelling K and Vodovotz Y. Assessment of PHB with varying hydroxyvalerate content for potential packaging applications. European Polymer Journal 2011. 47(2):179 -186

9. Gourmelon G. Global plastic production rises, recycling lags. New Worldwatch Institute analysis explores trends in global plastic consumption and recycling 2015. Available from: http://www.worldwatch.org [Accessed 2018]

10. Obruca S, Marova I and Snajdar O *et al.* Production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by *Cupriavidus necator* from waste rapeseed oil using propanol as a precursor of 3-hydroxyvalerate. Biotechnology letters 2010. 32(12):1925-1932

11. Transparencymarketresearch.com. Peanut Oil Market - Global Industry Analysis and Forecast 2017 - 2027. Available from: https://www.transparencymarketresearch.com/peanut-oil-market.html [Accessed 7th March 2018]

12. Zhao X, Chen J and Du F. Potential use of peanut by-products in food processing: a review. Journal of food science and technology 2012. 49(5):521-529

13. Wang L, Zhao B and Li F *et al.* Highly efficient production of D-lactate by Sporolactobacillus sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. Applied microbiology and biotechnology 2011. 89(4):1009-1017

14. Gunstone F D. Fatty acids—nomenclature, structure, isolation and structure determination, biosynthesis and chemical synthesis. In Fatty Acid and Lipid Chemistry. Springer MA Boston 1996:1-34

15. Jamieson G S, Baughman W F and Brauns D H. The chemical composition of peanut oil. Journal of the American Chemical Society 1921. 43(6):1372-1381

You might also be interested in Megan Johnson's article on her experience of plastic pollution at the Great Barrier Reef: see Reflections, page 14

Investigating the interaction between gemcitabine and capecitabine by analysis of synergy or antagonism of gemcitabine with capecitabine metabolites

Rebecca Cleator[1]

The chemotherapy gemcitabine is commonly used to treat people who have pancreatic cancer. A recent study showed that gemcitabine combined with another drug called capecitabine was more beneficial for cancer patients than gemcitabine alone. When capecitabine enters the body, it is broken down by the liver into a substance called DFCR, then into DFUR. Cancer cells have a protein called RRM1 which is linked to making cancer cells resistant to gemcitabine. This study treats cancer cells with gemcitabine plus DFCR or DFUR to look at the effects that these two different substances have when combined with gemcitabine. The cancer cells treated had varying levels of RRM1 protein. The impact of drug treatment was determined by measuring how many cancer cells were alive after being treated with these drugs. DFCR and DFUR were found to have different effects on cancer cells and that their actions may be affected by how much RRM1 a cancer cell has. The variability of RPM1 is particularly interesting because it means that in the future, measuring RRM1 levels in a cancer patient may be able to tell doctors which chemotherapy is best for the patient. This additional treatment guidance could potentially increase the survival rate of pancreatic cancer, which is currently very low.

Abstract

The chemotherapeutic agent gemcitabine is commonly used to treat pancreatic cancer. It is a fluorinated deoxycytidine and results in the inhibition of ribonucleotide reductase (RNR) and causes DNA breaks via stalled replication forks. During the ESPAC-4 trial, it was determined that gemcitabine combined with the fluoropyrimidine capecitabine (termed GEMCAP) provided better clinical benefit than gemcitabine alone. This study aimed to begin to understand why GEMCAP provides a higher benefit than gemcitabine monotherapy. SUIT-2 and SUIT-2 gemcitabine-resistant cell lines with varying levels of RRM1 were treated with either gemcitabine + 5'-deoxy-5-fluorocytidine (DFCR) or gemcitabine + 5'-deoxy-5-fluorouridine (DFUR). The effects of these combination therapies on each cell line were then evaluated by measuring cell viability via MTS assay after each drug treatment. DFCR is antagonistic in cells with high levels of the RNR subunit RRM1 and is synergistic in cells with low RRM1, whereas DFUR is antagonistic in cells with low RRM1 and verges on being synergistic with high RRM1. I hypothesise that in low RRM1 cells, DFCR works alongside gemcitabine to inhibit RNR, while DFUR binds to RNR but does not inhibit it. However, the binding of DFUR to RNR could block gemcitabine from being able to inhibit RNR. The different actions of each metabolite suggest that deamination of DFCR to DFUR is likely to take place outside of the cancer cell. The results from this study justify further research into the possible use of RRM1 as a predictive biomarker which could determine which therapy is best suited to each individual.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease which affects the exocrine component of the pancreas and is currently the fourth deadliest cancer in the United States (1). PDAC's high mortality rate is attributable to the fact that the early presenting symptoms for this disease are non-specific (2), resulting in diagnosis occurring after the cancer has already locally advanced or metastasised, meaning curative surgery is rarely an option for patients facing a late diagnosis (1).

Gemcitabine

The chemotherapeutic agent commonly used to treat pancreatic cancer is the nucleoside difluorodeoxycytidine (dFdC), more commonly known as gemcitabine.

After entering the cancer cells via nucleoside transporters such as hENT1, gemcitabine is converted to gemcitabine monophosphate (dFdCMP), diphosphate (dFdCDP) or triphosphate (dFdCTP) via phosphorylation, and becomes activated (3). The current consensus is that gemcitabine acts in a cytotoxic manner by inhibiting DNA synthesis in several ways, one of which includes inhibiting the enzyme ribonucleotide reductase (RNR). This inhibition reduces the function of RNR and the availability of deoxyribonucleotides for the synthesis of DNA, hence slowing the rate of DNA synthesis within the cancer cell. Gemcitabine also acts through direct incorporation of itself into newly synthesised DNA, causing termination of the chain (3).

[1]Third Year Biological & Medical Sciences Student, School of Life Sciences, Liverpool, UK, L69 7ZB

GEMCAP

Gemcitabine can be given as a single chemotherapy or in combination regimens alongside other cytotoxic compounds (4). The ESPAC-4 trial aimed to determine whether using gemcitabine plus capecitabine (GEMCAP) or gemcitabine alone would be more beneficial for patients who have had their cancer surgically resected (5).

Catabolising capecitabine, an oral fluoropyrimidine, via three metabolic steps produces its active metabolite 5-FU. Capecitabine bypasses the metabolite 5'-deoxy-5-fluorocytidine (DFCR) which is deaminated to 5'-deoxy-5-fluorouridine (DFUR) by the enzyme cytidine deaminase (CDA). DFUR is then converted, by thymidine phosphorylase, to the active form 5-FU (6). This trial determined that the use of gemcitabine and capecitabine in combination provides better clinical benefit than gemcitabine alone, following the removal of cancerous tissue (5). However, it remains unclear how exactly capecitabine potentiates gemcitabine.

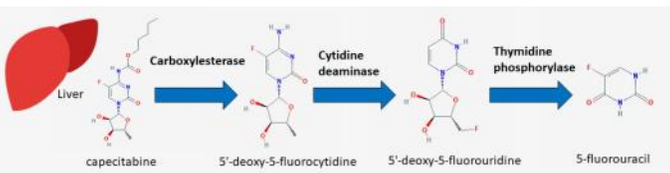


Figure 1. The metabolic pathway of the formation of 5-FU from capecitabine. The common consensus is that the first step of this pathway takes place mainly in the liver, although this is yet to be proven. It is unknown where the remainder of the metabolic path takes place, although with thymidine phosphorylase being expressed in higher concentration in neoplastic tissues than healthy tissues, it is hypothesised that this step may occur within the cancer cells. Chemical structures were taken directly from PubChem Open Chemistry Database (9).

RRM1

The ribonucleotide reductase subunit M1 (RRM1) gene encodes the regulatory subunit of the enzyme inhibited by gemcitabine; ribonucleotide reductase (RNR) (7). Previous work by our group has shown that gemcitabine-resistant cell lines, SUIT-2R and SUIT-2R2, derived from SUIT-2, have altered expression of RRM1 (Figure 2). SUIT-2R has lower RRM1 expression compared to SUIT-2, while SUIT-2R2 has higher RRM1 levels to SUIT-2. Patients expressing high levels of RRM1 had poor survival after gemcitabine treatment suggesting that there is an association between gemcitabine response and RRM1 expression (8). For this reason, RRM1 has been a molecule of interest, with the potential to be a predictive biomarker for gemcitabine treatment (8).

Study Importance

Based on the results from the ESPAC-4 trial, this study explores the results of combining either DFCR or DFUR with gemcitabine, by treating cell lines which have varying RRM1 expression levels. The effects of combining these different agents with gemcitabine will be compared to establish where the deamination stage of the

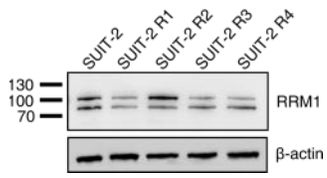


Figure 2. Western blot images showing the varying levels of RRM1 in SUIT-2 and SUIT-2 resistant cell lines. Images provided by Anthony Evans and Joshua Chapman.

metabolism of capecitabine takes place. We hypothesise that if both the DFCR and DFUR metabolite potentiate gemcitabine in these cell lines, then deamination is taking place within the cancer cell. If it is DFUR which potentiates gemcitabine, and the DFCR does not, we can confirm that the deamination stage is likely to be taking place outside of the cancer cell.

Methods

Tissue culture

Three human pancreatic cancer cell lines were used; SUIT-2, SUIT-2R and SUIT-2R2. The SUIT-2 tumour cell line was derived from a human metastatic liver tumour of pancreatic carcinoma (9). The cell lines were cultured in 10% FBS (Foetal Bovine Serum; Gibco®) supplemented Dulbecco's modified Eagle Medium (DMEM), and kept in T75 flasks (Thermo Scientific). Cells were fed three times a week and passaged with trypsin (Sigma-Aldrich) when they reached a confluence of ~70-80%.

Determining IC50s for DFCR and DFUR

Cell density optimisation was carried out before the synergy assay. From this, it was found that the optimal cell seeding density was 1500 cells/100 µL. Cells were seeded into 96-well plates at a density of 1500 cells per 100 µL, using the cell numbers previously counted using a Bio-Rad automated cell counter. Cells were left to adhere overnight prior to treatment. Each cell line was then treated with either DFCR or DFUR over a range of concentrations. The 50% inhibitory concentration (IC50) for each cell line, when exposed to DFCR and DFUR separately, was then calculated. Cell populations were then evaluated using an MTS assay (EZ4U assay, Biomedica Immunoassays) 24 hours after treatment and plates were read using a microplate reader at an absorbance of 450 nm and 620 nm.

The IC50 could not be determined for these cell lines, as the agent was not adequately toxic in this cell line in sole treatment. Therefore, a suitable concentration was chosen for each metabolite from the literature.

Synergy assay

SUIT-2, SUIT-2R and SUIT-2R2 cell lines were treated with gemcitabine (500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0 nM) plus either DFCR (Santa Cruz Biotechnology) or DFUR (Abcam) (320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0 µM) for 24 hours. The metabolites were dissolved in dimethyl sulfoxide (DMSO), so one column contained a vehicle DMSO control. Figure 3 shows the 96-well plate

layout. The assays were performed in 96-well plates, seeded with 1500 cells per 100 µL for all cell lines. Cells were adhered overnight prior to drug treatment. The effect on cell viability of gemcitabine combined with each metabolite was then investigated using the MTS assay. Absorbance, which in this assay represents the quantity of MTS tetrazolium reduced to formazan dye by the cells, for each plate was measured at 450 nm and 620 nm 3 hours after addition of the MTS reagent. Values from the plate reader were than analysed for synergy using an R software package called COMBIA. This software includes both Bliss (independence) and Loewe (additivity) analysis (10).

Results

Figures 4 (A-F) and 5 (A-F) display analysis of synergy/ antagonism, utilising the Bliss and Lowe methods conducted with the R package COMBIA. The Bliss independence method compares the observed combination response with the predicted combination response (11). This method assumes that the two drugs act through independent mechanisms, but contribute to the same result by addition of the independent interactions (12). Contrastingly, the Loewe additivity model assumes that the two drugs act through a similar mechanism, and because of this can be substituted for one another (12, 13).

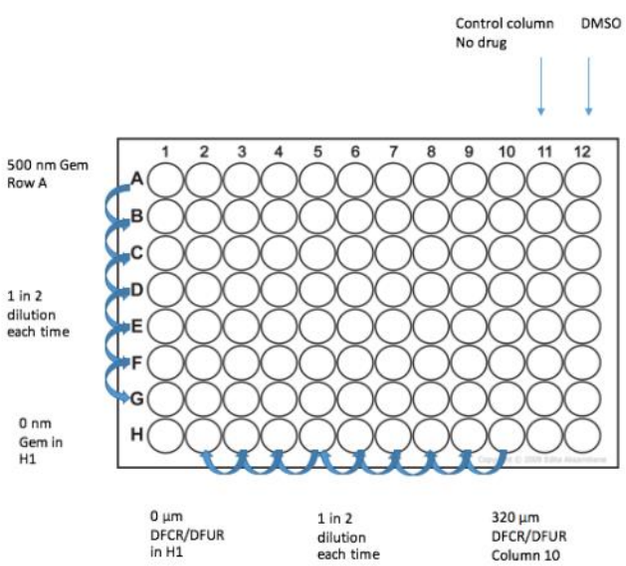


Figure 3. The layout of the 96-well plate during the synergy assay. The plate was prepared via serial dilutions of gemcitabine down from its initial starting concentration of 500 µM, and of either DFCR or DFUR down from initial starting concentration of 320 nM.

DFCR

From the pseudoisobolar analysis displayed in Figure 4, it can be seen that DFCR is most antagonistic in cells with high RRM1 (Figures 4E and 4F) and is synergistic in cells with low RRM1 (Figures 4C and 4D). From this, it can be concluded that DFCR has the same effects as gemcitabine in low RRM1 cells, and counteracts the effects of gemcitabine in cells expressing high levels of RRM1.

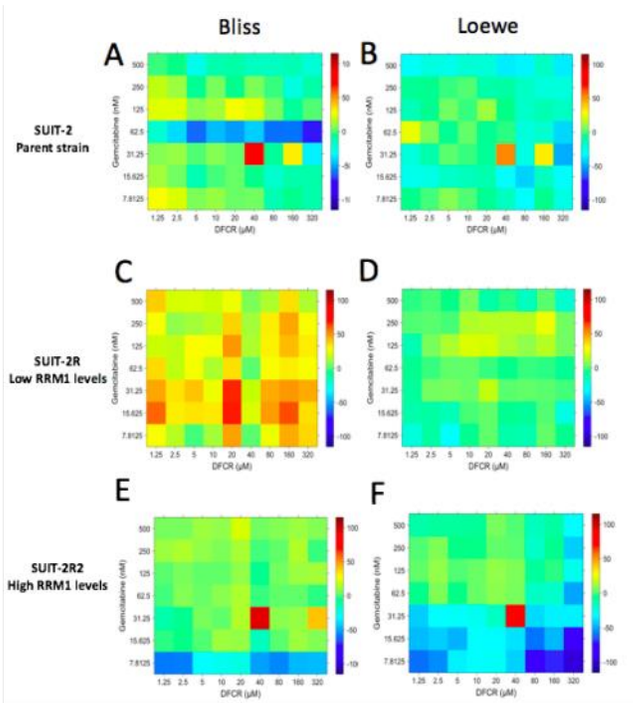


Figure 4. The results of testing for synergistic and antagonistic interactions between gemcitabine and DFCR in cell lines SUIT-2, SUIT-2R and SUIT-2R2. A positive value indicates synergy, a negative value indicates antagonism and zero indicates neither (shown in corresponding colour key) (10). A and B show the Bliss and Loewe synergy analysis in the SUIT-2 cell line. C and D show the Bliss and Loewe synergy in the SUIT-2R cell line. E and F show the Bliss and Loewe synergy analysis in the SUIT-2R2 cell line.

DFUR

From Figure 5 it can be seen that DFUR is most antagonistic in cells with low RRM1 (Figures 5C and 5D) and verges on being synergistic with high RRM1 (Figures 5E and 5F). This data is contrasting to what can be seen with DFCR. From the data, we can see that DFUR alone has limited effect, but is antagonistic in cells with low levels of RRM. It is assumed that DFUR does not inhibit RNR, as DFUR has no effect on cell viability as a single treatment. However, the act of DFUR binding to RNR could block gemcitabine from binding to RRM. The data in Figure 5 supports this hypothesis (N=1).

Discussion

The ESPAC-4 trial determined that GEMCAP provides better clinical benefit than gemcitabine alone, following surgical removal of pancreatic cancer (5). In this study, we aimed to gain a better understanding of why this combination therapy offers a clinical benefit, with the hope to improve treatment options, and the survival rate, for PDAC.

The contrasting effects of the two agents suggest deaminating DFCR to DFUR occurs outside of the cancer cell. If not, both of these metabolites would affect cell viability in the same manner. This conclusion is tentative due to a lack of liver or stromal CDA that is present *in vivo*. We also demonstrate that the two different capecitabine derivatives, in combination with gemcitabine, do not have the same effects on cell viability. However, each cell

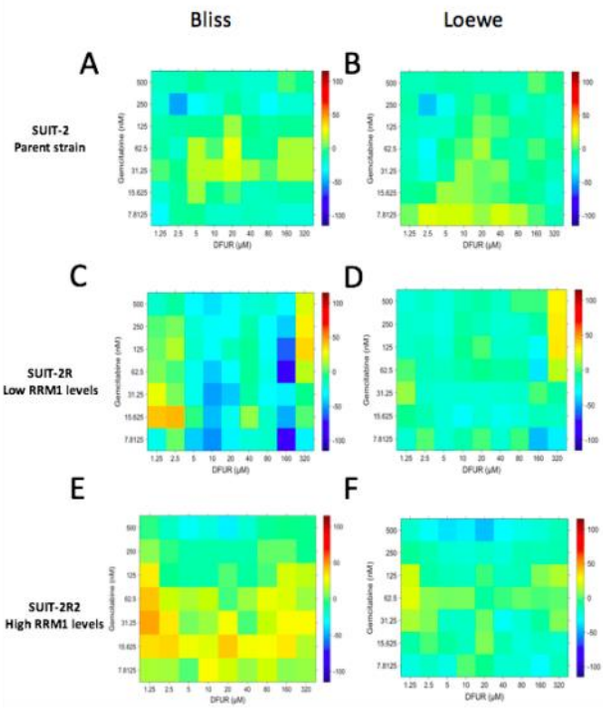


Figure 5. The results of testing for synergistic and antagonistic interactions between gemcitabine and DFUR in cell lines SUIT-2, SUIT-2R and SUIT-2R2. The colour scale next to each graph corresponds to each value (positive, negative or zero) to a colour. A positive value indicates synergy, a negative value suggests antagonism and a value of zero indicates neither (10). A and B show the Bliss and Loewe synergy analysis in the SUIT-2 cell line. C and D show the Bliss and Loewe synergy analysis in the SUIT-2R cell line. E and F show the Bliss and Loewe synergy analysis in the SUIT-2R2 cell line.

line showed an unexpectedly low response to gemcitabine limiting these conclusions. A possible explanation for this is that the 24-hour time treatment was insufficient time for gemcitabine to have an effect. To confirm our conclusions, the experiments in this study should be repeated with further time-points to ensure gemcitabine has optimum time to have a significant impact.

The RRM1 levels are one of the critical differences between the resistant cell lines used in this study, and the difference in RRM1 expression could lead to alterations in response to GEMCAP treatment. Future work involving these metabolites could directly target RRM1 either by inhibition or overexpression prior to drug treatment, to further dissect the relationship between RRM1 and GEMCAP. Other studies have already used gene-specific overexpression and RNA interference to determine the impact of RRM1 on various types of cytotoxic agents (14). However, using siRNA to knock down RRM1 is not the same as the reduction seen in the evolution of gemcitabine resistance. There is also the possibility of targeting RRM1 expression as a therapeutic option in patients before gemcitabine treatment. However, although RRM1 is related to gemcitabine resistance, it is unknown whether reversing the expression of RRM1 will alter this effect (7, 8, 15).

In this study, cells with variable levels of RRM1 responded differently to each capecitabine metabolite plus gemcitabine treatment, justifying further research into whether RRM1 levels alter effects on survival in response to GEMCAP. Using this information RRM1 expression levels could become a predictive biomarker for identifying appropriate treatment. However, a recent study has shown that intratumoural RRM1 protein expression levels in patient samples from trials involving treatment with 5-FU, folinic acid or gemcitabine showed no association with survival when analysed in isolation (18). Therefore, when considering RRM1 as a potential biomarker, RRM1 would likely need to be used in combination with other biomarkers such as hENT1 (19).

If deamination of DFCR to DFUR occurs outside of the cancer cell, as suggested here, intratumoural CDA may not be as important as previously thought. It may be that liver CDA is more relevant, as this is where the deamination step is likely to be taking place. Incorporating measurements of RRM1 and CDA expression could allow further comparison of patient tissue samples. These findings would allow future experiments to evaluate the effectiveness of these proteins as predictive biomarkers for treatment.

Using biomarkers to identify the most appropriate treatment for individuals would vastly increase survival rates in pancreatic cancer (20). Previous studies show that patients with a higher level of the nucleoside transporter hENT1 survive longer after treatment with gemcitabine. (21). hENT1 also allows gemcitabine transport into and out of the cell. Although this study only used tissue samples from 21 patients, it provides a basis for the idea of hENT1, acting as a biomarker (21). A more recent study which further explored this idea examined 434 patients from the ESPAC-3 trial and compared survival rates between groups expressing high hENT1 levels and low hENT1 levels (20). This study determined that administering gemcitabine in patients displaying a low level of hENT1 was detrimental, and there was no survival benefit for patients who had received 5-FU (20), further confirming that hENT1 levels could indeed be an indicator of which is the best treatment for each patient. These findings allow future research to explore the benefit of personalised treatment for individuals.

Future studies could explore the effects of using inhibitors of the specific metabolites in the capecitabine metabolic pathway, determining which of the metabolites is necessary for the potentiation of gemcitabine. Due to capecitabine being a pro-drug that is only active once it has converted to 5-FU, the efficacy of capecitabine should be affected by the levels of enzyme present at each stage of the pathway, and also by the levels of dihydropyrimidine dehydrogenase (DPD), which returns 5-FU to a less toxic variant (19). Blocking DPD by using a competitive inhibitor such as 5-chloro-2, 4-dihydroxypyridine (CDHP) would allow us to observe GEMCAP administration, without 5-FU converting to its non-toxic form (22). The following analysis could determine whether it is the accumulation of cytotoxic 5-FU within the cancer cells or an alternative step in the metabolic pathway that causes GEMCAP's increased clinical benefit. Research into these areas has already begun to take place, with one study

having already explored the relationship between levels of DPD and the response to 5-FU hepatic perfusion chemotherapy in PDAC patients to determine whether DPD levels could be used as a predictive marker for responsiveness to the therapy (23). A more recent study indicated that DPD status could be a useful predictive marker in PDAC patients who undergo surgical resection with S-1 adjuvant chemotherapy (24). S-1 consists of tegafur, gimeracil and oteracl potassium (25). Flores *et al.* discuss recent studies that have explored the idea of DPD expression being associated with response to various 5-FU based therapies, but data which nominates DPD as a useful biomarker for 5-FU treatment needs validation. Numerous pathways can be further researched in the future to understand why GEMCAP benefits certain patients.

Increased knowledge of exactly how and why GEMCAP provides clinical benefit for pancreatic cancer patients could lead to the use of other pyrimidine analogues with gemcitabine to give equivalent or even better results than GEMCAP. In conclusion, our findings provide hope for the development of a possible predictive biomarker which could provide us with the ability to identify which therapy is the most appropriate for each patient.

References

1. Teague A, Lim K-H and Wang-Gillam A. Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies. Therapeutic Advances in Medical Oncology 2015. 7(2):68-84

2. Oberstein P E and Olive K P. Pancreatic cancer: why is it so hard to treat? Therapeutic Advances in Gastroenterology 2013. 6(4):321-37

3. Mini E, Nobili S, Caciagli B, Landini I and Mazzei T. Cellular pharmacology of gemcitabine. Annals of oncology: official journal of the European Society for Medical Oncology 2006. 17 Suppl 5:v7-12

4. Toschi L, Finocchiaro G, Bartolini S, Gioia V and Cappuzzo F. Role of gemcitabine in cancer therapy. Future Oncology 2005. 1(1):7-17

5. Neoptolemos J P, Palmer D H and Ghaneh P *et al.* Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. The Lancet 2017. 389(10073):1011-24

6. Reigner B, Blesch K, Weidekamm E. Clinical pharmacokinetics of capecitabine. Clinical pharmacokinetics 2001. 40(2):85-104

7. Bepler G, Kusmartseva I and Sharma S *et al.* RRM1 modulated in vitro and in vivo efficacy of gemcitabine and platinum in non-small-cell lung cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology 2006. 24(29):4731-7

8. Nakahira S, Nakamori S and Tsujie M *et al.* Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. International journal of cancer 2007. 120(6):1355-63

9. Iwamura T, Katsuki T and Ide K. Establishment and characterization of a human pancreatic cancer cell line (SUIT-2) producing carcinoembryonic antigen and carbohydrate antigen 19-9. Japanese journal of cancer research: Gann. 1987. 78(1):54-62

10. Kashif M, Andersson C, Mansoori S, Larsson R, Nygren P, Gustafsson MG. Bliss and Loewe interaction analyses of clinically relevant drug combinations in human colon cancer cell lines reveal complex patterns of synergy and antagonism. Oncotarget 2017. 8(61):103952-67

11. Zhao W, Sachsenmeier K and Zhang L *et al.* A New Bliss Independence Model to Analyze Drug Combination Data. Journal of Biomolecular Screening 2014. 19(5):817-21

12. Lederer S, Dijkstra T M H and Heskes T. Additive Dose Response Models: Explicit Formulation and the Loewe Additivity Consistency Condition. Frontiers in Pharmacology 2018. 9(31)

13. Fitzgerald J B, Schoeberl B, Nielsen U B and Sorger P K. Systems biology and combination therapy in the quest for clinical efficacy. Nature chemical biology 2006. 2(9):458-66

14. Zhou J, Oliveira P, Li X, Chen Z and Bepler G. Modulation of the Ribonucleotide Reductase-Antimetabolite Drug Interaction in Cancer Cell Lines. Journal of Nucleic Acids 2010. 2010:597098

15. Davidson J D, Ma L and Flagella M *et al.* An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. Cancer research 2004. 64(11):3761-6

16. Mann K, Chatzipavli V and Neoptolemos J *et al.* Two contrasting gemcitabine resistance mechanisms involving ribonucleotide reductase and cyclin D1b. Pancreatology 2017. 17(3):S43

17. Geller L T, Barzily-Rokni M and Danino T *et al.* Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. Science 2017. 357(6356):1156

18. Elander N O, Aughton K and Ghaneh P *et al.* Intratumoural expression of deoxycytidylate deaminase or ribonuceotide reductase subunit M1 expression are not related to survival in patients with resected pancreatic cancer given adjuvant chemotherapy. British journal of cancer 2018. 118(8):1084-1088

19. Elander N, Aughton K and Greenhalf W. Development of Novel Therapeutic Response Biomarkers. In: Neoptolemos J P, Urrutia R, Abbruzzese J, Büchler M W, editors. Pancreatic Cancer. New York, NY: Springer New York; 2017. p. 1-32

20. Greenhalf W, Ghaneh P and Neoptolemos J P *et al.* Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. Journal of the National Cancer Institute 2014. 106(1):djt347

21. Spratlin J, Sangha R and Glubrecht D *et al.* The Absence of Human Equilibrative Nucleoside Transporter 1 Is Associated with Reduced Survival in Patients With Gemcitabine-Treated Pancreas Adenocarcinoma. Clinical Cancer Research 2004. 10(20):6956

22. Harada K, Ferdous T and Ueyama Y. Therapeutic strategies with oral fluoropyrimidine anticancer agent, S-1 against oral cancer. Japanese Dental Science Review 2017. 53(3):61-77

23. Nakayama S, Takeda S and Kawase Y *et al.* Clinical Significance of Dihydropyrimidine Dehydrogenase in Adjuvant 5-Fluorouracil Liver Perfusion Chemotherapy for Pancreatic Cancer. Annals of Surgery 2004. 240(5):840-4

24. Murakawa M, Aoyama T and Miyagi Y *et al.* Clinical implications of dihydropyrimidine dehydrogenase expression in patients with pancreatic cancer who undergo curative resection with S-1 adjuvant chemotherapy. Oncology Letters 2017. 14(2):1505-11

25. Kobayakawa M and Kojima Y. Tegafur/gimeracil/oteracil (S-1) approved for the treatment of advanced gastric cancer in adults when given in combination with cisplatin: a review comparing it with other fluoropyrimidine-based therapies. OncoTargets and therapy 2011. 4:193-201

You might also be interested the article by Francesca Jones on tumour heterogeneity in pancreatic cancer: see Insights, page 58

Investigating the influence of temperature and glucose concentration on ethanol production in *Saccharomyces cerevisiae*

Liam Barningham[1], Amelia Collette[1], Nitasha Manzoor[2] & Matthew Owen[1]*

Yeast is a well-known and common fungus naturally found in warm and moist environments. When placed in less favourable surroundings, such as those depleted of oxygen this small fungus can alter the way it generates energy allowing it to survive and grow. In doing so the fungus readily produces by-products of carbon dioxide and ethanol. This is the process of fermentation and the reason why yeast is so famous. Throughout human history this adaptation has been applied to produce food, alcoholic drinks and now biofuels. As an indication to the value of this technology the UK beer market alone is currently worth $8 billion and while the demand for renewable energy sources grows, ethanol-based biofuels are becoming a more viable source of power. Therefore, there is great interest in improving the efficiency and productivity of this process. This study looked to achieve just that; through altering the temperature and sugar concentrations of yeast's environment we hoped to deduce the optimal conditions required to produce maximum ethanol yields. To achieve this a sample of yeast was incubated over 2 days at temperatures of either 25 °C, 30 °C and 35 °C and in a sugar concentration of either 150 g/L, 180 g/L or 210 g/L before the ethanol concentration for each condition was measured. The results of this study suggest from the conditions we tested a combination of 35 °C in a sugar concentration of 180 g/L produced the largest yields of ethanol (3.09 mg/ml). These findings agree with various other studies, however other studies have also achieved much greater yields by also manipulating a range of other factors e.g. pH, nitrogen concentration and source of sugar.

Abstract

The production of ethanol using *Saccharomyces cerevisiae* is one of the oldest and most commonly used examples of biotechnology. In nature, the capability of yeast to replace oxygen with glucose as a terminal electron accepter allows this microorganism to continue to grow even in anoxic environments producing ethanol as a by-product. However, when applied to an industrial context this metabolic survival strategy has important applications in the production of alcoholic drinks, food and bioethanol. In order to investigate the influence of incubation temperature and glucose concentration on ethanol yield by *S. cerevisiae,* we prepared 9 unique batch fermentation conditions. Each sample of *S. cerevisiae* was incubated for 48 hours at a temperature ranging from 25 °C to 35 °C while inoculated in a glucose solution ranging from 150 g/L to 210 g/L. After this time the ethanol concentration for each sample was determined using an ethanol assay. The greatest mean yield of ethanol (3.09 mg/ml) was obtained when samples were incubated at a temperature of 35 °C in a glucose concentration of 180 g/L. While an increase of incubation temperature from 25 °C to 30 °C at all glucose concentrations produced a statistically significant increase in ethanol yield, no significant increase was generated when temperature was raised from 30 °C to 35 °C at each glucose concentration tested. Additionally, no significant increase in ethanol yield was found when glucose concentration increased. Therefore, while these conditions generate the highest mean ethanol yield we cannot conclude this was due to increasing incubation temperature from 30 °C to 35 °C or by variation of glucose concentration between 150 g/L and 210 g/L.

Introduction

S. cerevisiae, also known as Baker's yeast is a single-celled eukaryotic fungus typically found in warm and moist environments such as the skin of grapes (1). Its ease of handling and molecular similarities to humans, makes it a popular model organism for research. Typically, yeast plays a number of roles in industry acting as a protein supplement, animal feed and vector for the production of recombinant proteins (2). However, most notably the fermentation of sugars and production of ethanol using *S. cerevisiae* is one of the oldest and most commonly used examples of biotechnology. This microbial anaerobic oxidation of sugars has been exploited for millennia in the production of alcoholic drinks, food and more recently in the production of bioethanol (3). As an indication of the importance of this technology the UK beer market is now worth an estimated $8 Billion (4).

[1]Second Year Microbiology Student, School of Life Sciences, University of Liverpool, Liverpool, UK, L69 7ZB
[2]Second Year Biological Sciences Student, School of Life Sciences, University of Liverpool, Liverpool, UK, L69 7ZB
*Corresponding author

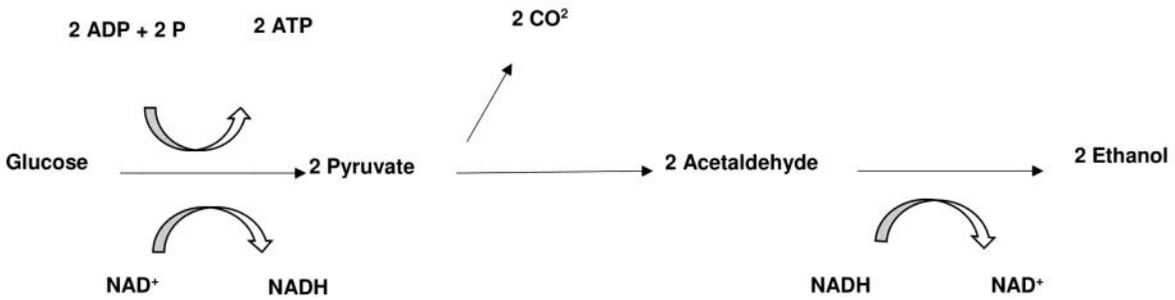


Figure 1 (above). Anaerobic fermentation of glucose sugar resulting in the production of ethanol and NAD^+ by *S. cerevisiae*.

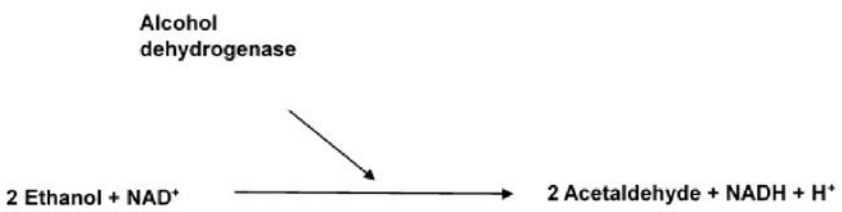


Figure 2 (left). Dehydrogenation of ethanol catalysed by alcohol dehydrogenase resulting in the production of acetaldehyde, hydrogen and NADH that can be assayed to give an indication of ethanol concentration.

Bioethanol production has become the largest consumer of corn in America. Naturally, the ability of *S. cerevisiae* to anaerobically ferment a range of hexose sugar substrates allows the fungi to survive in anoxic environments. However, it is this characteristic that makes the microorganism so attractive to industry. In the absence of oxygen *S. cerevisiae* can utilise sugars as a carbon source via glycolysis generating pyruvate and ATP. For this process to continue there must be a steady supply of the coenzyme NAD^+. To achieve this pyruvate is decarboxylated to acetaldehyde that is subsequently reduced to ethanol by NADH, regenerating NAD^+ and allowing glycolysis to continue (Figure 1) (5). Similarly, the decarboxylation of pyruvate releases CO_2 during the baking process creating air pockets within dough. *S. cerevisiae* is preferred to bacteria and other fungi in the industrial production of ethanol due to its various physiological advantages. It can operate in a large pH range with an acidic optimum meaning the process can naturally inhibit contaminating bacteria. Furthermore, the fungus has a higher ethanol tolerance (17% v/v) and is generally regarded as safe for human consumption enhancing its suitability for use in the consumables industry (6). The ability of *S. cerevisiae* to produce ethanol is heavily predicated on its fermentation conditions. The influence of factors such as carbon source, substrate concentration, temperature and pH have been heavily explored and the use of genetic manipulation is now being implemented to allow *S. cerevisiae* to ferment a greater range of substrates (7). Our study hypothesised that an increase in temperature and glucose concentration would increase the rate of fermentation by *S. cerevisiae* and therefore increase the concentration of ethanol produced. We aimed to deduce the optimal combination of glucose concentration and incubation temperature required to optimise batch production of ethanol using *S. cerevisiae*.

Materials and Methods

Broth preparation

A broth culture of *S. cerevisiae* was initially provided by the University of Liverpool (School of Life Sciences) and from this 1 ml of broth culture was plated onto several agar mediums including yeast extract-malt extract, mannitol salt agar, MacConkey agar and triple sugar iron agar that were all incubated at 37 °C overnight. Examination of the plates revealed the fungi had failed to grow on all

Table 1. Volumes of YEME broth and 300 g/L glucose stock solution required to produce 20 ml of mixed solutions with glucose concentrations of 150 g/L, 180 g/L and 210 g/L.

Volume of glucose 300 g/L stock (ml)	Volume of YEME broth (ml)	Final glucose concentration (g/L)
10	10	150
12	8	180
14	6	210

Table 2. Specific combinations of incubation temperature and substrate concentrations for batch glucose fermentation of *S. cerevisiae.*

	Glucose Concentration			
Temperature		150 g/L	180 g/L	210 g/L
	25 °C	25 °C & 150 g/L	25 °C & 180 g/L	25 °C & 210 g/L
	30 °C	30 °C & 150 g/L	30 °C & 180 g/L	30 °C & 210 g/L
	35 °C	35 °C & 150 g/L	35 °C & 180 g/L	35 °C & 210 g/L

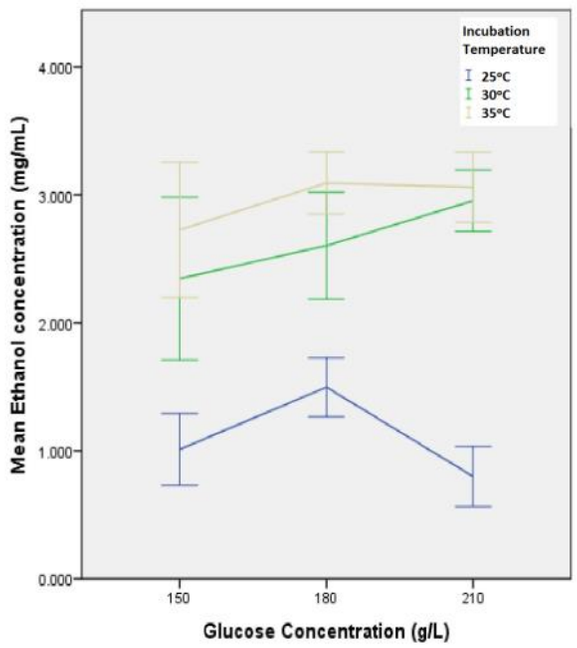


Figure 3. Ethanol yield obtained through batch glucose fermentation using *S. cerevisiae* at glucose concentrations of 150 g/L, 180 g/L and 210 g/L incubated for 48 hours at temperatures of 25 °C, 30 °C and 35 °C. The presence of overlapping error bars (± SE) between ethanol yields generated at incubation temperatures of 30 °C and 35 °C at all glucose concentrations suggests this difference is not significant. The error bars of ethanol yields generated at 25 °C at all glucose concentrations do not overlap with other incubation temperatures suggesting these produce significantly different ethanol yields.

excluding the yeast extract-malt extract (YEME) agar pate; because of this it was decided throughout the experiment *S. cerevisiae* would be inoculated into YEME broth. A 600 ml stock solution of YEME broth was mixed and from this 3 different volumes of broth (Table 1) were transferred to 20 ml glass bijoux bottles to be autoclaved. A 300 g/L glucose stock solution was purified using 0.22 µm filters removing any microbial contamination. From the stock, 3 separate volumes of solution (Table 1) were added to 20 ml glass bijoux bottles so that each one contained a combined total of 20 ml of YEME broth and glucose stock solution. The final proportions of glucose stock to YEME broth were calculated to produce solutions with glucose concentrations of 150 g/L, 180 g/L and 210 g/L (Table 1).

Incubation procedure

Each glass bijoux containing a YEME broth/glucose solution mix was inoculated with a single colony of *S. cerevisiae* approximately 1 mm in diameter using a sterile wire loop and incubated under static conditions for 48 hours at a temperature of either 25 °C, 30 °C or 35 °C in glucose concentrations of 150 g/L, 180 g/L and 210 g/L. This created 9 unique fermentation conditions (Table 2). Each fermentation condition was tested 9 times and a mean average ethanol yield was calculated.

Determination of ethanol concentration

After incubation each fermentation condition was individually assayed to determine its ethanol concentration. 0.1 ml of YEME broth now containing ethanol was added to 4.75 ml pyro buffer, 0.1 ml B-NAD and 0.05 ml alcohol dehydrogenase. After mixing and incubation at 37 °C for 25 minutes the level of NADH in each solution was measured at 340 nm using a spectrophotometer. The assay relies on the dehydrogenation of ethanol, converting NAD^+ to NADH catalysed by alcohol dehydrogenase (Figure 2). Using known ethanol concentrations of 5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml and 1 mg/ml a calibration curve was constructed. Values of ethanol yield were deduced via regression analysis using the linear equation y= mx + b derived from the calibration curve where y= A_{340} reading, m= gradient of the calibration curve, x= amount of ethanol present in the sample and b= the y intercept.

Data analysis

A repeated measures two-way ANOVA with a Greenhouse-Geisser correction was used to statistically analyse data obtained throughout the study. A pairwise comparison using a Bonferroni correction was later conducted to further analyse the results of the previous statistical test.

Results

By conducting an ethanol assay on the samples, we were able to deduce the ethanol concentration and make comparisons between the ethanol yields generated at different incubation temperatures and glucose concentrations. Higher ethanol yields indicate higher rates of fermentation. The greatest mean yield of ethanol (3.09 mg/ml) was obtained when samples were incubated at a temperature of 35 °C in a glucose concentration of 180 g/L. Incubation of samples at 35 °C and glucose concentrations of 210 g/L or 150 g/L produced decreased mean ethanol yields (Figure 3). Figure 3 demonstrates that when incubated at temperatures of 25 °C, 30 °C or 35 °C, increasing glucose concentration from 150 g/L to 180 g/L resulted in an increase of mean ethanol yield from 1.01 mg/ml to 1.44 mg/ml, 2.34 mg/ml to 2.62 mg/ml and 2.73 mg/ml to 3.09 mg/ml respectively. When samples were incubated at a temperature of 30 °C and glucose concentration was further increased to 210 g/L this trend was repeated producing a higher mean ethanol yield of 2.95 mg/ml. However, increasing glucose concentration from 180 g/L to 210 g/L in samples incubated at 25 °C and 35 °C resulted in decreased mean ethanol yields of 0.642 mg/ml and 3.06 mg/ml respectively. A repeated measures two-way ANOVA with a Greenhouse-Geisser correction showed that the mean ethanol concentration was not significantly different between glucose concentrations: $P>0.05$. A further ANOVA under the same conditions showed a significant difference in ethanol concentration between incubation temperatures: $P=0.00$ ($P<0.05$). Pairwise comparison using a Bonferroni correction showed that the ethanol concentration produced at 30 °C was increased

on average by 1.555 mg/ml compared to 25 °C ($P<0.05$). Between 30 °C and 35 °C, there was not a significant increase in ethanol production ($P>0.05$). Therefore, while a combination of 35 °C incubation in a glucose medium of 180 g/L produced the highest mean ethanol yield we cannot conclude this was a result of increasing incubation temperature from 30 °C to 35 °C or by our variations of glucose concentration between 150 g/L and 210 g/L.

Discussion

Fermentation plays a central role in many of the biotechnological processes that we rely on in various aspects of our lives e.g. food, alcoholic drinks and biofuels. By conducting research into this process, we can create specific fermentation conditions that maximise its efficiency and yield, subsequently leading to developments that both improve quality and reduce costs. Our study hypothesised that by increasing both glucose concentration and incubation temperature we would achieve higher ethanol concentrations, however the absence of a significant difference between ethanol yields at the tested glucose concentrations suggests variation between 150 g/L and 210 g/L has no influence on ethanol production during batch fermentation.

The presence of a significant difference between ethanol yields when incubated at temperatures ranging from 25 °C to 30 °C demonstrates increasing the incubation temperature of batch fermentations in this range will result in larger ethanol yields. However ethanol production at temperatures ranging from 30 °C to 35 °C are not significantly different indicating that any increase above 30 °C to 35 °C would have no influence on ethanol yield during batch fermentation. This finding can be explained by enzyme kinetics and the temperature dependent nature of enzyme activity. Increasing the incubation temperature from 25 °C to 30 °C during the fermentation process provides a greater level of kinetic energy to the reactants and enzymes involved in glucose fermentation and ethanol production. This higher level of kinetic energy increases the frequency of collisions between the enzymes and reactants increasing the rate of reaction leading to a greater ethanol yield. The conclusions drawn from these results remain consistent with the findings of previous studies that suggest achieving the greatest yield of ethanol during batch fermentation with *S. cerevisiae* incubation temperatures should range from 30 °C to 35 °C (6). Therefore, it is most likely that no significant difference was observed between ethanol yields at temperatures of 30 °C and 35 °C as the optimal temperature lies within this range.

Previous literature indicates a glucose concentration of 200 g/L is optimal for ethanol production, this is consistent with our findings (8). However, our results indicated variation of glucose concentration from 150 g/L to 210 g/L produced no significant change in ethanol yield during batch fermentation contradicting findings of previous studies. Past investigations of growth and fermentation characteristics of *S. cerevisiae* indicate significant differences are observed in ethanol yield between glucose concentrations of 20 g/L and 300 g/L when increased in intervals (8). It is possible the range of glucose concentrations tested throughout our study was too small to produce a significant change in ethanol yield and by testing a greater range of glucose concentrations the study would be improved.

Further investigation of previous studies highlights that when incubated at 30 °C for 24 hours with a glucose concentration of 200 g/L (pH 6.0) ethanol yield can reach 85.56 ± 1.13 mg/ml (8), much higher than our optimal ethanol yield of 3.09 mg/ml (35 °C with 180 g/L glucose). This finding indicates that while we were able to optimise ethanol production from our limited range of incubation temperatures and glucose concentrations there is massive potential for further optimisation. Based on a theoretical maximum yield of 0.51 g ethanol/g sugars (9) through variation of incubation temperature and glucose concentration we were able to achieve an ethanol yield of 2.9% of the theoretical maximum yield.

Numerous different strategies have been employed to improve ethanol yields from batch fermentation such as optimisation of pH, nitrogen supplementation, immobilisation, carbon source and inoculum size. These are all factors that could be further investigated in later studies to provide an insight into improving ethanol yields. A key limitation of our study is derived from the fact only 2 variables were manipulated, whereas to optimise ethanol yields a study would have to consider all of the factors previously listed. Furthermore, during incubation the inoculum of *S. cerevisiae* would settle to the bottom of the universal. Consequently, it is unlikely that all glucose substrate would be utilised during fermentation producing lower yields of ethanol. As a resolution to this problem in future investigations higher yields of ethanol may be obtained by regularly shaking incubation vessels to prevent the sedimentation of *S. cerevisiae.* Our findings indicate optimal ethanol yields are produced when incubated at temperatures ranging from 30 °C to 35 °C; In order to overcome this limitation and improve the specificity of the study it would be more appropriate to test incubation temperatures that increase in 1 °C intervals rather than 5 °C intervals.

Recently the use of genetic manipulation and synthetic biology is driving the future of ethanol production using *S. cerevisiae*. These techniques have enabled *S. cerevisiae* to utilise xylose, the second most abundant sugar present in plant biomass behind glucose. Xylose fermentation using *S. cerevisiae* has become a possibility due to the introduction heterologous xylose assimilation pathways into *S. cerevisiae*. Genetically engineered *S. cerevisiae* are capable of fermenting both glucose and xylose present in hemicellulose during ethanol production greatly improving utilisation of carbon sources during

ethanol production. This technology has massive applications in the microbial conversion of biomass to bioethanol as efficient xylose utilisation is seen as a key prerequisite for developing economic sources of biofuel (10). Alternative branches of research into optimisation of ethanol production focus on improving ethanol tolerance of *S. cerevisiae*. To achieve this a number of different approaches have been used however, via directed mutagenesis researchers have been able to modify RNA polymerase II subunit Rpb7 altering the transcription of its genome resulting in improved ethanol yields (11). Studies such as these pave the way for developments in ethanol fermentation using *S. cerevisiae*.

References

1. JoVE Science Education Database. An Introduction to Saccharomyces cerevisiae. Available from: https://www.jove.com/science-education/5081/an-introduction-to-saccharomyces-cerevisiae [Accessed 2018]

2. Moyad M. Brewer's/baker's yeast (*Saccharomyces cerevisiae*) and preventive medicine: Part II. Urologic Nursing Journal 2008. 73-75

3. Fakruddin M, Quayum A, Ahmed, M and Choudhury N. Analysis of Key Factors Affecting Ethanol Production by Saccharomyces cerevisiae IFST-072011. Biotechnology 2012. 11(4):248-252

4. IBIS World. Beer Production - UK Market Research Report. Available from: https://www.ibisworld.co.uk/industry-trends/market-research-reports/manufacturing/manufacture-of-beverages/beer-production.html [Accessed December 2017]

5. Kahn Academy. Fermentation and anaerobic respiration. Available from: https://www.khanacademy.org/science/biology/cellular-respiration-and-fermentation/variations-on-cellular-respiration/a/fermentation-and-anaerobic-respiration [Accessed 2016]

6. Tesfaw A and Assefa F. Current Trends in Bioethanol Production by *Saccharomyces cerevisiae*: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization. International Scholarly Research Notices 2014. 532852-532863

7. Yang S-T, Liu X and Zhang Y. Metabolic Engineering – Applications, Methods, and Challenges. In Yang S-T, Liu X and Zhang Y. Bioprocessing for Value-Added Products from Renewable Resources. Elsevier; 2006. p. 73-118

8. Lee J-S, Park E-H and Kim J-W. Growth and Fermentation Characteristics of *Saccharomyces cerevisiae* NK28 isolated from kiwi fruit. Journal of Microbiology and Biotechnology 2013. 23(9):1253-9

9. Krishnan M, Ho N and Tsao G. Fermentation kinetics of ethanol production from glucose and xylose by recombinant Saccharomyces 1400(pLNH33). Applied Biochemistry and Biotechnology 1999. 78:373–388

10. Kwak S and Jin Y-S. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. Microbial Cell Factories 2016. 16 (1):82

11. Qiu Z and Jiang R. Improving *Saccharomyces cerevisiae* ethanol production and tolerance via RNA polymerase II subunit Rpb7. Biotechnology for Biofuels 2017. 10:125

Nucleolar number but not characteristics are altered in chondrocyte ageing

Sophie Sutton[1], Ellice Wakelin[2], Mandy Peffers[3]*

Osteoarthritis (OA) is a prevalent age-related joint disease; however, the disease process is not yet fully understood. At the cartilage level it is caused by connective tissue breakdown with an imbalance between anabolism and catabolism of the connective tissue proteins. The nucleolus is the compartment of the cell in which proteins are made and we have previously found that the capacity of cartilage cells to translate proteins reduces in OA. The aim of this study was to investigate whether the nucleoli characteristics alter with ageing in horse cartilage cells since size, shape and number of nucleoli are a potent indicators of nucleoli activity. In order to measure these characteristics (area and circularity index), immunocytochemistry was performed by visualising an abundant nucleolar protein nucleophosmin (also called NPM1 or B23) in cartilage cells from young and old horses. The fluorescence of this protein thus enabled visualisation of the nucleoli within the nucleus. We then measured the area, circularity and number of nucleoli present between young and old horses. Finally, we measured the gene expression of nucleophosmin. This study found a significant increase in the number of nucleoli per nucleus in cartilage cells from older horses. There was no significant variation in area or circularity between the young and old cartilage cells. However, there was a reduction in nucleophosmin expression. Age is an important factor in the cause of OA. The changing number of nucleoli and nucleophosmin expression in ageing cartilage cells may have a role in the imbalance between connective tissue protein production and breakdown seen in cartilage tissue in OA.

Abstract

Osteoarthritis is a common degenerative musculoskeletal disease of ageing with a poorly understood disease process. As translational capacity is an important factor in chondrocyte proliferation in osteoarthritis the aim of this study was to measure nucleosome characteristics as an indicator of translational capacity in young and old equine chondrocytes. Nine young (≤8yrs) and eight old (≥15yrs) equine chondrocytes were grown and stained using B23/Nucleophosmin antibody and Alexafluor™ mouse IgG1 labelling reagent. Samples were counterstained with DAPI Vectashield®. Area (µm), circularity index and number of nucleoli per nucleolus were measured using immunocytochemistry and Photoshop™. Gene expression of nucleophosmin (NPM1) was measured using real-time qRT-PCR. The number of nucleoli per nucleus was significantly increased in old samples compared to young (P=0.042), the average number of nucleoli per nucleolus was 1.69 (SEM=0.034) for young and 1.81 (SEM=0.042) for old samples. There was no significant difference in nucleoli area (µm) or circularity index between young and old chondrocytes. There was a reduction in NPM1 expression in ageing (P=0.03). Ageing and OA have known associations with altered chondrocyte gene expression and an imbalance between protein anabolism and catabolism. Altered nucleoli number and NPM1 expression may have a role in age-related OA through changing ribosomal translational capacity. This study needs to be replicated in OA equine chondrocytes to determine these parameters in disease.

Introduction

Osteoarthritis (OA) is a common age-related joint disease, causing joint pain and stiffness and is an incurable cause of chronic disability, affecting 30% of the human population worldwide (1-2). The underlying disease process is multifactorial, characterised by the destruction of articular cartilage, pathological bone changes and inflammation of the synovium (3). Currently, the molecular mechanisms of the inflammatory process are unclear (4) and radiographic diagnosis of OA is only possible when cartilage degradation becomes too severe to prevent disease progression. It would be beneficial to develop a method of measuring OA progressions using markers for molecular changes within the body to provide an earlier diagnosis and thus more effective management (2).

Treatment options focus on symptoms and pain management but do not address the disease process itself (5). In recent decades, there has been increasing interest from researchers in the molecular and genetic mechanisms responsible for its pathogenesis (6) and if early deterioration in musculoskeletal health could be identified and treated, serious physical impairment may be avoided (7). Studies have shown reduced translational capacity in OA chondrocytes (8), whilst our unpublished work in ageing equine chondrocytes indicates there is no age-related change. OA is associated with altered chondrocyte gene expression and resulting changes in cell morphology (9).

OA is characterised by structural damage of articular car-

[1]Fourth Year Veterinary Sciences Student, Institute of Veterinary Science, Thompson Yates Building, University of Liverpool, Liverpool, UK, L69 3GB
[2]Third Year Bioveterninary Science Student, School of Life Sciences, University of Liverpool, Liverpool, UK, L69 7ZB
[3]Institute of Aging and Chronic Disease, University of Liverpool, West Derby Street Liverpool, UK, L7 8TZ
*Corresponding author

tilage caused by disturbances in homeostasis. Cartilage homeostasis is maintained by chondrocytes (the only cell type in cartilage), expressing extracellular matrix (ECM) components (e.g. type II collagen and proteoglycans) the principal output in chondrocyte anabolism (10). As cells age, anabolic activity is unable to match degenerative catabolic activity in the joint space (11), compounded by increased secretion of pro-inflammatory cytokines (12). Although accelerated catabolic processes in OA are well researched (8, 13), there is little information about the suppression of anabolic processes that occur with age and OA in cartilage (14).

In OA, chondrocytes require a higher translational capacity to meet the demands of proliferation, collagen matrix production and cellular hypertrophy within the growth plate (15). Ribosome biogenesis is an anabolic process integral to cell growth and proliferation through its roles in protein synthesis and translation of mRNAs; as ribosomes translate mRNA into protein to synthesize all protein within the cell (15). Ribosomes are an assembly of four rRNAs transcribed by RNA polymerase I and III, 80 ribosomal proteins, accessory proteins and around 70 snoRNAs (16).

The nucleoli are multifunctional domains containing hundreds of different types of proteins involved in many fundamental processes including ribosome biogenesis. Studying nucleosome characteristics as cells age could be an insightful indicator of their translational capacity potential and ability to maintain cartilage homeostasis in OA.

Small Nucleolar RNAs (snoRNAs) are 60-300 nucleotide non-coding guide RNAs in the nucleolus and form one of the largest classes of non-coding RNA (17). Nucleoli are one of the main centres of ribosomal biogenesis, RNA maturation and apoptosis regulation (18), therefore are integral to maintaining cell translational capacity and morphology.

Previous studies have shown that unusual expression of snoRNAs and morphological abnormality of nucleoli are both indicative of pathological processes such as cancer. (18, 20, 21). Recent research presented at Osteoarthritis Research Society International (OARSI) congress 2018 indicated differential expression of snoRNAs in ageing and OA cartilage (22). Peffers *et al*. (23) also found that dysregulation of certain snoRNAs in OA and ageing have been shown to alter ribosome biogenesis and translational capacity of chondrocytes.

The size, morphology, number and ultrastructural organisation of nucleoli are potent indicators of cellular nucleolar activity. Nucleoli can be visualized by immunofluorescence experiments using antibodies directed against nucleolar proteins, such as B23. B23/nucleophosmin is a nuclear acid chaperone encoded by NPM1 active in ribosome biogenesis, chromatin remodelling, mitosis, DNA repair, replication and transcription (24). Nucleophosmin is also responsible for nucleolar morphology (25).

Aims

The aim of this study was to measure nucleosome characteristics (by measuring area, circularity index and number of nucleoli per nucleolus) in young and old equine chondrocytes. To visualise nucleoli, chondrocytes were stained using immunocytochemistry with a B23 (nucleophosmin) antibody and a DAPI nuclear costain. Change in nuclear morphology between young and old samples is considered here as a measure of translational ability of the chondrocyte. We hypothesise that nucleosome characteristics will vary with age in equine chondrocytes.

Table 1. Equine chondrocyte samples used for the analysis.

Sample O	Age O
O6	16
O7	16
O8	18
Average	16.67
SEM	0.67

Sample Y	Age Y
Y4	8
Y6	5
Y7	4
Y9	3
Average	5.00
SEM	1.08

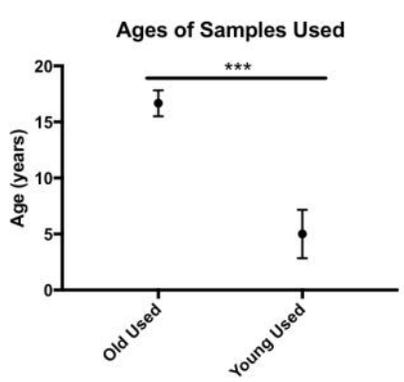


Figure 1. Equine chondrocyte samples were obtained the difference in age between groups was plotted (P=0.004). Error bars represent SEM.

Methods

Sample selection & tissue culture

Equine chondrocytes were harvested previously from the metacarpophalangeal joints of n=9 young (≤8yrs) and n=8 old (≥15yrs) healthy horses collected from an abattoir. Schedule 2 of the Animals (Scientific Procedures) Act 1986 does not define collection from these sources as a scientific procedure (26). Equine articular chondrocytes were isolated from macroscopically intact cartilage (27).

Chondrocytes were isolated and aseptically grown as monolayers at 37 °C in normaoxic conditions (20% O_2) in

Dulbecco's modified Eagle's medium (DMEM; catalogue no. 12430104, Invitrogen, Paisley, UK) containing 100 U/ml penicillin, 100 U/ml streptomycin, 10% foetal calf serum (FBS) and 50 ng/ml Amphotericin B (all from Invitrogen) (28).

The chondrocytes were thawed and seeded at 5,000-6,000 cells/cm^2. Cells were incubated to 80% confluency for 1 week and culture media was changed every other day. Chondrocytes were visualised using light microscopy to assess cell numbers and morphology.

Immunocytochemistry plating and staining

For immunofluorescence analysis, young and old equine chondrocyte cells were plated on 22 mm x 22 mm glass coverslips previously sterilised with 100% ethanol. 5 – 10,000 cells in 400 µl culture medium was plated per coverslip and left overnight to attach. Attachment density was checked using light microscopy and coverslips were washed with PBS to remove culture medium. Cells were then fixed in 1:1 ice cold acetone:methanol (Sigma-Aldrich, UK) for 5 minutes. Six coverslips were plated per sample.

Nucleolar protein B23 was localised by incubating coverslips in 150 µl of 1 µl/ml (1 in 500 dilution) monoclonal mouse B23 antibodies B0556 (Sigma-Aldrich, UK) for 1 hour at RT. B23 antibody was then aspirated and coverslips were washed 3 times in PBS for 5 minutes. Cells were then incubated in 180 µl (1 in 400 dilution) Alexafluor 488 Mouse IgG1 labelling reagent (ThermoFisher Scientific) for 1 hour in the dark (18).

Coverslips were rinsed once with PBS for 5 minutes before being mounted with 1 drop (approx. 25 µl) of Vectashield mounting medium with 4', 6'-diamidino-2-phenylindole (DAPI) counterstain (Vector Laboratories) as per manufacturer's instructions. Staining was visualised at room temperature by fluorescent microscopy using a Zeiss Axioimager 2 microscope. Images were captured using an Axiocam HR camera.

Nucleolar protein B23 was localised using mouse monoclonal B23 antibodies B0556 (Sigma, USA) alongside an Alexaflour 488 Mouse IgG labelling kit, specifically the IgG1 labelling reagent (ThermoFisher Scientific) as per (18). Coverslips were mounted using Vectashield mounting medium with 4', 6'-diamidino-2-phenylindole (DAPI) counterstain (Vector laboratories) per manufacturer's instructions. Staining was visualised at room temperature by fluorescent microscopy (Zeiss Axioimager 2). Images were captured using an AxioCam HR camera.

Nucleoli counting

Number of nucleoli per nucleolus were counted for 100 cells by first visualising the DAPI stained nuclei with a blue fluorescent filter and then the B23 stain under the green fluorescent filter of the Zeiss microscope. Once cells were localised, they were counted in a battlement pattern until 100 cells containing nucleoli had been counted.

Six slides were produced and visualised for each sample and each sample was considered of adequate quality for analysis if three correctly stained slides were obtained.

Number of nucleoli was counted in n=4 young and n=3 old samples. Area and circularity index were evaluated from n=106 young and n=98 old nucleoli obtained from n=4 young and n=3 old samples.

Image Analysis and Statistical Analysis

Out of the n=9 young and n=8 old samples, only n=4 young and n=3 old samples were considered of a sufficient quality (Table 1). When comparing young versus old age groups using a T-test, they were considered significantly different ages with $P<0.05$ (Figure 1).

Images were analysed in Photoshop™ (Adobe, UK) using the lasso tool to select nucleoli for area measurement and circularity analysis (29). Measurements were recorded in pixels and later converted to micrometres.

NPM1 expression in ageing chondrocytes

As NPM1 participates in ribosome biogenesis (30) we decided to also measure its expression in ageing equine chondrocytes. RNA was extracted from equine chondrocytes using the Trizol method and converted to cDNA as previously described. Relative expression levels were normalised to GAPDH and calculated using the $2^{\Delta\Delta Ct}$ method (31).

Statistical Analysis

Nucleosome data was tested for normality in GraphPad Prism (GraphPad Software, USA) and found to be non-parametric and could not be normalised. A Mann-Whitney U test was chosen (32) and was performed on area (µm^2) and circularity index values in addition to nucleoli counts to assess if values were significantly different in the old versus young samples with $P<0.05$. For gene expression data was Mann-Whitney U test was undertaken in GraphPad Prism.

Results

As per Belin *et al.* (18), detection of the B23 signal can be seen in Figure 2. The nucleus is revealed by DAPI staining. Overall area of nucleoli and circularity index results indicated that the morphology of cells was not significantly different. There was a significant increase in the number of nucleoli per nucleus in the ageing.

Area

A Mann-Whitney U test of area values for the young and old sample groups (Figure 3A) indicated no significant difference in nucleoli size between the groups ($P=0.9957$). The average area of nucleoli was 1.65 µm (±SEM 0.21) for young samples and 1.82 µm (±SEM 0.18) for old samples (Table 2).

Circularity index

Testing for circularity index for young and old sample groups (Figure 3B) indicated no significant difference in

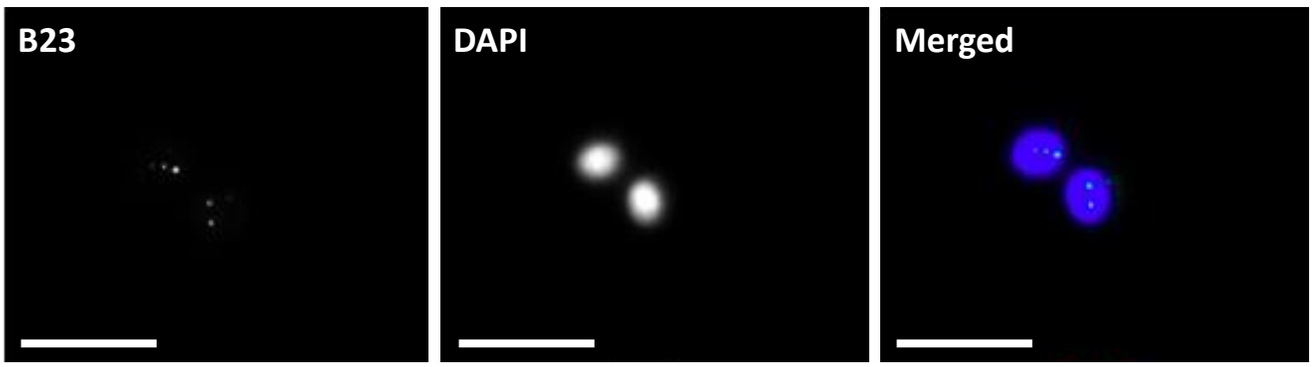


Figure 2. Immunocytochemistry Images. Images showing detection of B23 signal (left), the nucleolus revealed by DAPI staining (centre) and the merged image (right). 5 µm marker lines are displayed for scale.

nucleoli size between the groups (P=0.5383). The average circularity index of nucleoli was 0.81 (SEM=0.0094) for young samples and 0.80 (SEM=0.011) for old samples (Table 2).

Number of nucleoli

A Mann-Whitney U test of the number of nucleoli per nucleus (Figure 3C) showed a significantly higher number of nucleoli per nucleus in old samples versus young samples (P=0.042). The average number of nucleoli per nucleus was 1.69 (SEM=0.034) for young and 1.81 (SEM=0.042) for old samples (Table 2).

NPM1 gene expression

There was a significant reduction in NPM1 expression in ageing chondrocytes (p=0.03) (Figure 4).

Discussion

The size, morphology, number and structural organisation of nucleoli are considered potent indicators of nucleolar activity. The aim of this study was to characterise the nucleoli in ageing chondrocytes by measuring area, circularity index and number of nucleoli per nucleolus. Nucleoli were visualised by immunofluorescence experiments using antibodies against nucleolar protein B23 (Figure 2). Additionally, we measured the expression of NPM1 which was shown to be over expressed in actively proliferating cells.

Figure 3 shows that whilst the area and circularity index of nucleoli did not vary with ageing the number of nucleoli per nucleolus was significantly higher in old samples compared to young samples. Recent studies have shown that changes to translational apparatus are prominent in early stage OA, persisting through to the later stages (8).

Initially, Image J (NIH, USA) was used to quantify the area of nucleoli in cells as per Belin *et al.* (18) however due to inaccurate thresholding, it was not possible to accurately select individual nucleoli to measure area and circularity. However, using Photoshop™ meant that images did not require thresholding and it was easier to distinguish pixels that were to be counted as 'nucleoli'. In addition, area measurements were faster as multiple nucleoli in an image could be measured individually and exported to excel. Limitations to this method were due to hand selection of nucleoli rather than using an algorithm. We counteracted this by having one person analyse all images using the same equipment (33). All images were of the same magnification and size in pixels therefore although measurement bias may exist, it is consistent throughout. Due to the user bias of this method, it would be beneficial to measure area and circularity values twice or have two people separately measure area and circularity and use a Cappa Kohen statistic to measure interrater or intrarater reliability respectively (34).

Others have described morphological changes in nucleoli such as increasing size with age (35, 36), and multiple studies have documented morphological change in older specimens (18, 37). Our results showed no significant difference with age. Due to a lack of useable samples, analysis was made on a relatively small data set and increasing the number of samples plus the addition of OA samples would have been beneficial.

Others have shown a link between age and expression of the NPM1 gene resulting in changing morphology of nucleophosmin due to differences in protein-RNA interactions (38, 39). We therefore also measured NPM1 expression and found it was reduced in ageing. As NPM1 is involved in several cellular processes including ribosome biogenesis, nucleocytoplasmic transport and transcriptional regulation, its reduced expression in ageing cells could be indicative of its role in the regulation of cell metabolism (40). We would expect however that with reduced NPM1 expression with age, there would be fewer nucleoli in older cells however this was not the case in this study.

NPM1 gene which codes for B23/Nucleophosmin is a driver of conformational changes in the nucleoli (39). As NPM1 expression was shown to be significantly decreased in old samples, we would expect that B23 would therefore be less abundant in those samples and that morphology would be affected, however, it was not found in this study.

There was an increase in the number of nucleoli per nucleus in the older cell samples. An explanation for this may be due to the rapidly assembly and disassembly of nucleoli inside the nucleus in response to changes in transcriptional activity, which is critical for cellular integrity and homeostasis. The creation and structural integrity of nucleoli depends exclusively on protein-protein,

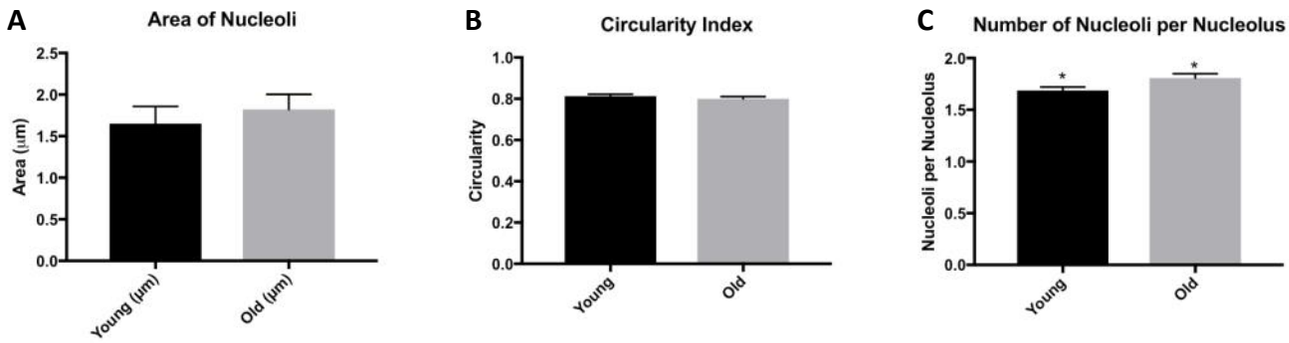


Figure 3. Graphs of Area, Circularity Index and Number of Nucleoli per Nucleolus. We measured the number & morphology of nucleoli in young and old chondrocytes using signal detection of staining by Photoshop™. The area (A), the circularity index (B) and the nucleoli were evaluated per 100 cells from each donor using the n=4 young and n=3 old mean±SEM ages in years, young 5±1.08, old 16.6±0.67. These analyses showed that there were no statistically significant differences between circularity index or nucleoli area in ageing. Quantitation of the number of nucleoli per cell, evaluated in 100 cells per donor (C) demonstrated that the number of nucleoli varied from 1 to 4 per chondrocytes and increased by 10% in ageing chondrocytes (P=0.004). Altogether, these results suggested that during chondrocyte ageing, the nucleus may undergo adaptive changes to support changing nucleolar activity.

protein-RNA and/or DNA interactions and NPM1 coding for nucleophosmin protein is a driver of conformational changes upon RNA binding (39). Theoretically, if changes in NPM1 expression are present in cartilage due to ageing (41), this will in turn affect the assembly/disassembly of nucleoli in chondrocytes. This study found that NPM1 was decreased with age and that more nucleoli were assembled in old samples. There was however no significant difference in morphology of cells due to the changing NPM1 expression.

Buchwalter and Hetzer (35) showed that increased ribosome biogenesis and activity are a hallmark of premature ageing, therefore we can hypothesise that in the cartilage increase number of nucleoli may indicate increased ribosomal biogenesis and that this may be indicative of ageing or degeneration. Another measurement to consider for future analysis would be to measure the overall area of all nucleoli per 100 cells and then compare if there is an increased overall area of nucleoli to compensate for the less abundant nucleoli in young samples.

Chondrocytes are known to require a high translational capacity to undertake anabolic processes (15). Ageing and degenerative diseases of ageing such as OA are known to be associated with altered chondrocyte gene expression and thus morphological changes (9). These changes in morphology influence chondrocyte metabolism (42). In this study, we studied the nucleosome characteristics of young and old equine chondrocytes as an indicator of translational capacity by visualising nucleoli by immunofluorescence of nucleolar protein B23. Thus, we could use this information as an indicator of their translational capacity and ability to maintain cartilage homeostasis (42) in ageing

We found there to be no morphological variation (in area and circularity index) between young and old chondrocytes, contrary to our hypothesis that morphology would vary with age (43). We did however, find there to be a larger number of nucleoli present in old samples. Future studies are required in OA chondrocytes to ascertain if they behave similarly.

Table 2. Measurements for area, circularity index and number of nucleoli per nucleolus. ns meaning P>0.05 and * meaning P≤0.05.

Area	Young	Old	Old vs. Young
Mean	1.651	1.822	
SEM	0.206	0.1821	
P-Value			0.0975
Significance			ns

Circularity Index	Young	Old	Old vs. Young
Mean	0.8119	0.7992	
SEM	0.0094	0.01106	
P-Value			0.5383
Significance			ns

No. Nucleoli	Young	Old	Old vs. Young
Mean	1.687	1.807	
SEM	0.0339	0.04207	
P-Value			0.0419
Significance			*

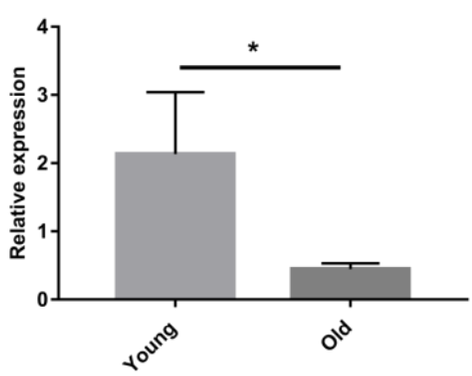


Figure 4. Relative gene expression of NPM1 in ageing equine chondrocytes. Data are represented as the relative expression compared to GAPDH of young n=9 and old n=8 donors. Histograms represent means ±SEM (* = P<0.05).

Conclusion

Ageing and OA have known associations with altered chondrocyte gene expression and an imbalance between protein anabolism and catabolism. Altered nucleoli number and NPM1 expression may have a role in age-related OA through changing ribosomal translational capacity. This study needs to be replicated in OA equine chondrocytes to determine these parameters in disease

Acknowledgements

I would like to thank Dr Mandy Peffers for giving me the opportunity to undertake this work and supervising me throughout the duration of this project.

References

1. Woolf A D and Pfleger B. Burden of major musculoskeletal conditions. Bull World Health Organ 2003. 81(9):646-656

2. Li Y, Tavallaee G, Tokar T and Nakamura A *et al.* Identification of synovial fluid microRNA signature in knee osteoarthritis: differentiating early-and late-stage knee osteoarthritis. Osteoarthritis and Cartilage 2016. 24(9):1577-1586

3. Goldring M B and Otero M. Inflammation in osteoarthritis. Curr Opin Rheumatol 2011. 23(5):471-478

4. Yang J and Wang N. Genome-wide expression and methylation profiles reveal candidate genes and biological processes underlying synovial inflammatory tissue of patients with osteoarthritis. International journal of rheumatic diseases 2015. 18(7):783-790

5. Loeser R F. Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. Osteoarthritis and Cartilage 2009. 17(8):971-979

6. Nugent M. MicroRNAs: exploring new horizons in osteoarthritis. Osteoarthritis and Cartilage 2016. 24(4):573-580

7. Steinbusch M M, Fang Y and Milner P I *et al.* Serum snoRNAs as biomarkers for joint ageing and post traumatic osteoarthritis. Scientific Reports 2017. 7:43558

8. Katsara O, Attur M, Ruoff R, Abramson S B and Kolupaeva V. Increased activity of the chondrocyte translational apparatus accompanies osteoarthritic changes in human and rodent knee cartilage. Arthritis & Rheumatology 2017. 69(3):586-597

9. Reginato A M and Olsen B R. The role of structural genes in the pathogenesis of osteoarthritic disorders. Arthritis Research & Therapy 2002. 4(6):337

10. Cartilage proteoglycans. Seminars in cell & developmental biology: Elsevier; 2001

11. Sandell L J and Aigner T. Articular cartilage and changes in arthritis: cell biology of osteoarthritis. Arthritis Research & Therapy 2001. 3(2):107

12. Goldring M B. The role of the chondrocyte in osteoarthritis. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology 2000. 43(9):1916-1926

13. Sloan K E, Warda A S and Sharma S *et al.* Tuning the ribosome: The influence of rRNA modification on eukaryotic ribosome biogenesis and function. RNA biology 2017. 14 (9):1138-1152

14. Kim H, Kang D, Cho Y and Kim J. Epigenetic regulation of chondrocyte catabolism and anabolism in osteoarthritis. Mol Cells 2015. 38(8):677

15. Trainor P A and Merrill A E. Ribosome biogenesis in skeletal development and the pathogenesis of skeletal disorders. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 2014. 1842(6):769-778

16. Lafontaine D L and Tollervey D. The function and synthesis of ribosomes. Nature Reviews Molecular Cell Biology 2001. 2(7):514

17. Gardner P P, Bateman A, and Poole AM. SnoPatrol: how many snoRNA genes are there? Journal of biology 2010. 9(1):4

18. Belin S, Beghin A and Solano-Gonzàlez E *et al.* Dysregulation of ribosome biogenesis and translational capacity is associated with tumor progression of human breast cancer cells. PLOS ONE 2009. 4(9):e7147

19. Egger G and Arimondo P. Drug Discovery in Cancer Epigenetics. Academic Press; 2015.

20. Montanaro L, Trerè D and Derenzini M. Nucleolus, ribosomes, and cancer. The American journal of pathology 2008. 173(2):301-310

21. Okugawa Y, Toiyama Y and Toden S *et al.* Clinical significance of SNORA42 as an oncogene and a prognostic biomarker in colorectal cancer. Gut 2017. 66(1):107-117

22. Peffers M J, Ripmeester E and Caron M *et al.* A role for the snoRNA U3 in the altered translational capacity of ageing and osteoarthritic chondrocytes. Osteoarthritis and Cartilage 2018. 26:S46

23. Peffers M J, Balaska P, Dyers P, Welting T and Cremers A. A role for small nucleolar RNA host genes in cartilage ageing and osteoarthritis. Osteoarthritis and Cartilage 2018. 26:S161

24. Lindström M S. NPM1/B23: a multifunctional chaperone in ribosome biogenesis and chromatin remodeling. Biochemistry research international 2011. 2011

25. Mitrea D M, Cika J A and Guy C S *et al.* Nucleophosmin integrates within the nucleolus via multi-modal interactions with proteins displaying R-rich linear motifs and rRNA. Elife 2016. 5e13571

26. Hollands C. The animals (scientific procedures) act 1986. The Lancet 1986. 328 (8497):32-33

27. Tew S R, Murdoch A D, Rauchenberg R P and Hardingham T E. Cellular methods in cartilage research: primary human chondrocytes in culture and chondrogenesis in human bone marrow stem cells. Methods 2008. 45(1):2-9

28. Tew S R, Peffers M J and McKay T R *et al.* Hyperosmolarity regulates SOX9 mRNA posttranscriptionally in human articular chondrocytes. American Journal of Physiology-Cell Physiology 2009. 297(4):C906

29. Agley C C, Velloso C P, Lazarus N R and Harridge S D. An image analysis method for the precise selection and quantitation of fluorescently labelled cellular constituents: application to the measurement of human muscle cells in culture. Journal of Histochemistry & Cytochemistry 2012. 60(6):428-438

30. Peffers M J, Milner P I, Tew S R and Clegg P D. Regulation of SOX9 in normal and osteoarthritic equine articular chondrocytes by hyperosmotic loading. Osteoarthritis and cartilage 2010. 18(11):1502-1508

31. Livak K J and Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 2001. 25(4):402-408

32. Parab S and Bhalerao S. Choosing statistical test. International journal of Ayurveda research 2010. 1(3):187

33. Burton-Jones A. Minimizing method bias through programmatic research. Mis Quarterly 2009. 445-471

34. McHugh M L. Interrater reliability: the kappa statistic. Biochemia medica: Biochemia medica 2012. 22(3):276-282

35. Buchwalter A and Hetzer M W. Nucleolar expansion and elevated protein translation in premature aging. Nature communications 2017. 8(1):328

36. Tiku V, Jain C and Raz Y *et al.* Small nucleoli are a cellular hallmark of longevity. Nature Communications 2017. 8:16083

37. Guarente L. Link between aging and the nucleolus. Genes Dev 1997. 11(19):2449-2455

38. Sahoo T, del Gaudio D and German J R *et al.* Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster. Nat Genet 2008. 40(6):719

39. Németh A and Grummt I. Dynamic regulation of nucleolar architecture. Curr Opin Cell Biol 2018. 52:105-111

40. Zhu Y, Shi M and Chen H *et al.* NPM1 activates metabolic changes by inhibiting FBP1 while promoting the tumorigenicity of pancreatic cancer cells. Oncotarget 2015. 6 (25):21443

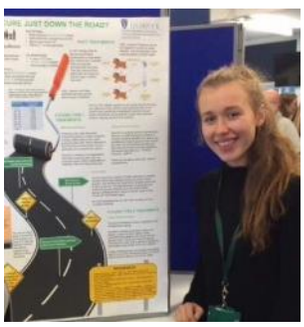
41. Lee N, Kim D and Kim E *et al.* Comparative interactomes of SIRT6 and SIRT7: Implication of functional links to aging. Proteomics 2014. 14(13-14):1610-1622

42. Ingber D E, Dike L and Hansen L *et al.* Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulate cell growth, migration, and tissue pattern during morphogenesis. International review of cytology. Elsevier: 1994. P. 173-224

43. Peffers M J, Liu X and Clegg P D. Transcriptomic signatures in cartilage ageing. Arthritis research & therapy 2013. 15(4):R98

Development of a high-throughput mass spectrometry-based method for the detection and localisation of protein tyrosine sulfation, in proteins extracted from human cell lysate

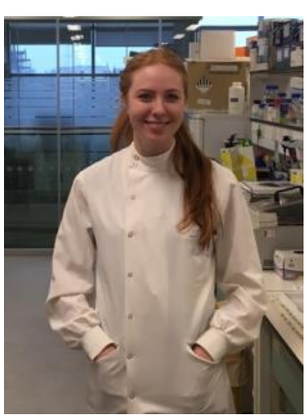
Anna Birrell, BSc Biological & Medical Sciences Supervisor: Prof Claire Eyers



Cells have extensive regulatory flexibility that enable them to survive dynamic environmental changes. Their ability to sense and adapt to extracellular signals is fundamental to the maintenance of their homeostasis. Whilst modulation of gene expression plays a key role, this mechanism alone is too slow. Cells therefore rely on additional switches to ensure fast and reversible regulation. Protein post-translational modifications (PTMs) fulfil this requirement. Their biological importance is reflected in the large number of corresponding regulatory enzymes encoded in the human genome, performing over 200 types of modifications. Whilst the best-studied PTM is phosphorylation, there has been recent interest in tyrosine sulfation, a less common yet biologically significant modification. It is believed that sulfation may be necessary for the bioactivation of many secreted and membrane-bound proteins, perhaps critical for chemokine signalling and hemostasis. Despite this, the extent of this PTM remains unclear, most likely due to there being no conventional high-throughput method to distinguish phosphate groups from sulfate. With a mass difference of only 0.009 daltons, it is possible that tyrosine sulfation is being mistaken for phosphorylation. Since targeting protein sulfation pathways could offer potential strategies to treat inflammatory diseases and steroid hormone-dependent cancers, this less understood modification is rightfully attracting attention in proteomic research. The aim of this project is to develop a high-throughput mass spectrometry-based method that will successfully detect and localise tyrosine sulfation, in proteins extracted from human cell lysate. Until an optimised approach is found, the complex sulfoproteome will remain unexplored. So with some luck, this project will provide the essential foundation for many future studies.

Glutathione transferase responses to ivermectin in *Haemonchus contortus*

Shannan Summers, BSc Tropical Disease Biology Supervisor: Dr James LaCourse



The most economically important gastrointestinal nematode of sheep is *Haemonchus contortus* (the Barber's Pole Worm). This is a parasitic nematode which can cause severe clinical manifestations in the sheep host. Symptoms can include severe anaemia and 'bottle jaw', an accumulation of fluid under the jaw, whilst sheep suffering from high intensity infections of the parasite can succumb and die. It has become increasingly difficult to control infections of *H. contortus* within the global sheep industry due to the rapid development of widespread anthelmintic resistance against multiple drug classes. There are concerns that the worldwide resistance observed against the macrocyclic lactone ivermectin in particular may significantly threaten the feasibility of the sheep industry. Ivermectin is also widely used to treat a range of human parasitic infections including lymphatic filariasis and concerns over resistance in these worms are also reported. However, the mechanisms for anthelmintic drug resistance are poorly understood and prior studies have focused on a range of potential mechanisms and drug targets. Amongst these potential mechanisms are the glutathione transferases (GSTs), a superfamily of detoxification enzymes which may play a role in aiding nematode survival against anthelmintic drugs. However, little is known of the biochemical profile of GSTs in *H. contortus*. This project aims to biochemically characterise the GSTs present in *H. contortus* and establish the potential role GSTs may play in resistance mechanisms. Thus, the findings from this investigation can be applied to combat the inevitable development of resistance in parasitic nematodes infecting both livestock and humans.

Do some bird species receive more aggression than others in a multi-species enclosure?

Kate Smith, BSc Zoology Supervisor: Dr Carl Larsen

Captive breeding is a form of conservation that is performed by many organisations worldwide, from zoos and aquariums, to wetland centres like the Wildlife and Wetland Trust (WWT) in Martin Mere. Captive breeding aims to create a stable and healthy population of the endangered species in question in order to reintroduce the species back into their natural habitat (1). A form of captive breeding is a mixed species enclosure, like that within the aviary at Martin Mere where this project will take place. The species enclosed include: Crowned crane (*Balearica regulorum),* Avocet (*Recurvirostra avosetta),* White-faced whistling duck (*Dendrocygna viduata)* and, Comb duck (*Sarkidiornis melanotos).* Multi-species enclosures are common and often encouraged to provide a more dynamic and enriching environment. They often display species sharing similar ecological or geographical themes (2). Snyder *et al.* (3) rightly stated that those who have captive breeding programmes should operate to prevent disease and have genetic and behavioural management. This project will allow for the aggression behaviour of the species kept within the aviary to be monitored. Overall, the data collected will be used to further manage their enclosure, to provide the best conditions and welfare for the birds involved. This is important as space in such organisations is becoming limited. Therefore, by maintaining the welfare within this enclosure, space can be maintained and there can be an increase in conservation return on space and infrastructure by keeping multiple species in one enclosure and not separate.

References

1. National Geographic Society. Introduction to Captive Breeding. Available from: https://www.nationalgeographic.org/activity/introduction-captive-breeding/ [Accessed 5th Dec. 2018]
2. Campbell-Palmer R and Rosell F. Captive care and welfare considerations for beavers. Zoo Biology 2015. 34(2):101-109
3. Snyder N, Derrickson S and Beissinger S *et al.* Limitations of Captive Breeding in Endangered Species Recovery. Conservation Biology 1996. 10 (2):338-348

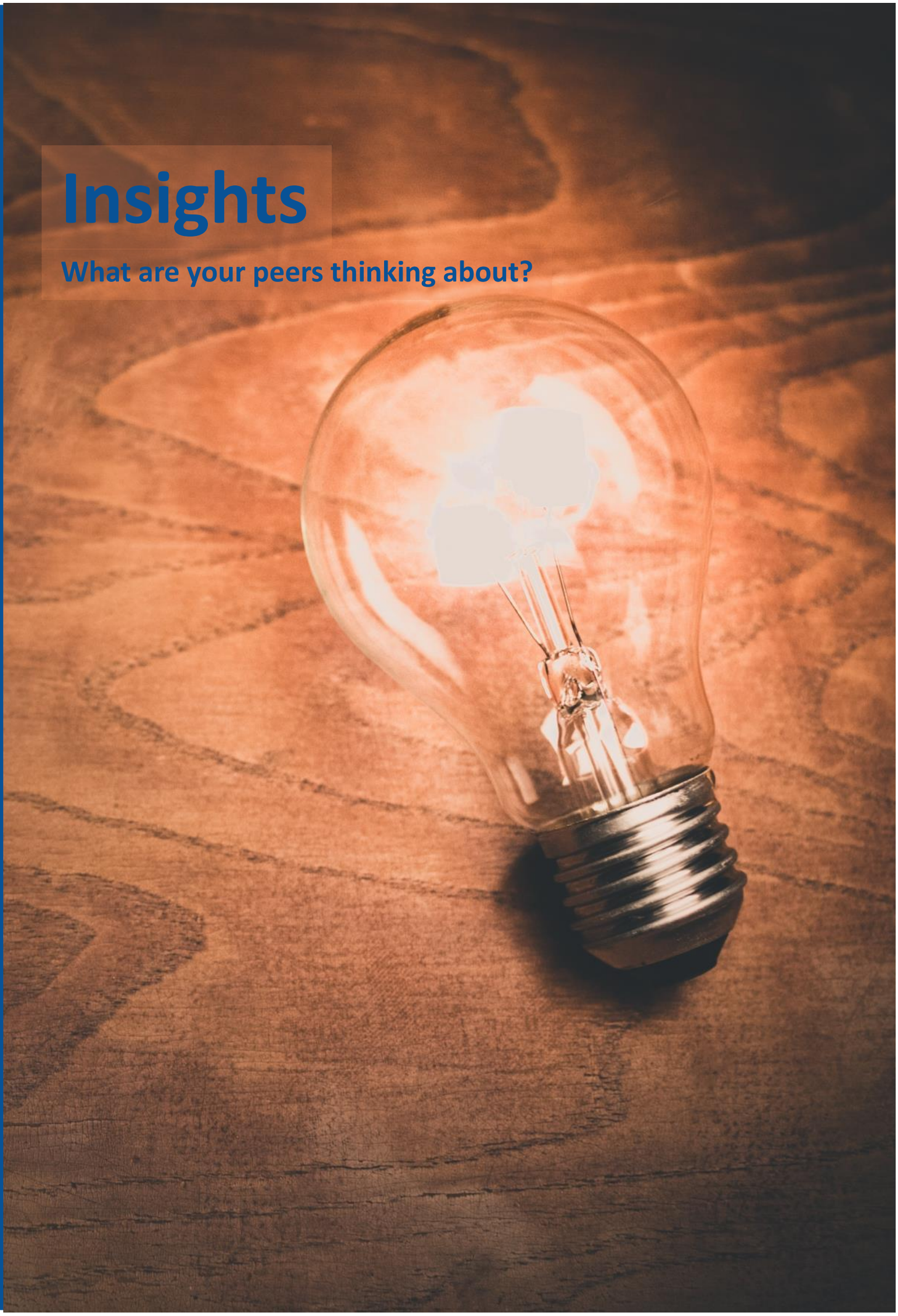
The effect of nitisinone on mosquito survival

Lucy Wust, BSc Tropical Disease Biology Supervisor: Dr Alvaro Acosta Serrano

Mosquito-borne diseases, such as malaria and dengue, continue to be one of the leading causes of mortality across the tropical regions. Reducing these high mortality rates is focused primarily on vector control, highlighting the importance of developing novel control methods that will effectively reduce the population of mosquitoes, thus reducing the transmission rate of these deadly diseases and the devastating social and economic burdens which follow. My project investigates a novel mosquito control method that has had minimal research to date. I will be investigating the effect of the drug, nitisinone, on the tyrosine degradation pathway and determining whether this affects the survival of mosquitoes. Tyrosine is an essential amino acid but is toxic when present in high concentrations, which is shown in the human genetic diseases, Tyrosinemia type I and Alkaptonuria. Female mosquitoes must take blood meals to ensure their reproductive success and survival. When a blood meal is digested, toxic concentrations of tyrosine are produced in the mosquito, however, they can detoxify the excess tyrosine. We will be investigating the optimal concentration of nitisinone, which is an inhibitor of an enzyme used in the tyrosine degradation pathway; and determining if it causes tyrosine to accumulate to a toxic concentration, thus killing the mosquito. The rise of insecticide resistance makes this investigation imperative as novel control methods must be found to keep the incidence rates of these diseases low, prevent epidemics, and allow time for effective drugs and vaccinations to be developed, which present more long-term solutions.

Insights

What are your peers thinking about?



The benefits of probiotics are outweighed by faeces

Jessica Buddle, 3rd Year Microbiology BSc

It has long since been established that the microbiome – the various microbial species inhabiting the body – and its diversity is paramount for human health. Studies have repeatedly demonstrated antibiotic impact on gut dysbiosis (imbalance), causing altered microbial community diversity and metabolism (1). Antibiotics reduce colonisation resistance; the phenomenon of microbiome colonisers preventing establishment of opportunistic pathogens and increase the risk of antibiotic-associated diarrhoea (2). More recently, the use of probiotics – beneficial bacteria which claim to enhance wellbeing – have become widely acknowledged as a treatment for antibiotic-associated dysbiosis (3,4). Such treatments include probiotics (available in yoghurts or over-the-counter supplements); or autologous faecal microbiome transplantation (aFMT), which refers to the provision of one's pre-dysbiosis faeces (containing a healthy microbiome) to reduce dysbiosis. Suez *et al.* (5) present data suggesting probiotics actually delay reconstitution of both stool and mucosal native microbiomes, and do not restore the species diversity important for health (5).

The therapeutic aim of any probiotic is to restore the gut to a pre-antibiotic state of diversity (6). Since research may under report adverse effects, efficacy of treatment is relatively unknown. Additionally, much probiotic research focuses on animal models and stool samples as a representation of the gut microbiome. Suez *et al.* (5) investigated colonisation of probiotics (using faecal and mucosal surface samples) in both mice and humans. Their data show that aFMT restores mucosal microbiome and gut transcriptome reconstitution. On the other hand, probiotics delay gut microbiome and transcriptome reconstitution.

Suez *et al*. (5) first investigated colonisation of probiotic bacteria following antibiotic treatment in mice and humans. In mice, they found a milder colonisation of probiotic bacteria, suggesting antibiotics only somewhat decrease colonisation resistance. In humans however, a significant decrease in colonisation resistance was observed. Probiotic species, particularly *Bifidobacterium spp.,* were elevated in stools, and shedding still occurred 5 months post-probiotic treatment. This finding raises questions regarding the effectiveness of animal models for microbiome study. Notably, person-specific variation in response to probiotic treatment was also found, suggesting specific microbiome compositions and host factors also influence colonisation resistance.

As shown previously, the authors found antibiotic consumption led to decreased α-diversity – species diversity within the microbiome. In mice, aFMT was the best treatment for recovering species richness (number), with diversity being indistinguishable from non-antibiotic controls 8 days after treatment, and microbiome composition returning to normal within 4 weeks. This suggests aFMT can successfully reconstitute faecal, lower and upper gastrointestinal tract native microbiomes; adding to the accumulating body of evidence in favour of FMT. This was not the case for spontaneous recovery, or indeed probiotic treatment. The authors found probiotic treatment failed to restore α-diversity. Significantly, probiotics were associated with the slowest recovery rate, potentially due to 5 taxa which bloomed in the microbiome inhibiting reconstitution.

Through endoscopies, the authors revealed the impact of probiotic treatments on human microbiome reconstitution. aFMT was again the best treatment, showing the fastest restoration of native microbiome species. Comparing pre-antibiotic and post-treatment microbiomes, the authors found the smallest species diversity difference was achieved using aFMT. Surprisingly, spontaneous recovery allowed reconstitution within 21 days. The authors found probiotic treatment prevented reconstitution, and dysbiosis was maintained for over 5 months. α-diversity was low for this group, attributed to the blooming of 4 probiotic genera (*Enterococcus, Akkermansia, Bifidobacterium* and *Blautia)*, which also inhibited native genera such as *Clostridiales.* This suggested that probiotics, despite colonising well in the post-antibiotic gut, delayed return to normal microbiome diversity.

Suez *et al*. (5) went on to investigate the metatranscriptome – all genes expressed in the cells of the gastrointestinal tract – in humans, to determine the impact of treatments on the host. Interestingly, both aFMT and spontaneous recovery lead to

reversion toward normal gene expression profiles. Probiotics increased expression of antimicrobial and inflammatory genes, suggesting they negatively impact both the host and its microbiome.

Suez *et al*. (5) provide key insights into the post-antibiotic microbiome, regarding colonisation tendencies and host gene expression effects. aFMT shows rapid microbiome reconstitution, reversion to normal host gene expression, and protection against opportunistic pathogens. However, whilst probiotic bacteria colonise well, they alter the microbiome composition and function, ultimately prolonging dysbiosis. Since only one antibiotic treatment and one probiotic species mixture was used, the generalisability of these data remains unknown. Importantly, risk-group populations (such as immunocompromised individuals and the elderly) likely to develop opportunistic infections following antibiotic treatment are likely to benefit most from aFMT (7). Such groups are not assessed in this study, meriting further research in the context of disease.

Looking forward, the success of aFMT demonstrated by Suez *et al.* (5) may give rise to its widespread use as an antibiotic adjuvant; however, this is likely to be technically challenging and unsustainable economically. Future research concerning a "personalised probiotic" approach, whereby patients receive an individualised probiotic combination, may be more feasible. This would reduce antibiotic-associated dysbiosis, whilst minimising the delayed reconstitution effects of current indiscriminate probiotics.

References

1. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci USA 2011. 108 (Suppl. 1):4554–61

2. Lewis B B, Buffie C G, Carter R A *et al*. Loss of Microbiota-Mediated Colonization Resistance to *Clostridium difficile* Infection With Oral Vancomycin Compared With Metronidazole. J Infect Disease 2015. 212 (10):1656–65

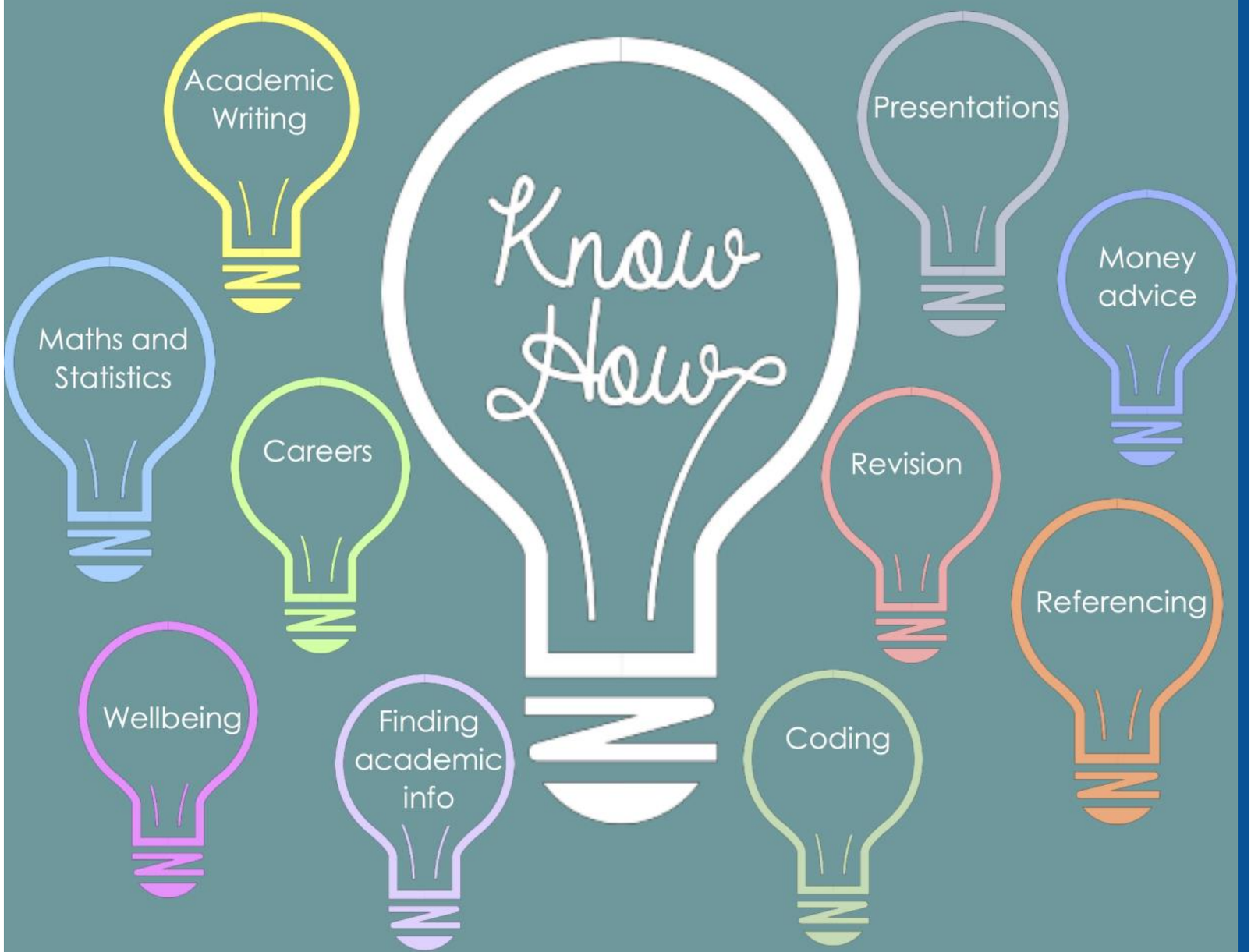
3. Zoppi G, Cinquetti M, Benini A, Bonamini E, Minelli E B. Modulation of the intestinal ecosystem by probiotics and lactulose in children during treatment with ceftriaxone. Current Therapeutic Research 2001. 62 (5):418–35

4. Islam S U. Medicine (Baltimore): Clinical Uses of Probiotics. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4 748908 [Accessed October 2018]

5. Suez J, Zmora N, Zilberman-Schapira G *et al.* Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by Probiotics and Improved by Autologous FMT. Cell 2018. 174(6):1406–1423.e16

6. Korpela K, Salonen A, Vepsäläinen O *et al.* Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. Microbiome 2018. 6(1):182

7. Moayyedi P, Surette M G, Kim P T *et al.* Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. Gastroenterology 2015. 149(1):102–109.e6



Come along to one of our **workshops** – find out what is coming up and book your place at https://libguides.liverpool.ac.uk/knowhow

Explore our **online resources** in our VITAL module KnowHow Academic Success

knowhow@liverpool.ac.uk @livuniknowhow

CRISPR: The new genetic modification machine

Luisa Silva, 3rd Year Genetics BSc

The native CRISPR/Cas9 system

CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats) were first identified in *E.coli* bacteria, where they played a role in eliminating exogenous genetic material such as viral DNA (1). They consist of small fragments of identical interspaced DNA repeats. Each of these repeats is adjacent to a spacer DNA (non-repetitive region) which is a sequence that has been inserted due to a previous exposure to exogenous DNA (2). Additionally, there are other genes associated with CRISPR, termed CRISPR-associated genes (Cas). These genes generate Cas proteins which carry helicase and nuclease activities, allowing them to unwind and cut DNA, respectively. Transcription of CRISPR will result in the formation of CRISPR RNAs (crRNAs) which have the ability to recognise and bind, for example, viral DNA and act as guides for Cas endonucleases to cleave and remove the exogenous DNA (2).

Exploiting CRISPR/Cas9

CRISPR-mediated genome editing exploits the CRISPR/Cas9 system, a complex delivered into the cell's genome comprised of Cas9 endonucleases (made up of HNH endonuclease and RuvC-like endonuclease) and a synthetic RNA (gRNA) to guide the Cas9 endonuclease complex (2). crRNA associates with trans-activating crRNA (tracrRNA) to form a gRNA complex which guides Cas9 to the target locus. In order to achieve sequence-specific cleavage, crRNA must complement the protospacer sequence (target sequence) and the PAM (protospacer adjacent motif) sequence (1). When bound to the target site, both DNA strands are cleaved by HNH and RuvC-like endonucleases (Figure 1). This creates a double-strand break which can be repaired by the cell's DNA repair mechanisms. Here, a DNA sequence may be incorporated or deleted, in order to repair defective genes which cause disease; to regulate transcription of a certain gene; or to correct DNA repair mistakes which cause deletions or point mutations (2).

Recent applications of the CRISPR/Cas9 system

The CRISPR/Cas9 system shows major promise as an anti-viral therapy, assisting humans to eliminate exogenous DNA introduced into host cells by a foreign viral pathogen. Human Immunodeficiency Virus (HIV) infection, still a prevalent health condition worldwide, was investigated using this approach (4). HIV is a pathogen which infects and kills cells of the immune system, particularly CD4+ helper T cells (immune cells which help to fight off infections) (5). In 2016, scientists were able to delete entire copies of the *HIV-1* gene from *HIV-1* infected primary cultured human CD4+ T-lymphoid cells using the CRISPR/Cas9 system (4). Repression of viral replication was achieved in peripheral blood mononuclear cells (PBMCs) and also in some latently infected T cells (4). Kaminski *et al.* (4) also observed that *HIV-1* gene deleted T cells, as long as Cas9 and RNA-guided CRISPR were present, showed resistance to new HIV infections. This gene excision did not cause any disruption to cell viability and the cell cycle, did not cause any off-target side effects or disrupt any local or distant genes (4). This experiment provided evidence that, in the future, this system could be used as therapy for HIV-1 patients if safe delivery mechanisms can be formulated.

The CRISPR/Cas9 editing system may be

References

1. Rath D, Amlinger L, Rath, A and Lundgren, M. The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie 2015. 117(1):119-128

2. Zhang F, Wen Y and Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. Human Molecular Genetics 2014. 23 (1):40-46

3. Peng R, Lin G and Li J. Potential pitfalls of CRISPR/Cas9-mediated genome editing. The FEBS Journal 2015. 283(7):1218-1231.

4. Kaminski R, Chen I, Fischer T *et al*. Elimination of HIV-1 Genomes from Human T-lymphoid Cells by CRISPR/Cas9 Gene Editing. Scientific Reports 2016. 6(1):*22555*

5. Web M D. HIV (Human Immunodeficiency Virus) Infection – Topic Overview. Available from: https://www.webmd.com/hiv-aids/tc/human-immunodeficiency-virus-hiv-infection-topic-overview#1 [Accessed: 20 October 2018]

6. Flynn R, Grundmann A and Renz P *et al*. CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells. Experimental Hematology 2016. 43 (10):838-848.

7. Cox, D B T, Platt R J and Zhang F. Therapeutic Genome Editing: prospects and challenges. Nature Medicine 2015. 21(1):121-131

8. Sigma-Aldrich. CRISPR Cas9 Nuclease RNA-guided Genome Editing. Available from: https://www.sigmaaldrich.com/technical-documents/articles/biology/crispr-cas9-genome-editing.html [Accessed: 28 October 2018]

9. Rodriguez E. Ethical Issues in Genome Editing using Crispr/Cas9 System. Journal of Clinical Research & Bioethics 2016. 7(2):1000266

10. Fox M. Chinese researcher says he is 'proud' of gene-editing twins. Available from: https://www.nbcnews.com/health/health-news/chinese-researcher-says-he-proud-gene-editing-twins-n941201 [Accessed: 10 December 2018]

used as a treatment for some genetic diseases by introducing precise genetic alterations: removing, inserting or substituting single or multiple bases to repair a targeted mutation (2). For example, chronic granulomatous disease (CGD) is a rare genetic disorder in which phagocytic cells do not carry out oxidative burst processes to allow the destruction of invading pathogens (6). Therefore patients with this disease acquire constant infections, especially when they are younger (6). This condition is usually caused by a single base pair mutation in the CYBB protein (expressed in phagocytes), which leads to an ineffective oxidative burst mechanism. Flynn *et al.* (6) used the CRISPR/Cas9 system to repair a point mutation in the heavy chain portion of this protein. Employing the use of induced pluripotent stem cells (iPS), derived from the patient and then differentiated into phagocytes, this group were able to insert the correct copy of the gene using the CRISPR/Cas9 system. Ultimately, these stem cells could be introduced back to the patient through autologous bone marrow transplantation (6). It was shown that the CRISPR/Cas9 system was highly efficient in correcting CYBB function, recovering oxidative burst mechanisms in some phagocytes (6). Therefore, this technique might be used as a gene therapy approach for treating CGD and for other genetic disorders, as long as knowledge of exactly what mutations cause the disease and methods to safely introduce "edited" cells back into the patient are established.

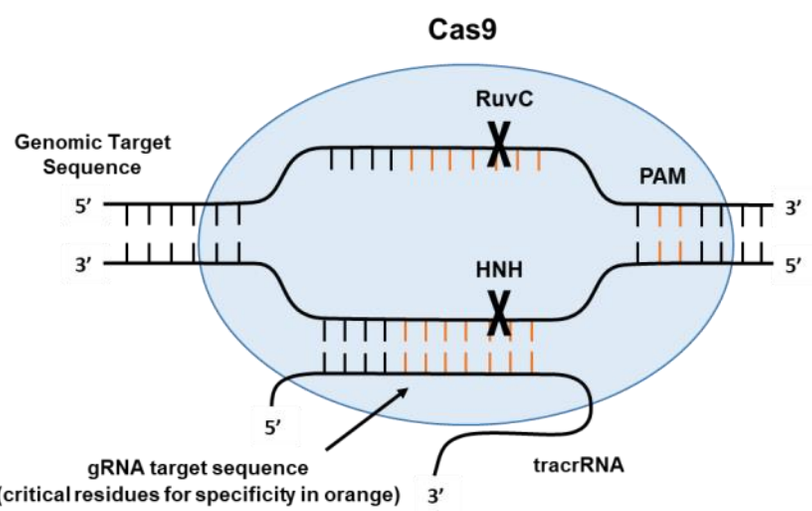


Figure 1. CRISPR/Cas9 genome editing system cleavage. gRNA (crRNA and tracrRNA) guides Cas9 to the target locus. The crRNA aligns with the protospacer (target sequence in orange) and PAM sequences. Both strands are cleaved by HNH and RuvC-like endonucleases, creating a double strand break (8).

Issues to be resolved with the CRISPR/Cas9 technology

The CRISPR/Cas9 system could potentially be widely employed in biomedicine, RNA editing, gene inactivation and therapy for genetic diseases in humans. Despite its major potential as a gene editing system, there are some problems associated with the efficiency and specificity of this system that need to be taken into account such as reduced Cas9 activity and off-target effects (3). These problems need to be overcome before being employed in humans.

Normally single-guided RNA (sgRNA) binds to the target sequence, guiding Cas9, which recognises it. However, it was found that in the presence of mismatches close to the cleavage site between the gRNA and the target DNA sequence, endonuclease activity of Cas9 was diminished (3). In addition, the presence of large amounts of Cas9-sgRNA complexes may lead to off-target effects due to binding of this complex to non-specific loci in the genome (3). Therefore, Cas9 activity, sgRNA sequence design and concentration of Cas9-sgRNA complexes need to be taken into account to improve CRISPR/Cas9 system.

CRISPR/Cas9 delivery

Another challenge of this system is considering the delivery methods: common approaches include electroporation of plasmids incorporating the CRISPR system. Some plasmids integrate randomly into the host cell genome and that can lead to not only off-target effects (disruption of other genes through Cas9 cleavage and integration) but also to host immune responses which might inhibit the gene editing process (3). In *in vivo* approaches, adeno-associated virus (AAV) is used. Despite being very successful in delivery, their expression is sometimes long-lasting, inducing toxicity (7). Further, patients previously exposed to AAV may have developed immunity to it and therefore expression may be stopped (7).

CRISPR raises important questions on genome editing

Ethical issues have emerged regarding the use of the CRISPR genome editing system in the inheritable human germline (9). Somatic genome editing has been widely accepted since there is a favourable equilibrium between advantages and risks and there is informed authorization from the patient. Germline editing, on the other hand, carries unpredictable risks which may be transmitted through several generations and obtaining informed authorization from multiple generations is impossible (9). Recently, a Chinese scientist was responsible for editing the genome of two embryos so that when born, the twins would never develop HIV in their lifetime, which raised a lot of ethical issues and criticism from the scientific community (10). In addition, ethical issues have arisen regarding the use of this system for non-medical purposes, for example to improve the performance of a professional sportsperson or a child's cognitive ability (9). In society, this presents a problem where some people may extend competitive advantages over others. The CRISPR/Cas9 system raises the question of whether it is ethical to use gene-editing technologies for non-medical purposes, in any context.

To conclude, the CRISPR/Cas9 gene editing system is a remarkable tool which can be used to delete an integrated copy of foreign DNA or RNA. It may be used for carrying out precise changes to the nucleotide sequence of a gene that has been mutated or has an association with a non-functional protein and this can be used for the treatment of genetic diseases such as HIV and CGD. Although CRISPR has many potential applications, problems regarding its specificity and efficiency, and also the ethical issues it raises, must be addressed and dealt with before it can be employed safely in humans.

Butterflies in Summer

Aleksa Vidic, 1st Year Biological Sciences BSc

I discovered a large group of butterflies clustered around an elderflower bush above a trickling stream in the Tara National Park in Serbia during June, two years ago. I had to lay flat on the planks of a small wooden bridge, my arms dangling over the side, in order to reach them. They fluttered below me, and I managed to capture two in focus.

With the forest's verdant mid-summer foliage awash in sunlight behind these two butterflies, I feel this photo captures the beauty and variety of life, and reminds me why I love studying Biology so much.

Identified as *Erebia* species *Pers. comm.: J N Greatorex-Davies; J Delf, Hon. Research Fellow, University of Liverpool; C Larsen, Lecturer, University of Liverpool*

Commerce, imperialism, biology and tea: how the East India Company helped discover new species (and brought tea to India)

Jonathan Roberts, 3rd Year Biological Sciences and History BSc

On the 3rd of May 1852, Frederick Smith read out his latest paper to the Royal Entomological Society of London, describing two dozen new species of Hymenoptera, mostly wasps, ichneumons and bumblebees from northern China (1). Two things made these little insects different from the thousands of others described and catalogued by the society: at the time, foreigners were not allowed beyond 30 miles from six Chinese Treaty Ports; and the man who collected them, Robert Fortune, appeared to have acquired them while on a mission to smuggle tea plants out of China.

Before the 1850s, China, under the Qing Dynasty, held a monopoly on tea. While many ordinary Chinese people grew their own tea plants (2), no-one had been able to establish them outside of China. Tea was an extraordinarily valuable commodity; the British Tea Tax accounted for around 9% of the British Government's income in the years 1835-1858 (3). This meant that the Tea Tax alone paid for almost all of the Royal Navy's yearly spending (3). The British Empire really did run on tea! The Chinese Empire found this out the hard way in 1840, when gunboats that their tea had paid for sailed into their ports during the First Opium War. The resulting Treaty of Nanking (Nanjing) of 1842 created six Treaty Ports, governed under British law and open to British trade.

Robert Fortune, a Scottish botanist, took this opportunity to travel to China in order to acquire samples on behalf of the Horticultural Society of London (2). From 1843 to 1846 he toured the treaty ports and the surrounding countryside, visiting villages, towns, farms and temples (2). During this he visited the tea plantations in Chekiang and Fokien and observed the drying process through which drinking teas were created (2). While on this journey he became probably the first westerner to suggest that both black and green teas were derived from the same species of tea tree, although different species were grown in different areas of the country (2). He suggested these to be *Thea viridis* in the north and *Thea bohea* in Canton (2).

In 1848 he returned to China, this time contracted by the East India Company to acquire the seeds and shrubs of tea plants which the company could use to establish its own tea plantations (4). Fortune was of the opinion that the tea produced around the treaty port of Ningbo (Ning-po) was unsuitable for the foreign market, and so resolved to also visit Hwuy-chow (probably Huangshan*) (4). He was familiar with the areas he was legally allowed to visit such as the Chusan (Zhoushan) archipelago, Ningbo and the temple at Teintung (20 miles inland), and it was from these that he procured the specimens named by Smith (1,4; Table 1). Fortune admitted to being extremely untrusting of Chinese people – despite having twice been mugged on his previous visit after ignoring warnings by a Chinese soldier and his servant to avoid certain

*It is not at all clear from Fortune's text where 'Hwuy-chow' is located, but Huangshan is in the same region and could plausibly be anglicised to Hwuy-chow.

areas (2,4). Because of this, instead of employing local agents to procure tea from Hwuy-chow, he decided to adopt the local dress and travel in disguise (4). He claimed this was suggested by his servants (4). He then employed, via an interpreter, a boat and crew to take him upriver from Shanghai to Hangzhou (4).

From Hangzhou he travelled further up the Quiantang and onto the Fuchan River. While the boatmen were navigating the rapids of the Fuchan and the Xin'an Rivers, Fortune took the opportunity to engage in botany on the hills above. He found several species in abundance that were rare elsewhere, such as 'the curious and much-prized' *Edgeworthia chrysantha* (4). Fortune was so impressed by the local species of palm that he sent specimens to the Royal Gardens at Kew for cultivation, where they apparently thrived, in the hope that they would eventually be found 'ornamenting the hill-sides in the south of England' (4). Around the same time, he also acquired seeds of the previously undescribed Funereal Cypress (*Cupressus funebris*) from an innkeeper who owned a particularly impressive tree (4).

After some time, he arrived in Sung-lo (Shandouxiang) where, according to legend, the tea plant had first been cultivated (4). Here he spent some time at the house of his servant Wang's parents, collecting tea seeds and learning all he could about their cultivation (4). During this time, he also acquired shrubs of a species of *Berberis* which he had not encountered before and was particularly enamoured with (4). Fortune also investigated the dyed green teas which were popular in Britain at the time,

Table 1. Species described by Smith, and where they were collected by Fortune.

Species	Location collected
Ancistrocerus flavo-punctatus	Ningbo
Bombus atripes	Zhoushan
Bombus breviceps	Zhoushan
Bombus flavescens	Zhoushan
Bombus haemorrhoidalis	Zhoushan
Bombus nasutus	Zhoushan
Bombus trifasciatus	Zhoushan
Bombus tunicatus	Zhoushan
Cerceris zonalis	Northern China
Crocisa decora	Not recorded
Cryptus purpuratus (now Chlorocryptus purpuratus)	Ningbo
Eumenes decoratus	Teintung
Eumenes quadratus	Near Ningbo
Pison regalis	Ningbo
Polistes sulcatus	Near Ningbo
Rhynchium flavo-marginatum	Not recorded – the specimen Smith used hosted two parasitic *Stylops*
Rhynchium ornatum	Teintung
Trogus pepsoides	Ningbo
Vespa craboniformis	Not recorded
Vespa ducalis	Teintung
Vespa mandarinia	Teintung
Xylocopa appendiculata	Near Ningbo
Xylocopa pictifrons	Zhoushan
Xylocopa rufipes	Not Recorded

References

1. Smith F. Descriptions of some new and apparently undescribed species of Hymenopterous insects from North China, collected by Robert Fortune, Esq. Transactions of the Royal Entomological Society of London 1852. **7**(2)

2. Fortune R. Three Year's Wanderings in the Northern Provinces of China. London: John Murray; 1847

3. Mather J. Botanising in a Sinocentric world: Robert Fortune's travels to China. *Studies in Travel Writing* 2010. **14**(3) 257-270

4. Fortune R. A Journey to the Tea Countries of China. London: John Murray; 1852

5. USC US-China Institute. Treaty of Nanjing (Nanking) 1842 Available from: https://china.usc.edu/treaty-nanjing-nanking-1842 [Accessed October 2018]

discovering that Prussian Blue and Gypsum dyes were added to green teas for the international market: the manufacturers did not drink it themselves, but saw no problem in adding it for foreigners, as dyed teas fetched a higher price (4). After this he returned to Ningbo, collecting further seeds on Silver Island, Chusan, before returning to Shanghai to cultivate his seedlings, which were then shipped to India via Hong Kong (4).

After a journey of thousands of miles, the wasps and bees that Fortune had collected arrived at the British Museum. Here Smith sat down to classify them, and to admire their beauty (1). Smith even consulted with Fortune upon his return to Britain to gain the best possible understanding of where the Hymenoptera were collected (1). With the seeds and (probably more importantly) the knowledge that Fortune brought back from China, the East India Company was able to establish its first tea plantations. This broke the Chinese monopoly and paved the way for the Indian teas which we drink today (3,4). This was, however, never solely a commercial venture: the plants and insects that Fortune brought back to Britain were very well-received by British scientists, who lost no time in adding them to their taxonomic schemes. Without this (illegal) expedition, much of China's flora and fauna would have been unknown to western science for many more years.

It should not be forgotten that nearly every cup of tea we drink exists as a consequence of the Opium War and Fortune's expedition and that modern science is a product of the Imperial Age. We like to think that science is something value-neutral, if not actively positive, but it must be acknowledged that the Life Sciences in particular benefitted hugely from Empire. Our understanding of evolution was aided by experiments on Batesian and Mullerian mimic butterflies carried out from African colonies such as Natal, for example. Without the Empire, would there even have been a voyage of the Beagle? This is not a call to disown science: though we may be uncomfortable about the circumstances in which our knowledge was acquired, knowledge and understanding of the world is a good in itself. Rather, we should be mindful that everything in modern Britain is a consequence of what came before: we are one of the richest countries in the world because of the slave trade and because of brutal exploitation of both native and foreign working classes, as well as the careless disregard of the governments of foreign nations exemplified by Fortune's mission. Not even science is free of that legacy.

Tumour heterogeneity in pancreatic cancer

Francesca Jones, 3rd Year Genetics BSc

Summary

Cancer is a relentless disease defined by uncontrolled cell division. In pancreatic cancer, cells of the pancreas grow and divide rapidly. Cancerous cells can invade and destroy adjacent healthy tissue. Tumours are comprised of cancerous and non-cancerous cells and the original tumour is referred to as the primary tumour. As the disease progresses, different cells in the tumour acquire different mutations resulting in the main body of the tumour consisting of cells possessing molecular differences termed tumour heterogeneity. Pancreatic cancer is an aggressive disease with a high mortality rate. Various pancreatic tumour subtypes have been suggested and as such a 4 type classification system has been devised which includes the following pancreatic tumour subtypes: squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine. These subtypes are each involved in slightly different genetic processes. Some of the subtypes are associated with a more severe clinical outcome than others such as the squamous subtype which is linked to metastatic disease. Metastatic disease is when the cancer spreads from the primary tumour to other tissue forming a secondary tumour. There are few treatment options available for pancreatic cancer. However, studies have looked at how cancerous cells communicate with each other and identified potential therapeutic sites in these cell communication pathways. Cells from 3 metastatic sites (lung, liver and peritoneum) have been analysed and a cell surface receptor involved in cell communication called Axl was found to be hypophosphorylated in cells derived from lung and liver metastatic cell lines but not in cells from the peritoneal cell lines. Cells derived from these different metastatic sites could possess varying levels of sensitivity to drugs that inhibit these cell communication pathways from working properly. These drugs are called pathway inhibitors and examples include R428 and lapatinib. Tumour heterogeneity in pancreatic cancer provides problems for clinicians and the treatment of the disease. However, a greater understanding of this heterogeneity could lead to the discovery of novel therapeutic targets.

Cancer is defined as uncontrollable cell proliferation (1) as it is a disease involving relentless clonal expansion of somatic cells to result in the killing of 'healthy' tissues through cell invasion and erosion (2). Cancer is thought to be a dynamic disease due to the fact that as the disease progresses the tumours will tend to become more heterogeneous which eventually results in the main body of the tumour consisting of multiple cell-types with differing molecular signatures which have variable levels of sensitivity to treatment (3). Tumour heterogeneity can be divided into intratumoural heterogeneity and intertumoural heterogeneity. Intratumoural heterogeneity defines the heterogeneity within the tumour cells of one patient. These differences between tumour cells may have arisen from phenotypic, genetic and epigenetic features (3).

For the purposes of this review article, I will define intertumoural heterogeneity as the heterogeneity between tumours in different patients where the tumour is of the same stage and grade. Intertumoural heterogeneity arises from patient specific factors such as differing somatic mutation profiles and genetic variation in the germline cells and environmental factors (3). Cancers can spread locally from the primary tumour into the surrounding healthy tissue. This disease is also able to spread to other regions in the body via the blood and lymphatic system to form new tumours. This is known as metastasis and it has been proposed that metastatic tumours may be of the same type as the primary tumour (5). Intermetastatic heterogeneity also exists due to the possibility of metastatic lesions being comprised of different cell populations from the primary tumour. Another possibility is intrametastatic heterogeneity which arises due to the metastatic lesion's ability to obtain new mutations with each round of cell division (3). These differences between inter and intra heterogeneity are all outlined in Figure 1.

As defined previously, intratumoural heterogeneity is the heterogeneity found within tumour cells of an individual patient. Intratumoural heterogeneity is further segregated into spatial or temporal heterogeneity as

References

1. Sherr C. Cancer Cell Cycles. Science 1996. 274(5293):1672-1677

2. Evan G and Vousden K. Proliferation, cell cycle and apoptosis in cancer. Nature 2001. 411(342-348)

3. Dagogo-Jack I and Shaw A. Tumour heterogeneity and resistance to cancer therapies. Nature Reviews Clinical Oncology 2017. 15(2):81-94

4. Jamal-Hanjani M, Quezada S, Larkin J and Swanton C. Translational Implications of Tumour Heterogeneity. Clinical Cancer Research 2015. 21(6):1258-1266

5. National Cancer Institute. Metastatic Cancer. Available from: https://www.cancer.gov/types/metastatic-cancer [Accessed December 2018]

6. International Cancer Genome Consortium. Available from: https://icgc.org/ [Accessed December 2018]

7. Bedard P, Hansen A, Ratain M and Siu L. Tumour heterogeneity in the clinic. Nature 2013. 501 (7467):355-364

8. Cancer Research UK. Pancreatic cancer statistics. Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/pancreatic-cancer#heading-One [Accessed December 2018]

9. Lewis A, Valle J and McNamara M. Pancreatic cancer: Are "liquid biopsies" ready for prime-time?. World Journal of Gastroenterology 2016. 22(32):7175

10. Adamo P, Cowley C and Neal C *et al.* Profiling tumour heterogeneity through circulating tumour DNA in patients with pancreatic cancer. Oncotarget 2017. 8(50)

11. Diehl F, Schmidt K and Choti M *et al.* Circulating mutant DNA to assess tumour dynamics. Nature Medicine 2008. 14(9):985-990

12. Bailey P, Chang D and Grimmond S. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature 2018. 531:47-52

13. Pancreatic Cancer UK. Types of pancreatic cancer. Available from: https:/www.pancreaticcancer.org.uk/ media/315869/types-of-pancreatic-cancer-factsheet-2016.pdf [Accessed December 2018]

14. Imaoka H, Shimizu Y and Mizuno N *et al.* Clinical Characteristics of Adenosquamous Carcinoma of the Pancreas. Pancreas 2014. 43(2):287-290

15. La Rosa S, Sessa F and Capella C. Acinar Cell Carcinoma of the Pancreas: Overview of Clinicopathologic Features and Insights into the Molecular Pathology. Frontiers in Medicine 2015. 2(41):1-13

demonstrated in Figure 2. Spatial heterogeneity defines the unequal distribution of genetically different tumour subpopulations located within a lone tumour or across multiple tumour sites. Temporal heterogeneity differs from the former since it describes the variability of the genetic diversity of a single tumour over a time period (6).

Tumour heterogeneity causes numerous clinical implications that can have an impact on the prognosis of the patient. Tumour heterogeneity results in tumours consisting of multiple cells with varying molecular signatures, each with varying levels of sensitivity to treatment. Due to this, resistance to targeted therapies can arise from the evolution of drug tolerant cells (3). Subsequently, there has been a shift in treatment to a more personalised and genotypically guided approach which takes into consideration the genetically diverse tumour subpopulations both within and between cancer patients (3). In 2012 there were an estimated 8.2 million deaths worldwide from cancer (7). This high mortality rate emphasises the importance of developing efficient treatment options for the various cancers.

The use of circulating tumour DNA as a biomarker in pancreatic cancer

Pancreatic cancer is the eleventh most prevalent cancer in the UK and is the sixth most common cause of cancer death in the UK and it accounted for 6% of all cancer deaths in 2016 (8). Pancreatic cancer has one of the worst prognoses out of all the malignancies (9). Since the early 1990's the prevalence rate of pancreatic cancer has increased by approximatley 15% in the UK (8). At present, the only potentially curative treatment is surgery but only around 15-20% of pancreatic cancer patients have resectable disease at presentation in the clinic (9). Unfortunately, in patients who undergo both surgery and adjuvant chemotherapy the 5 year survival rate is incredibly low – just 16.3%-28.9% (9). This shows that this resective surgery is rarely curative.

The most common cause of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) which makes up 80% of all pancreatic cancer cases (10). PDAC tumours tend to have a high stromal content which creates a strong hypovascular barrier that is considered to hinder the delivery of chemotherapeutics and encourage aggressive neoplastic cell behaviour (10). Earlier detection of PDAC would lead to a more positive patient prognosis as it could increase the probability of successful treatment and improve survival rates (10).

The diagnosis of pancreatic cancer can involve performing a tissue biopsy but it is possible to achieve false negative results due to the high stromal content which is characteristic of many pancreatic cancers. Alongside tissue biopsies being expensive and potentially being painful for the patient, another potential issue with tissue biopsies is that they only give site specific information and will not provide an accurate representation of the entire genomic landscape of the tumour (10). Pancreatic cancer is highly heterogenous and so it is important to consider these limitations of tissue biopsies when conducting mutational profiling as the results from targeted next generation sequencing would not be reliable due to the heterogeneity (10). A way to possibly combat this is to take multiple tissue biopsies from the PDAC tumour as these will take into account the heterogeneity within the tumour to give an accurate representation of the entire tumour's genomic landscape. An alternative method that is currently being investigated is the use of circulating tumour DNA (ctDNA) taken as a liquid biopsy. CtDNA is derived from tumour cells undergoing necrosis or apoptosis which is occurring in the natural development of the cancer from a primary tumour to a metastatic lesion (10). Therefore,

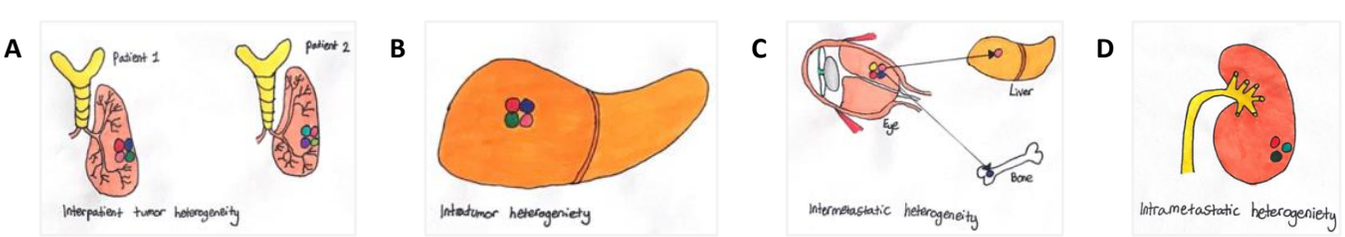


Figure 1. A comparison of intertumoural heterogeneity and intratumoural heterogeneity. A: Intratumoural heterogeneity which is the existence of numerous sub clones in a primary tumour. This results in heterogeneity amongst tumour cells. B: Intratumoral heterogeneity which is heterogeneity among the tumour cells of an individual patient. C: Intermetastatic heterogeneity which is the existence of differing sub clones in separate metastatic lesions of one patient. D: Intrametastatic heterogeneity which is the occurrence of numerous sub clones within a single metastatic lesion. The distinct coloured dots signify the different sub clones. Figure adapted from Jamal-Hanjani *et al.* (4).

ctDNA is thought to be less affected by intratumour heterogeneity than a single tissue biopsy is. This ctDNA also allows for rapid evaluation of tumour changes in a matter of hours since the half-life of ctDNA is approximately 2 hours. This makes the use of liquid ctDNA biopsies ideal for monitoring the effectiveness of treatment and disease progression (11). Furthermore, liquid biopsies are more cost effective, quicker to perform, more comfortable for the patients since only a blood sample is taken and they are easy to repeat.

The use of a liquid biopsy containing ctDNA allows for detection of circulating tumour derived biomarkers in the blood which is a non-invasive approach that could provide an earlier detection of PDAC and other pancreatic cancers (10). These circulating tumour derived biomarkers such as point mutations in key cancer genes can be detected by targeted amplicon sequencing of circulating free DNA (cfDNA) that has been isolated from blood plasma (10). Once these findings have been validated, these circulating tumour biomarkers can be used to detect early PDAC and potentially even premalignant lesions (10). This is hugely beneficial as patient prognosis can be improved through the use of a more effective treatment that is targeted to the genomic landscape of the patients' tumours.

As shown above, tumours can be quantified through the use of liquid biopsies of patient blood. Liquid biopsies contain ctDNA to allow for the detection of circulating tumour derived biomarkers such as mutations of key cancer genes (KRAS, CDKN2A, SMAD4 and TP53). Therefore, in order to quantify tumours by their molecular subtype through liquid biopsies, it will be necessary to detect these circulating tumour biomarkers for each of the 4 molecular subtypes. Each of the 4 molecular subtypes have differing molecular signatures and so it is important to detect the mutations responsible for these differences and utilise them as biomarkers.

Molecular subtypes of pancreatic cancer

Analysis of the genomic landscape of pancreatic cancer has revealed a varied mutational landscape with four common oncogenic mutations in the cancer genes: KRAS, CDKN2A, SMAD4 and TP53 alongside other less commonly mutated genes (12). It has been discovered that oncogenic point mutations of individual genes accumulate into essential molecular pathways which include cell cycle regulation, axonal guidance, DNA damage repair, chromatin regulation and TGF-β signalling.

In a paper by Bailey *et al.* (12), 382 pancreatic cancer patients and their associated histopathological variants were analysed through deep exome sequencing, whole genome sequencing and copy number analysis in order to identify genomic events and key mutations that contribute to disease progression. The study participants had suffered from PDAC and its associated histological variants which were colloid, PDAC associated with intraductal papillary mucinous neoplasm (IPMN), adenosquamous and acinar cell carcinomas (12). Adenosquamous carcinoma, acinar cell carcinomas and colloid carcinoma are rare variants of pancreatic cancer (13). Adenosquamous carcinoma is considered to be more aggressive than PDAC and as a result, patients with this histological subtype have a poorer prognosis (14). It has been found that acinar cell carcinoma tumours show varying morphological characteristics, molecular signatures, patient prognosis and clinical symptoms at presentation in the clinic (15). This varied heterogeneity in the clinicopathological spectrum produces difficulties for the pathological diagnosis of the tumour which in turn has implications on the efficiency of treatment and patient prognosis (15).

The patients who participated in the Bailey *et al.* (12) study were treatment naïve and had undergone resective surgery. Analysis

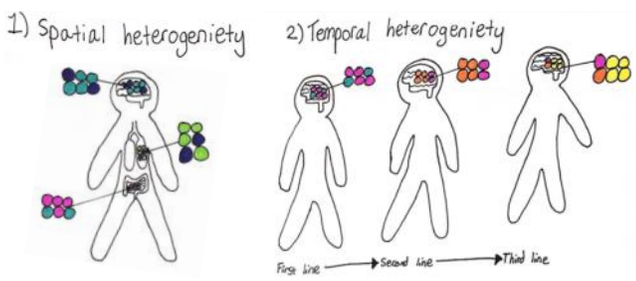


Figure 2. A comparison between spatial and temporal heterogeneity. 1 depicts spatial heterogeneity which is shown by the uneven distribution of cancer sub clones across different regions of the metastatic sites and primary tumour. 2 illustrates temporal heterogeneity which is depicted by the change in the genomic landscape of a single lesion over time due to the tumour's natural progression or exposure to selective pressure such as chemotherapy or immunotherapy. The individual coloured dots signify different sub clones. Figure adapted from Dagogo-Jack *et al.* (3).

16. Cancer genome atlas research network. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. Cancer Cell 2017. 32(2):185-203

17. Dreyer S, Jamieson N and Upstill -Goddard R *et al.* Defining the molecular pathology of pancreatic body and tail adenocarcinoma. British Journal of Surgery 2018. 105(2):183-191

18. TeachMeAnatomy. The Pancreas. Available from: https://teachmeanatomy.info/abdomen/viscera/pancreas/ [Accessed 14th December 2018]

19. Beijersbergen R, Wessels L and Bernards R. Abstract IA5: Synthetic lethality in cancer therapeutics. Molecular Cancer Therapeutics 2017. 1:141-161

20. Campbell P, Yachida S and Mudie L *et al.* The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature 2010. 467:1109-1113

21. Kim M, Zhong Y and Yachida S *et al.* Heterogeneity of Pancreatic Cancer Metastases in a Single Patient Revealed by Quantitative Proteomics. Molecular & Cellular Proteomics 2014. 13(11):2803-2811

22. Yachida S, Jones S and Bozic I *et al.* Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 2010. 467 (7319):1114-1117

identified 23,538 coding mutations and 7,377 of these mutations were verified through a statistical approach. 21,208 genomic rearrangements were also identified (12). Further analysis of bulk tumour tissue was undertaken in order to gain an understanding of the molecular mechanisms and transcriptional networks that underline the tumour microenvironment. Unsupervised clustering of RNA-seq data for 96 tumours identified 4 molecular classes which were: Squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX) (12). These 4 classes were based on the differential expression of transcription factors and downstream targets that are essential for lineage specification and differentiation in pancreas development and regeneration (12). An alternative proposal for the molecular subtypes of pancreatic cancer is outlined by The Cancer Genome Atlas Research Network and it confirmed 2 pancreatic cancer tumour subtypes which were: classical/ pancreatic progenitor and basal-like squamous (16).

The squamous subtype is characterised by four core gene programmes which includes gene networks involved in metabolic reprogramming, hypoxia response, inflammation, autophagy, MYC pathway activation, TGF-β signalling and upregulated expression of TP63ΔN and its target genes (12). The pancreatic squamous subtype is associated with mutations in KDM6A and TP53. TP63ΔN in the presence of mutated TP53 regulates epithelial cell plasticity, tumorigenicity and epithelial to mesenchymal transition in numerous solid tumours (12). This particular subtype has a notably poor prognosis (17). The pancreatic progenitor subtype is defined by transcriptional networks containing the following transcription factors: HES1, PDX1, FOXA3, FOXA2, MNX1, HNF1A, HNF1B, HNF4G and HNF4A (12). These transcription factors play a hugely important role in pancreatic endoderm cell-fate determination towards a pancreatic lineage (12). The ADEX molecular subtype is characterised by transcriptional networks that play a key role in the latter stages of pancreatic development and differentiation (12). The ADEX subtype is also a subtype of pancreatic progenitor tumours (12). The final subtype is the immunogenic subtype which shares numerous characteristics with the pancreatic progenitor class but it differs in the fact that it exhibits a notable immune infiltrate (12). The immunological genes associated with the immunogenic subtype are genes involved in antigen presentation, toll-like receptor signalling pathways, B cell signalling pathways, CD8 + T cell and CD4+ T cell (12).

Molecular heterogeneity in pancreatic cancer

Recent large-scale genomic studies have identified the transcriptomic and genomic landscape of pancreatic cancer. However, not much is known about the molecular heterogeneity that results from differing tumour locations in the pancreas (17).

The pancreas is located deep in the upper abdomen. It consists of 4 main anatomical structures as shown by

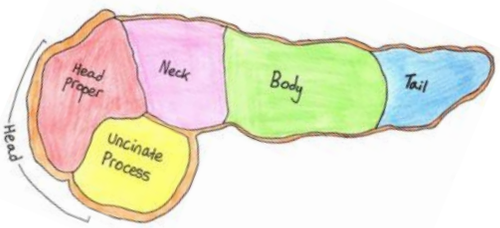


Figure 3. The anatomical structures of the pancreas. The pancreas can be divided into 4 main parts; the tail, the body, the neck and the head which can be further divided into the head proper and the uncinate process. Figure adapted from teachmeanatomy (18).

Figure 3. These structures are: The tail, body, neck and head which can be further segregated into the uncinate process and head proper (19). Studies have revealed notable differences in the prognosis of cancers situated in the head of the pancreas compared to those located in the tail and body (17). Around 15% of PDAC arise in the tail and body of the pancreatic tumour which are characterised by a late presentation in the clinic. Patients with PDAC in the body and tail display symptoms characteristic of advanced disease such as pain and extreme weight loss. Tumours of the pancreatic head are often presented far earlier in the course of the disease since some patients with these tumours often have a comorbid diagnosis of jaundice (17).

Studies have identified that PDAC harbours substantial interpatient genomic heterogeneity. Whole genome sequencing performed on 100 resected PDAC tumours showed novel structural variation subtypes defined by chromosomal rearrangement patterns and numbers which are able to predict the reaction to platinum based chemotherapy through synthetic lethality (15). The resulting mutation produced from the perturbation of two genes by synthetic lethality produces a vulnerability that can be targeted therapeutically (19).

The relationship between body and tail pancreatic cancers and the squamous molecular subtype

Through mRNA microarray sequencing and whole-transcriptome sequencing it has been identified that pancreatic cancers of the tail and body co-segregate with the squamous molecular subtype (17). It has been discovered that there are significantly poorer clinical outcomes for the advanced and resectable disease stages in both tail and body pancreatic cancer (15). A study by Dreyer *et al.* (17) has identified molecular differences amongst resectable PDAC from head, body and tail of the pancreas. The squamous molecular subtype seems to be more advanced on the molecular clock compared to the other molecular subtypes which suggests a further level of genomic instability which results in the accumulation of

DNA damage that influences the novel transcriptome of tumours with this subtype. Therefore, it seems that body and tail pancreatic cancer has a greater chance to be of a squamous subtype thus insinuating a more aggressive disease at the time of diagnosis than cancer of the pancreatic head (15). Dreyer *et al.* (17) defined the squamous molecular subtype on the basis of metastasis of the liver, this metastasis of the liver was associated with a considerably poorer prognosis when compared to additional local and metastatic recurrence patterns.

Metastatic disease in pancreatic cancer

Pancreatic cancer is an aggressive malignancy with a five year mortality rate of 97%-98% (20). This high mortality rate is usually as a result of widespread metastatic disease (20). Metastatic disease is the most frequent cause of death in cancer patients (22). Tumour heterogeneity can be studied in patients with multiple sites of metastatic disease (21). Kim *et al.* (21) hypothesised that heterogeneity in metastatic pancreatic cancer causes heterogeneity at the proteomic level. At the proteomic level it was found that a class of cell surface receptors had a highly altered expression amongst the three metastatic cell lines examined (liver, peritoneum and lung). In this study there was a particular focus on the changes in tyrosine phosphorylation based signalling pathways amongst clones (21). Mass spectrometry was undertaken on purified tyrosine phosphorylated peptides that were enhanced using anti-phosphotyrosine antibodies. Differential activation of the tyrosine kinases was observed in the 3 metastatic subclones (21). Axl receptor tyrosine kinase was hypophosphorylated in the liver and lung metastatic cell lines but not in the peritoneal cell line (21). Due to the differing patterns of cell signalling pathways in the subclones it could be possible that tumours derived from separate sites have the potential to have varying sensitivities to pathway inhibitors (21). Tumour cells derived from the lung metastatic cell line showed a greater sensitivity to the Axl inhibitor R428 whereas cells obtained from the peritoneal metastasis showed an increased sensitivity to lapatinib (21).

Discussion

Pancreatic cancer is a highly heterogenous disease that is continually evolving due to the accumulation of new mutations throughout the course of the disease. Pancreatic cancer is an aggressive disease with a high mortality rate. It often presents late in the clinic when the disease is in its advanced stages. There is little in the way of curative treatment aside from resective surgery which is rarely successful in terms of survival as shown by the low 5 year survival rate. One of the main issues with pancreatic cancer is the fact it is often diagnosed in the latter stages of the disease when patients have multiple sites of metastatic disease and treatment options are limited. If the disease could be detected earlier then perhaps it would lead to a more positive prognosis for the patient; research efforts should be focused on screening methods to allow for the earlier diagnosis of patients. If the use of liquid biopsies containing ctDNA became more widespread then changes in a tumour would be able to be rapidly evaluated regularly thus allowing for a more efficient monitoring of the effectiveness of treatment and disease progression. Tissue biopsies can be further improved by taking biopsies from multiple sites in the primary tumour to give an accurate representation of the genomic landscape of the tumour that takes into account any heterogeneity that may exist. It is hugely important to take tumour heterogeneity into consideration when choosing suitable therapeutics. Conventional blanket chemotherapy provides a very crude treatment approach and can lead to resistance to therapeutics arising from drug tolerant cells which can make further treatment difficult. As death from metastatic disease is common in cancer patients, particularly in pancreatic cancer due to no efficient treatment option existing, it seems sensible that treatment options to target the metastatic disease are developed. Studies at the proteomic level have identified differing patterns in the cell signalling between different metastatic cell lines. Tumours derived from varying sites in the subclones have the potential to be sensitive to different pathway inhibitors and this provides another potential therapeutic target. Tumoral heterogeneity provides numerous issues to clinicians, however, it seems as if targeting this heterogeneity may be the key to developing successful treatment options with the use of a personalised genotypically guided treatment based on the genomic landscape of the individual's tumour. However, it is important to remember that the majority of cancers will become resistant to these targeted therapies. Creating a dual treatment approach that comprises of a targeted approach to the drug tolerant cells and a personalised approach that takes into account the tumoral heterogeneity could lead to huge advances in the treatment of pancreatic cancer. Overall, having more of an understanding of heterogeneity in pancreatic cancer could lead to the discovery of more therapeutic targets.

You might also be interested in the research article by Rebecca Cleator on the interactions between chemotherapy agents used to treat pancreatic cancer: see Research, page 30

Publish with Insider Imprint!

Here are just a few reasons why it is excellent to publish with *Insider Imprint*:

* Show-case your achievements
* Boost your CV
* Inspire other students
* Wow your future employer
* Published online open access for the world to see
* High quality & peer-reviewed
* Gain real-life experience working with a journal to publish your work

Who can write for us?

If you are an **undergraduate or masters student** in the **Faculty of Health and Life Science**, you can write for us!

That includes first, second and final year students from the Life Sciences, Health Sciences, Veterinary, Dentistry, Medical and Psychology Schools.

Insider Imprint

If you have something you want to write about, we want to hear from you!

Meerkat behaviour in the wild versus captivity

David Chalmers, MSc in Advanced Biological Sciences (Host:Parasite Biology)

Meerkats (*Suricata suricatta*) are a species of small omnivorous, social members of the mongoose family. They are found in arid and semi-arid deserts around Africa, and live in social grouping with a single dominant pair and several subordinate pairs (1). Group members will often forage together in close proximity subsisting on a diet of arthropods and small vertebrates (1). Groups sleep in underground burrows at night and are fiercely territorial marking their territory with specialised odour glands, urine and faeces; after defecation a meerkat may drag their anus across the ground to further rub in its scent (1). They breed annually, however group membership is not always permanent with group size sometimes shifting (2, 3). Within a group subordinate meerkats will often engage in baby-sitting and provisioning, with the degree to which these behaviours are performed varying with sex, age and social status (4). Meerkats are known for their sophisticated alarm call system, which features different alarm calls for different predators (5). As well as special details concerning the predator's location and distance from the group, even captive meerkats are shown to have the same call repertoire as their wild cousins, and are shown to display these calls in response to perceived predators and to educate their young (5). This essay looks into the meerkat behaviours and how captivity can cause variation in their behaviour using the behaviours observed in a set of captive meerkats as an example. These captive meerkats were a set of nine male meerkats belonging to Croxteth Country Farm which were donated by Knowsley Safari Park.

Behaviours of wild meerkats

Meerkat behaviour can be summarised into a series of categories which can assist with visualising the kind of behaviours they perform (Table 1). Pups start engaging in foraging with the adults at 30 days old but will remain dependent on the adults to feed them until 90 days (6). After this, they will reach independence in food acquisition, and pups who show signs of high foraging behaviour will be better at foraging as adults (6). Meerkats have been the subject of numerous social learning studies; meerkat pups show great exploratory behaviour but will rely on adults for learning (7). Individuals in meerkat groups can show great innovation and may be responsible for new foraging techniques being introduced within the group: it seems the more subordinate a meerkat is the more innovative they can be seen to be while more dominant individuals show higher aggression to new ideas (7). While it is unknown how many families are in a single colony, it can be assumed that there is at least some weak relation between all meerkats (8). Play behaviour is more commonly seen between male parents and siblings rather than mothers (8). This early play can aid in developing social standing when the pups reach adulthood. Until a dominant breeding pair is established, female meerkats will be in an intense competition to get themselves and their mates set up as the dominant pair (9). Female personality can lead to certain individuals naturally becoming more dominant than their sisters while more submissive females will have a clearer disposition towards being helpers (9). This makes meer-

References

1. Golabek K A, Jordan. N. R and Clutton-Brock T H. Radiocollars do not affect the survival or foraging behaviour of wild meerkats. Journal of Zoology 2007. Doi: 10.111/j.1469-7998.2007.00377

2. Bateman A W, Lewis M A, Gall G, Manser M B and Clutton-Brock T H. Territoriality and home-range dynamics in meerkats *Suricata suricatta*: a mechanistic modelling approach. Journal of Animal Ecology 2014. 84:260-271

3. Moran G and Sorensen L. Scent marking behaviour in a captive group of meerkats (*Suricata suricatta*). Journal of Mammalogy 1986. 67:120-132

4. English S, Nakagawa S and Clutton -Brock T H. Consistent individual differences in cooperative behaviour in Meerkats (*Suricata suricatta*). Journal of Evolutionary Biology 2010. 23:1597-1604

kats a popular model to study niche specialisations, as early life behaviours can predict life trajectories within their colony (9).

It is so far unclear whether or not there is a direct link between the size of a female and her status as a dominant female in meerkat colonies. Dominant females will produce up to four litters a year with subordinate females only permitted to breed sparingly (10). Dominant females may simply become larger due to reductions in energy expenditure through helper subordinates assisting in daily activities (10). Dominants have also been shown to have an increase of estradiol and progesterone, which may account for their larger size (10). It is thought that such changes are the result of gaining alpha status as opposed to larger individuals initially gaining the alpha status (10). The majority of aggression in a meerkat colony comes from dominant females disciplining subordinate females who attempt to breed without their consent (11). The dominant female will groom the dominant male more than she will the subordinate males possibly to reinforce their bond (11). Subordinate females may also groom the dominant female to placate her and this may help to earn breeding rights to an extent (11). There is some aggression between dominant males and subordinate males, this is usually in correlation with large group sizes, and this is in particular to non-offspring subordinates (12).

Behaviours of captive meerkats

Croxteth farm houses a set of nine young male meerkats which were removed from their parental group at a young age. During a 6-week observation study, this group displayed numerous behavioural similarities to those of wild meerkats; one of the most obvious of these behaviours was the iconic sentry behaviour. Throughout the day one or more of the nine meerkats would stand on its hind legs and look around, and occasionally sudden noises triggered sudden bouts of this sentry behaviour. Digging behaviour was also observed, though only minimal, and no more than small temporary ditches were created. This suggests that vestiges of the meerkats natural digging behaviour is carried over into captivity. The presence of shelter provided by the farm may negate the meerkats' need to create subterranean shelters as well as the possibility of them not being taught properly to dig full shelters. Some communal activities where observed in the meerkats' daily routines, such as group sleeping. Foraging behaviour was carried out frequently and peaked during designated feeding times at the farm. Several forms of social behaviour

Table 1. Behavioural categories which can be seen in meerkats.

Behaviour	Definition
Foraging	Eating
Maintenance	Actions which maintain bodies inner working e.g., drinking, defecation
Social interaction	Interaction with another meerkats e.g. allogrooming, play fighting
Grooming	Any form of self-cleaning
Locomotion	Movement not connected with other behaviours
Vigilance	Standing still looking around habitat
Rest	Absence of activity

5. Hollen L I and Manser M B. Persistence of alarm-call behaviour in the absence of predators: A comparison between wild and captive-born meerkats (*Suricata suricatta*). Ethology 2007. 113:1038-1047

6. Thornton A. Early body condition, time budgets and the acquisition of foraging skills in meerkats. Animal Behaviour 2007. 75:951-962

7. Thornton A and Samson J. Innovative problem solving in wild meerkats. Animal Behaviour 2012. 83:1459-1468

8. Wemmer C and Fleming M J. Ontogeny of playful contact in a social mongoose, the meerkat, *Suricata suricatta*. American Zoology 1974. 14:415-426

9. Carter A J, English S and Clutton-Brock T H. Cooperative personalities and social niche specialization in female meerkats. Journal of Evolu-

were also observed such as allogrooming, where one meerkat would lick and remove dirt from another. Grooming can help reduce conflict with dominant individuals and can help gain access to better resources (13). It has been found that removing key individuals from a colony can disrupt natural allogrooming patterns, and these disruptions could lead to increased levels of antagonism in captive meerkats (13). Play fighting would occur in seemingly random bursts and these would only last for short periods with the two meerkats involved darting in different directions.

Wemmer and Flemming (14) observed that keeping meerkats in small groups can prevent them expressing the full range of their natural behaviour - it was thought that a minimum of 25 meerkats would be required for natural behaviour to be maintained. Captive meerkats will generally act aggressively if new meerkats are introduced to a group making initially small groups hard to rectify (14). During the 6-weeks of observation at Croxteth Farm, examples of more unnatural behaviour were seen in the captive meerkats; most of these behaviours are harder to explain and do not seem to have any explanations in the literature. One such behaviour was a phenomenon of a single meerkat escaping the enclosure and running away from the group; this happened several times. This behaviour is strange as meerkats are highly social animals whose survival depends on group living. The meerkats did appear to learn some new behaviours from their captive environment, for example meerkats learnt to recognise the Croxteth Farm employees as well as those dressing in similar clothing, responding by approaching such individuals with the expectation of being fed. Meerkats also displayed climbing behaviour including climbing on those feeding them; this may have been some new form of competition developed in captivity, as they seemed to be almost racing to gain food before the others.

Final remarks

Observing captive meerkats at Croxteth Farm supported reports in the literature that there can be alterations in behaviour such as increased aggression as well as changes in natural social structure compared to that seen in the wild. The most common behaviour observed in captivity was vigilance for the farm staff, implying that the meerkats had learned (most likely through operant conditioning (15)) to associate the presence of staff members with being fed. Since this phenomenon occurred soon after the first observation, it is easy to believe that meerkats are able to recognise certain individuals very quickly. It is important to keep in mind that variation of visitor presence may have caused anomalies in behaviour patterns, as it has been shown that unfamiliar human presence can affect meerkat behaviour (16, 17). The observations at Croxteth indicate that meerkat behaviour is partially instinctual, while other behaviours may be taught by prior generations.

tionary Biology 2014. 27:815-825

10. Russel A F, Carlson A A, McIlrath G M, Jordan N R and Clutton-Brock T H. Adaptive size modification by dominant female meerkats. Evolution 2004. 58(7)

11. Kutsukake N and Clutton-Brock T H. Social functions of allogrooming in cooperatively breeding meerkats. Animal Behaviour 2006. 72:1059-1068

12. Kutsukake N and Clutton-Brock T H. The number of subordinates moderates intrasexual competition among males in cooperatively breeding meerkats. Proceedings, Royal Society of Biology 2008. 275: 209-216

13. Madden J R and Clutton-Brock T H. Manipulating grooming by decreasing ectoparasite load causes unpredicted changes in antagonism. Proceedings, the Royal Society of Biology 2009. 276:1263-1268

14. Wemmer C and Flemming M J. Management of Meerkats, Suricata suricatta. Small mammals in captivity. International Zoo Yearbook 1975. 5:73-77

15. Hoppitt W, Samson J, Laland K N and Thornton A. Identification of learning mechanisms in a wild meerkat population. PLoS ONE 2012. 7:E42044

16. Martin R A and Melfi V. A comparison of zoo animal behaviour in the presence of familiar and unfamiliar people. Journal of Applied Animal Welfare 2016. 19:234-244

17. Sherwen S L, Magrath M J L, Butler K L, Phillips C J C and Hemsworth P H. A multi-enclosure study investigating the behavioural response of meerkats to zoo visitors. Applied Animal Behaviour Science 2014. 156:70-77

MICROBIOLOGY SOCIETY

Visit the Microbiology Society website to learn about...

Grants

Networking opportunities

Professional development

Publishing your research

@MicrobioSoc

microbiologysociety.org

How effective is eye screening for diabetic retinopathy?

Emma Khoury, 3rd Year Bachelor of Medicine & Bachelor of Surgery

Abstract

This review has been conducted in order to assess whether eye screening for diabetic retinopathy is effective and whether it would be safe to extend the annual screening interval beyond one year. Approximately 285 million people in the world have diabetes, of which 95 million have signs of diabetic retinopathy. This number is expected to increase due to advances in treatment of diabetes and an aging population. Eighteen papers have been critically analysed in order to assess whether the current screening programme is cost effective and whether or not it would be safe to extend the current annual screening interval beyond one year. No date restrictions were enforced and only articles in the English language were considered. Increasing the diabetic eye - screening interval is safe and cost effective for lower risk patients, however high-risk patients should continue to receive annual treatment. In an environment with limited resources it is important to make the screening programme cost effective. This can be achieved provided that the screening interval is decided on by risk stratification; it seems appropriate that high risk patients should continue to receive annual screening with the lower risk diabetic patients having screening at longer intervals of up to 5 years.

Introduction and background

Diabetic retinopathy is one of the leading causes of blindness in the UK (1, 2), alongside glaucoma, cataracts and age-related macular degeneration (AMD). Those with diabetes are 25 times more likely to become blind than those unaffected individuals (3). Individuals with either type 1 or type 2 diabetes are both at risk of developing diabetic retinopathy. Individuals aged 12 and over are eligible to be screened for diabetic retinopathy (4).

Diabetic retinopathy is a condition caused by very high blood glucose levels. If the blood glucose levels are very high, the blood vessels, which supply blood to the retina, become damaged (5). These vessels may 'leak fluid' and blood, which can result in swelling, known as oedema. The vessels then begin to close off, which leads to ischemia (6). This process is known as non-proliferative diabetic retinopathy. Over time, if not treated, abnormal blood vessels may develop in the retina by a process known as neovascularization, which can damage the retina. This is known as proliferative diabetic retinopathy (7).

Diabetic retinopathy is prevalent throughout the world. Results from 35 population-based studies show that approximately 93 million people worldwide have diabetic retinopathy, of which 17 million have proliferative diabetic retinopathy, 21 million have diabetic macular oedema (DME), and 28 million have sight-threating diabetic retinopathy (8). The prevalence of diabetic retinopathy for those affected by type 1 diabetes is 77.3% and 25.2% for those with type 2 diabetes. The prevalence of proliferative diabetic retinopathy was 32.4% for those with type 1 diabetes and 3.0% for those with type 2 diabetes.

The screening process is approximately half an hour long (10). The process involves administering eye drops in order to dilate the pupils and then photographs of the retina are taken. When the eyes are screened, the results are graded into five stages (11). Grading is achieved by looking at the digital photographs of the retina. The first stage is no retinopathy, in which no signs of diabetic retinopathy were identified. Stage two retinopathy is known as background retinopathy, which signifies that micro aneurysms are present in the retina, which could result in blood leakage (12). Stage two does not require treatment except for lifestyle changes. Stage three diabetic retinopathy is known as pre-proliferative retinopathy,

References

1. Bowen A, Hazelton C, Pollock A and Lincoln N B. Cognitive rehabilitation for spatial neglect following stroke. Cochrane Database of Systematic Reviews 2013. 7:CD003586

2. NHS Birmingham, Solihull and Black Coventry Diabetic Eye Screening. Retinopathy and Screening Visit. Available from: www.retinalscreening.co.uk/patient-information/about-retinopathy-and-your-screening-visit/

3. Williams R, Airey M and Baxter H *et al.* Epidemiology of diabetic retinopathy and macular oedema: a systematic review. Eye 2004. 18 (10):963-83

4. Tye A, Wharton H and Wright A *et al.* Evaluating digital diabetic retinopathy screening in people aged 90 years and over. Eye 2015. 29 (11):1442-5

5. Giorgi R G, Woods R L and Peli E. Clinical and laboratory evaluation of peripheral prism glasses for hemian-

opia. Optometry and Vision Science 2009. 86(5):492-502

6. Wang W and Lo A. Diabetic Retinopathy: Pathophysiology and Treatments. International Journal of Molecular Sciences 2018. 16 (6):1816

7. Boyd K. Diabetic Retinopathy Causes. Available from: https://www.aao.org/salud-ocular/consejos/diabetic-retinopathy-causes [Accessed September 1st 2013]

8. Hogg R. Diapedia: Epidemiology of diabetic retinopathy. Available from: www.diapedia.org/71040851101/rev/11 [Accessed 2018]

9. Yorston D. Diabetic retinopathy (DR): management and referral. Community Eye Health Journal 2011. 24(75):12-3

10. National Health Service Choices. Diabetic eye screening 2016 Available from: www.nhs.uk/conditions/diabetic-eye-screening/Pages/Introduction.aspx [Accessed 2018]

11. National Health Service. Diabetic retinopathy - Stages NHS choices2016. Available from: www.nhs.uk/conditions/diabetic-retinopathy/pages/symptoms.aspx [Accessed 2018]

12. Broadbent D. Diabetic retinopathy: Fundamentals for primary care. Diabetes & Primary Care 2013. 15:201-10

13. Taylor-Phillips S, Mistry H and Leslie R *et al.* Extending the diabetic retinopathy screening interval beyond 1 year: Systematic review. British Journal of Ophthalmology 2016. 100(1):105-14

14. Jones S and Edwards R T. Diabetic retinopathy screening: a systematic review of the economic evidence. Diabetic Medicine :a journal of the British Diabetic Association 2010. 27(3):249-56

15. Wu B, Li J and Wu H. Strategies to Screen for Diabetic Retinopathy in Chinese Patients with Newly

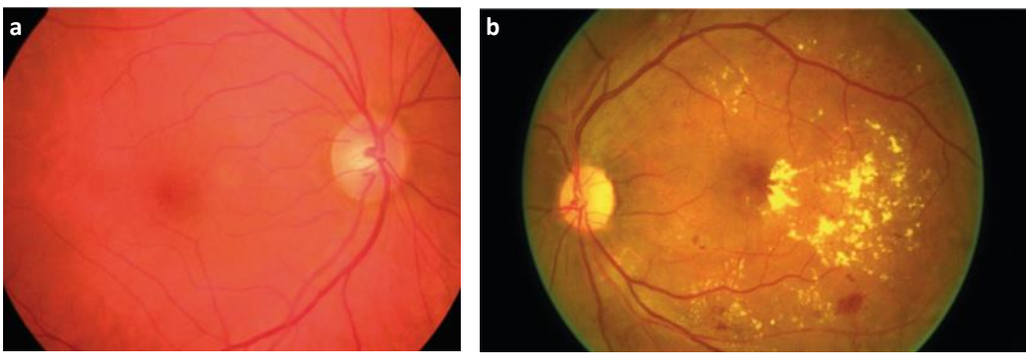


Figure 1a and b. Comparison of the differences in the fundus retinal photographs of an eye with no abnormalities identified (a) with the eye of a diabetic person who has diabetic maculopathy (b) (9). The image on the right (b) shows hard lipid exudates surrounding the blood vessels (yellow spots). Micro-aneurysms (identified as red spots) can also be seen in image (a). These are swellings of the blood vessels as a result of macular oedema.

where there may be bleeding in the retina (12). These individuals are clearly at risk of progressing to proliferative retinopathy with risk to their eyesight. Stage four is known as proliferative retinopathy in which there is excessive bleeding due to scarring and the formation of new blood vessels in the retina, which may lead to retinal detachment (12). At this stage, treatment is given to try to stabilise vision. The final stage is diabetic maculopathy, which is a result of the blood vessels in the macula leaking or becoming blocked (12). This stage requires treatment and referral to a specialist.

Aims

The main aims of this paper are to decide on how effective eye screening is for diabetic retinopathy and whether it is safe to extend the screening interval beyond one year. The studies that have been conducted will be assessed and the work critically analyzed resulting in the production of a well-informed decision as to whether or not it would be safe to extend the screening interval.

This paper will address the following:

- What is the evidence to support the screening programme?
- How cost effective is the screening programme?
- Is it safe to extend the screening interval beyond one year for diabetic retinopathy?

Methodology

This paper will be looking at a broad range of papers chosen from three main databases PubMed, Medline and Scopus. No date restrictions were enforced however articles which were not in English were not included in this paper, which helped to narrow down search results. The main key phrases that were used were 'diabet* retinopathy', 'screening interval', 'screening period', 'economics' and 'cost*-benefit analysis'.

Results

Using the PubMed database, the results were reduced from 32440 to 56 papers, from 36355 to 24 papers with Scopus and from 25131 to 5 papers with the MEDLINE database. Table 1 summarizes these results.

The 85 remaining papers were used and assessed on their content, from which 18 papers were selected for my discussion (Table 2). The 18 papers were selected after reading through the titles and abstracts of each of the papers and then deciding which papers were most relevant in order to address the aims. The databases picked up two systematic review papers (13, 14), from which papers discussed in the review were used for the discussion.

Table 1. Search terms with number of results from three different databases.

Phrases	PubMed	Scopus	MEDLINE
'Diabet* Retinopathy'	32440	36355	25131
'Diabet* Retinopathy' and 'Screening interval' or 'Screening period'	1093	447	25
'Diabet* Retinopathy' and 'Screening interval' or 'Screening period' and 'Cost*-benefit analysis' or 'Economics'	56	24	5

Discussion

What is the evidence to support the screening programme?

There have been several studies to support the diabetic retinopathy screening programme. A study conducted by Prasad *et al.* (32) has shown that screening with slit-lamp indirect ophthalmoscopy by suitably trained optometrists is an effective and efficient way of screening for diabetic retinopathy. With all screening programmes there are likely to be a certain number of false positive and false negative cases. The diabetic eye screening programme abides by 'Exeter Standards' for sensitivity, the extent to which true positives are detected, which is recorded at 80% and specificity, the extent to which false positives are recorded, which is set at 95% (33). This method of screening had a sensitivity of 76% and specificity of 95% (32). This same study had a very low false negative rate of 1.16% and a false positive rate of 30.18%. There was a high ratio of 16.54 that a positive test indicates sight-threatening retinopathy and a ratio of 0.25 for a negative test. Additionally, the technical failure rate was very low at 0.2% (32).

The study mentioned above highlights the merits of the diabetic eye screening programme and there have also been several studies published that have provided evidence in support of a yearly diabetic eye screening programme. For example, a study by Backlund *et al.* (28) in a very large database study in Stockholm showed that the risk of developing severe diabetic retinopathy causing blindness has been reduced by an average of 11% per year by early intervention as a result of regular screening of the eyes. This is quite a significant find, which seems to validate the benefits of annual screening.

On the other hand, some authors have challenged this, suggesting that a blanket approach to screening those with diabetic retinopathy may not be appropriate. For example those who have just been diagnosed with diabetes are probably unlikely to attend screening for a few years (19) and compliance may be weaker with younger patients. This has been backed up by Tung *et al.* (19), who looked at the non-attendance of patients at the diabetic retinopathy clinic. This study has identified factors such as patients who have been treated for diabetes for a long time as well as older patients as having better compliance. Saadine *et al.* (20) also concluded that patients who are older or have poorer eyesight are more likely to be compliant with the annual screening programme. Of the 5000 diabetic patients, 1/3 (1667) were diagnosed with diabetic retinopathy and did not attend any screening appointments with an ophthalmologist. Having a yearly diabetic screening appointment may be viewed as a burden by those patients who are already dealing with quite a complex condition (20). Saadine *et al.* (20) looked at the compliance rate of diabetic eye screening; only 27.6% of 2412 patients, who received an eye examination, returned for a follow up yearly eye screening appointment.

Numerous studies have shown that regular eye screening in diabetic patients is highly effective in diagnosing eye disease at an early stage when it is amenable to treatment. These studies have also shown that early interventions have reduced the incidence of blindness in diabetic patients.

Diagnosed Type 2 Diabetes: A Cost-Effectiveness Analysis. Medicine 2015. 94(45):e1989

16. Scanlon P H, Aldington S J and Leal J *et al.* Development of a cost-effectiveness model for optimisation of the screening interval in diabetic retinopathy screening. Health technology assessment (Winchester, England) 2015. 19(74):1-116

17. Kawasaki R, Akune Y and Hiratsuka Y *et al.* Cost-utility analysis of screening for diabetic retinopathy in Japan: a probabilistic Markov modeling study. Ophthalmic epidemiology 2015. 22(1):4-12

18. Yeo S T, Edwards R T and Luzio S D *et al.* Diabetic retinopathy screening: perspectives of people with diabetes, screening intervals and costs of attending screening. Diabetic medicine : a journal of the British Diabetic Association 2012. 29 (7):878-85

19. Tung T H, Shih H C and Chen S J *et al.* Economic evaluation of screening for diabetic retinopathy among Chinese type 2 diabetics: a community-based study in Kinmen, Taiwan. Journal of epidemiology 2008. 18 (5):225-33

20. Saadine J B, Fong D S and Yao J. Factors associated with follow-up eye examinations among persons with diabetes. Retina (Philadelphia, Pa) 2008. 28(2):195-200

21. Klein R. Screening interval for retinopathy in type 2 diabetes. Lancet (London, England) 2003. 361 (9353):190-1

22. Dutra Medeiros M, Mesquita E and Gardete-Correia L *et al.* First Incidence and Progression Study for Diabetic Retinopathy in Portugal, the RETINODIAB Study: Evaluation of the Screening Program for Lisbon Region. Ophthalmology 2015. 122 (12):2473-81

23. Vijan S, Hofer T P and Hayward R A. Cost-utility analysis of screening intervals for diabetic retinopathy in

Table 2. Papers which were assessed, used and referenced in the discussion.

Title	Author/Journal	Key findings
Strategies to Screen for Diabetic Retinopathy in Chinese Patients with newly diagnosed Type 2 Diabetes: A Cost-Effective Analysis (15)	Wu B, Li J, Wu H. /Medicine (Baltimore)	$7,485 per Quality-Adjusted Life Year (QALY) for four yearly intervals
Development of a cost-effectiveness model for optimization of the screening interval in diabetic retinopathy screening (16)	Scanlon PH, Aldington SJ, Leal J, Luengo-Fernandez R, Oke J, Sivaprasad S, Gazis A, Stratton IM. / Health Technology Assessment	Those at low risk screened every 5 year (99-100% effective), medium every 2 years (43-48%) and high risk every 2 years (55-59%) at £30,000 per QALY
Cost-utility analysis of screening for diabetic retinopathy in Japan: a probabilistic Markov modeling study (17)	Kawasaki R, Akune Y, Hiratsuka Y, Fukuhara S, Yamada M. /Ophthalmic Epidemiology	Effective at $11,857 per QALY if screened every 3 years (within £20,000 threshold)
Diabetic retinopathy screening: perspectives of people with diabetes, screening intervals and costs of attending screening (18)	Yeo ST, Edwards RT, Luzio SD, Charles JM, Thomas RL, Peters JM, Owens DR. /Diabetic Medicine	Patients happy with longer intervals so long as they are reassured
Economic evaluation of screening for diabetic retinopathy amongst Chinese type 2 diabetics: a community-based study in Kinmen, Taiwan (19)	Tung SH, Shih HC, Chen SJ, Chou P, Liu CM, Liu JH. /Journal of Epidemiology	New Taiwan (NT) $21,924 (£563) per QALY per year, NT $25,319 (£650) per QALY every two years. Any screening interval more cost effective than no screening, NT $61,542 (£1,580)
Factors associated with follow-up eye examinations among persons with diabetes (20)	Saadine JB, Fong DS, Yao J. /Retina	27.6% compliance rate of annual screening programme
Screening interval for retinopathy in type 2 diabetes (21)	Klein R. /Lancet	Incidence of sight-threating diabetic retinopathy, 0.3% in year 1 to 1.8% at end of 5 years in those with no retinopathy
First incidence and progression study for diabetic retinopathy in Portugal, the RETINODIAB study: Evaluation of the screening programme for Lisbon region (22)	Dutra M, Mesquita E, Gardete-Correla L, Amaral- Turkman A, Raposo JF. /Ophthalmology	1.18 to 0.52% decrease in incidence of referable diabetic retinopathy in those with mild diabetic retinopathy. (109,000 tests)
Cost utility analysis of screening intervals for diabetic retinopathy in patients with type 2 diabetes mellitus (23)	Vijan S, Hofer TP, Hayward RA. /The Journal of the American Medical Association (JAMA)	$40,530 more per QALY if screened every year as opposed to every two years
Incidence of diabetic retinopathy in people with type 2 diabetes mellitus attending the Diabetic Retinopathy Screening Service for Wales: retrospective analysis (24)	Thomas RL, Dunstan F, Luzio SD, Chowdury SR, Hale SL, North RV, Gibbins RL, Owens DR. /British Medical Journal (BMJ)	Not safe to increase interval if on insulin treatment or had diagnoses of diabetes for over 10 years
Cost effectiveness of treating and detecting diabetic retinopathy (25)	Javitt JC, Aiello LP. /Annals of Internal Medicine	$1,996 for insulin dependent and $3,530 for non insulin dependent per QALY
Cost-Effectiveness of strategies for detecting Diabetic Retinopathy (26)	Dasbach EJ, Fryback DG, Newcomb PA, Klein R, Klein BEK. /Medical Care	319 eyes sights saved from a cohort of 1000 in their lifetime
Incidence of sight-threatening retinopathy in Type 1 diabetes in a systematic screening programme (27)	Younis N, Broadbent DM, Harding SP and Vora JP. /Diabetic Medicine	Incidence of sight-threatening diabetic retinopathy 9.9% increase in those with mild and background retinopathy over 5 years (501 patients)
New Blindness in Diabetes Reduced by More Than One-Third in Stockholm County (28)	Backlund LB, Algvere PV, Rosenqvist U. /Diabetic Medicine	Sight-threatening diabetic retinopathy progression reduced by 11%
Six-Year Retrospective Follow-Up Study of Safe Screening Intervals for Sight-Threatening Retinopathy in Patients with Diabetes Mellitus (29)	Soto-Pedre E, Herneaz-Ortega MC, Vazquez JA. /Journal of Diabetes Science and Technology	Type 2 diabetes with mild non-proliferative diabetic retinopathy had 8% chance of progressing to sight-threatening retinopathy after 2 yearly screening intervals
Screening for diabetic retinopathy in James Bay, Ontario: a cost-effectiveness analysis (30)	Maberley D, Walker H, Khoushik A, Cruess A. /Canadian Medical Association Journal	Camera method was $15,000 per QALY, much less than NICE guidance threshold of £20,000
Cost of a Community-Based Diabetic Retinopathy Screening Program (31)	Byrne M, Parker D, Tannenbaum S, Ocasio M, Lam B, Zimmer-Galler I, Lee D. /Diabetes Care	58% of total cost equated to staff wages
Effectiveness of optometrist screening for diabetic retinopathy using slit-lamp biomicroscopy (32)	Prasad S, Kamath GG, Jones K, Clearkin L, Phillips R. /Nature	76% sensitivity and 95% specificity according to Exeter Standards

How cost effective is the screening programme?

A number of authors have reported that screening for diabetic retinopathy is one of the most cost-effective medical interventions. A small scale screening programme of 607 economically disadvantaged Americans was reported to cost $188 per abnormality identified and it is interesting to note that 58% of the total cost of running the programme ($91,294) was related to staff wages (31). Other authors have looked at the cost per Quality Adjusted Life Year (QALY) and again reported that screening is a highly cost effective medical intervention and it has been reported that the cost for insulin dependent diabetics is $1,996 and for non-insulin dependent diabetics $3,530 per QALY (25). This is well within what is considered cost effective by the National Institute for Health and Care Excellence (NICE), the normal procedures that cost less than £30,000 per QALY are deemed to be cost effective and well worth supporting.

Similar modelling undertaken in China advised that screening at 4 yearly intervals is cost effective ($7,485 per QALY) (34). Similarly, Kawasaki *et al.* (17) who looked at the Japanese health service concluded that 3-yearly screening is cost effective at $11,857 per QALY. However, one can criticize this study as the patients were not stratified by risk and the study had a low compliance rate of 37%.

Moreover, it is important to appreciate that the cost effectiveness and the cost per QALY is very dependent on the screening interval as an extreme example for patients identified as high risk, if the annual screening interval were to be reduced to 6 months the additional cost per QALY would be £272,000, which is significantly higher than the Department of Health's £30,000 maximum per QALY (35).

Other approaches aimed at reducing the costs of screening have included the use of retinal photography with a digital portable camera which has been shown to be much more cost effective in hard-to-reach populations (30) at a cost of $3,900 per sight years ($15,000 per QALY) compared to $9,800 per sight years ($37,000 per QALY) for those examined by retina specialists.

The studies reported here confirm that screening for diabetic retinopathy is cost effective if it is targeted with the interval of screening stratified by the risk of developing serious eye complications. However, it is not cost effective if such risk stratification is not used and the same screening interval is used for patients with different risk factors or if all diabetic patients have annual screening.

Is it safe to extend the screening interval beyond one year for diabetic retinopathy?

NICE guidance (published in 2015) recommends that those with type 2 diabetes, registered blind or partially sighted should be examined and screened annually (36). The majority of investigators have recommended assigning screening intervals according to risk stratification; it is clear that not every diabetic patient requires annual ophthalmic examination. Indeed, annual screening for low risk patients is highly inefficient and not cost effective. For those patients who are categorized as low risk, screening every two years as opposed to annually would reduce the cost per QALY by £404,000 (35).

Similarly, Scanlon *et al.* (16) recommended that patients at low risk should be screened at 5 yearly intervals, with a probability of this being cost effective at £30,000 per QALY being 99-100%. Those in the medium risk category, every 3 years with a probability of 43-48% and those who are at high risk should be screened every two years with a probability of 55-59% (16). This report supports the need for risk stratification.

Additionally, most patients with low risk can have screening intervals of greater than 12 months. However, patients considered at higher risk (those whose diabetes was diagnosed more than 10 years ago or patients dependent on insulin) continuation with annual screening is advisable, as was shown by Thomas *et al.* (24). Some authors have argued that it is not cost effective to screen diabetic patients who are not insulin dependent (26).

patients with type 2 diabetes mellitus. Jama 2000. 283(7):889-96

24. Thomas R L, Dunstan F and Luzio S D *et al.* Incidence of diabetic retinopathy in people with type 2 diabetes mellitus attending the Diabetic Retinopathy Screening Service for Wales: retrospective analysis. BMJ 2012. 344:e874

25. Javitt J C and Aiello L P. Cost-effectiveness of detecting and treating diabetic retinopathy. Ann Intern Med 1996. 124(1 Pt 2):164-9

26. Dasbach E J, Fryback D G and Newcomb P A *et al.* Cost-effectiveness of strategies for detecting diabetic retinopathy. Medical care 1991. 29(1):20-39

27. Younis N, Broadbent D M, Harding S P and Vora J P. Incidence of sight-threatening retinopathy in Type 1 diabetes in a systematic screening programme. Diabetic medicine: a journal of the British Diabetic Association 2003. 20(9):758-65

28. Backlund L B, Algvere P V and Rosenqvist U. New blindness in diabetes reduced by more than one-third in Stockholm County. Diabetic medicine: a journal of the British Diabetic Association 1997. 14(9):732-40

29. Soto-Pedre E, Hernaez-Ortega M C and Vazquez J A. Six-year retrospective follow-up study of safe screening intervals for sight-threatening retinopathy in patients with diabetes mellitus. Journal of diabetes science and technology 2009. 3(4):812-8

30. Maberley D, Walker H, Koushik A and Cruess A. Screening for diabetic retinopathy in James Bay, Ontario: a cost-effectiveness analysis. CMAJ : Canadian Medical Association journal 2003. 168(2):160-4

31. Byrne M M, Parker D F and Tannenbaum S L *et al.* Cost of a community-based diabetic retinopathy screening program. Diabetes care 2014. 37(11):e236-7

32. Prasad S, Kamath G G, Jones K, Clearkin L G and Phillips R P. Effectiveness of optometrist screening for diabetic retinopathy using slit-lamp biomicroscopy. Eye (London, England) 2001. 15(Pt 5):595-601

33. Scanlon P H. The English National Screening Programme For Diabetic Retinopathy 2003-2016. Acta Diabetol 2017. 54(6):515-525

34. Wu B, Li J and Wu H. Strategies to Screen for Diabetic Retinopathy in Chinese Patients with Newly Diagnosed Type 2 Diabetes: A Cost-Effectiveness Analysis. Medicine 2015. 94(45):e1989

35. Scholes M, Reed E and Bagland S. Diabetic eye screening interval - a review of evidence 2014 Available from: https://legacyscreening.phe.org.uk/policydb_download.php?doc=550

36. National Institute of Clinical Care and Excellence. Type 2 diabetes in adults: management: National Institute for Health and Care Excellence; London 2015

Published results have shown that it is safe as well as cost effective to increase the screening interval to 3 years (27). Younis *et al.* (27) documented that in type 1 diabetics with no retinopathy on enrolment into the study, the annual incidence of sight-threatening diabetic retinopathy is 0.3% which would increase to 1.8% when screened at 5 years. They found that in patients with no significant ophthalmic abnormalities on fundal photographs, it is safe to arrange a screening for diabetic patients at 2 to 3 yearly intervals rather than annually. The above data would support the concept of having annual screening for high risk patients and screening at three to five years for lower risk patients. This model has received wide acceptance and support by specialists in ophthalmology (21). One potential drawback to the longer screening interval is that it could lead to the patient believing that visual loss is unlikely and therefore may lead to decreased compliance.

Clearly for any screening programme to be effective it has to have the confidence and support of the patient. Provided that the patients can be reassured that it is safe to extend the screening interval to two to three years, most patients would be satisfied and happy to follow this suggestion, as shown in the form of a written questionnaire given to the patients (18).

In support of extending the screening interval is the work by Dutra Medeiros *et al.* (22) who advocated a personalised schedule of screening as the most beneficial in terms of health costs. The scheduling was based on each patient's individualised risk factors, including the duration of diabetes, age at diagnosis and insulin treatment (22).

Dutra Medeiros *et al.* (22) screened individuals without retinopathy and the incidence of diabetic retinopathy was 4.6%, after a further screening interval of 5 years, the incidence decreased to 3.87%. Those who had mild proliferative diabetic retinopathy, in the first year there was a 1.18% increase to referable diabetic retinopathy, which then decreased to 0.52% after a 5-year interval (22). Vijan *et al.* (23) reached the same conclusion of personalised risk stratification. In order to assess cost effectiveness they investigated patients aged 40 years and above and stated that a patient who is stratified as a 'high risk patient' would gain an additional 21 days of sight if they were to be screened annually as opposed to being screened once every 3 years compared to a 'low risk patient' who would only gain an additional 3 days of sight (23). Patients who are classed, as 'high risk' would cost an extra $40,530 per QALY gained if they were screened every year as opposed to every 3 years (23). Whereas, those in the 'low risk' category would cost an additional $211,570 per QALY if screened annually (23). The above work supports extending the interval of screening for 'low risk' patients.

Furthermore, Soto-Pedre *et al.* (29) looked at two cohorts of patients. If there was no retinopathy at presentation; the risk of developing sight-threatening diabetic retinopathy was 3% at 4 years whereas those with mild non-proliferative diabetic retinopathy is double that, at 6%, at 4 years. For those with mild non-proliferative diabetic retinopathy annual screening is advised. In patients with good metabolic control, screening every two years would be appropriate. However, extending the interval to three to four years would be appropriate for those diabetic patients with no retinopathy at enrolment (29).

Conclusion

Based on my review and reading, diabetic eye screening reduces the risk of developing sight-threatening diabetic retinopathy by detecting changes at an early stage whilst they are still amenable to treatment. In an environment with limited resources it is important to make the screening programme cost effective. This can be achieved provided that the screening interval is decided on by risk stratification; it seems appropriate that high risk patients should continue to receive annual screening with the lower risk diabetic patients having screening at longer intervals of up to 5 years.

You might also be interested in the research article by Melissa Leak on malarial retinopathy: see Research, page 19

Employability

Interviews with Professionals

Insider Imprint **have interviewed professionals from five different career paths finding out what their job involves, and how they got there. Find out about interesting careers straight from the source over the next five pages!**

There are so many employment options open to Life Sciences graduates that we couldn't cover them all here! You can find out about a lot of them at the *School of Life Sciences careers fair* that runs every year, and on the school employability pages here:

www.liverpool.ac.uk/life-sciences/employability

Considering Marketing?

Find out more from *Jordan Parke*

“What is your job title?”

“Field marketing manager at GradTouch.”

“What is GradTouch?”

“A graduate jobs website that champions a more transparent application process.”

“What 3 - 5 words best describe you?”

“Serious, at times office clown.”

“How did you get to your current position?”

“At university I used to study 5 days a week but also work 6 days a week. By the time I left university, I had already worked for some of the world’s biggest blue-chip companies, including Samsung, Pepsi, Nestle, Guinness and Carling Lager. I worked in various roles from a sales consultant to a branding specialist. I even managed 209 stores around the UK for Pepsi at 18. I met the founders of my current company at a tech industry event in Manchester and really liked the sound of their company culture. A few coffee's and sofa chats later - I was on the team.”

“What does a typical day look like for you?”

“I usually wake up around 8am, have a coffee or two and then make my way to the office. My day consists of liaising with universities, my team of brand managers and ambassadors as well as coming up with new ways to push our message on the ground. I also visit universities and do various events throughout the year to engage with students.”

“What are the 3 main things you do in your job?”

“Manage the field marketing division of GradTouch. Develop relationships with key university contacts including staff, societies and students. Visit different student cities and universities with my team.”

“What are the skills & attributes valued most by employers in your field?”

“A good sense of humour is key, especially when interacting with so many people on a daily basis. A good work ethic and the ability to roll up your sleeves and get stuck in helps too.”

What are the top 3 things you love about your job?

“1. The ability to choose my own team. 2. The flexibility to work when I want and how I want. 3. The unlimited holiday allowance we all get. Yes, that’s right. Unlimited holidays!”

“What advice would you give to a current student?”

“Just go for it. Sometimes students get bogged down with a job description and feel like they may not fit the criteria because they don't have experience. Think about the skills gained from your degree. That is an experience. The best advice I could give as cheesy as it sounds is just be yourself. Don't put on a 'game face' at an interview! If employers don't like the real you then why would you want to work there anyway?”

“Is there anything else you want to tell our students?”

“Sign up to gradtouch.com!”

Considering Student Engagement?

Find out more from *Sally Bracegirdle*

“What is your job title?”

“Head of Student Engagement at GradTouch, a graduate jobs website that champions a more transparent application process”

“Describe your field in 3 words”

“Creative, competitive & inspiring”

“How did you get to your current position?”

“I graduated with a degree in French Studies and no real idea of what I wanted to do next. After a few months of job hunting, I got an internship with GradTouch where I ended up working full-time, and have since climbed up to be their Head of Student Engagement.”

“What is one thing that most surprised you about your company/career?”

“My career probably isn't what I would've predicted back when I was at university, and that's actually a good thing! I've ended up learning so much, gained so many new skills and achieved things I never thought I would. Keeping an open mind is so important when it comes to your career.”

“What are the 3 main things you do in your job?

Write marketing content that is relevant and helpful to students and graduates. Run social media channels. Manage paid social media campaigns.”

“What might a downside to your job be?”

“Trying to maintain a creative mindset when you're suffering from writer's block, or you're just having a bad day!”

“What are the skills & attributes valued most by employers in your field?”

“Passion, personality and creativity”

“What are the top 3 things you love about your job?”

“No two days are ever the same - my job is very varied and certainly never boring! I get to play a part in helping students and graduates to find a career that's right for them. I also love that I can be creative while I'm helping people, whether that's through the content I write, or events I go to.”

“What advice would you give to a current student?”

“It's the same in any area really - always make sure that you do your research into the company you're applying to because you need to prove not just that you're the right person for the job, but also the right person for the company.”

“Is there anything else you want to tell our students?”

“My best advice for the job hunt is: don't compare yourself to others - always ask for feedback and learn from it - always be yourself in your job applications, your personality is the only thing that truly differentiates you from your competitors.”

Considering being a Zoo Ranger?

Find out more from *Emily Lake*

"What is your job title?"

"Zoo Ranger at Chester Zoo"

"Describe your field in 5 words"

"Public engagement in a zoo"

"What 3 words best describe you?"

"Outgoing, inquisitive & adventurous"

"How did you get to your current position?"

"I grew up in Australia, and I did animal and veterinary biosciences as my undergraduate degree. I then did an honours degree in evolutionary genetics of marsupials but realised I didn't want to work in a lab. All through university, I had been volunteering at zoos around Australia as part of my degree and also to gain experience in the industry to see if it was something I could do as a career. I trained at my local zoo as a zookeeper, but there was no paid work available. So I moved to the UK to do an unpaid internship at Chester Zoo. Since then, I have been employed by several zoos across the UK as both a zookeeper and a zoo educator. I have also completed a masters by research in biological anthropology at Durham University here in the UK. I am now based permanently at Chester Zoo as a member of the education team where I am a Zoo Ranger (public engagement officer)."

"What does a typical day look like for you?"

"Our shifts are 9 - 5, but that's where the similarities to an office job end. At 9am I check emails and catch up with my colleagues about what activities we are running that day. Zoo Rangers work across the whole zoo offering animal talks, educational sessions for visitors and school groups and supervising walk-through exhibits like the free flight fruit bat house. There is no such thing as a typical day, however, a basic zoo ranger shift might involve a couple of hours in the fruit bat exhibit, followed by 1 or 2 animal talks and an educational 'discover session' at an exhibit. We have some office time to research and design our activities alongside the other members of the education team."

"What are the 3 main things you do in your job?"

"Develop educational activities for zoo visitors. Deliver those activities across the whole zoo site. Work in collaboration with the wider education team on joint projects."

"What are the skills & attributes valued most by employers in your field?"

"Being able to translate complex topics into language a visitor with limited knowledge of the natural world will understand is a difficult but valued skill and one that is crafted over time."

"What are the top 3 things you love about your job?"

"Talking to visitors about all the amazing animals at Chester Zoo. Working in one of the best zoos in the world. Best of all getting people excited and enthusiastic about conservation and what they can do to help!"

"What advice would you give to a current student?"

"Experience, experience, experience! Your degree is an essential stepping stone but, you need things to help you stand out from the crowd and a well-rounded knowledge of the natural world. I have volunteered with conservation organisations, on farms, at veterinary clinics, zoos and wildlife reserves just to name a few. If you are motivated enough to seek out these unique opportunities that will show through on your CV and at an interview."

"Is there anything else you want to tell our students?"

"Come and visit Chester Zoo, the Zoo Rangers are always happy to chat to people about what we do and how we got to where we are!"

Considering Technology Development?

Find out more from *Dr Rocky Cranenburgh*

"What is your job title?"

"Chief Technology Officer at Prokarium Ltd."

"Describe your field in 5 words"

"Bacterially vectored recombinant vaccines & immunotherapies"

"What 3 - 5 words best describe you?"

"An amiable boffin, mostly harmless."

"How did you get to your current position?"

"I completed a PhD in genomics of cyanobacteria at Newcastle University in 1997 and joined the biotechnology company Cobra Biologics later that year. I spent two years from 1998 as a postdoc at the University of Oxford for a collaborative project and relocated in 2000 to Cobra's headquarters at Keele, Staffordshire. I managed Cobra's molecular biology group, worked on internal R&D projects and contracts for biopharmaceutical companies in the field of recombinant protein and DNA production. I co-founded Prokarium Ltd as a spin-out company from Cobra in 2012, to use live attenuated Salmonella for oral delivery of recombinant vaccines. In 2018 we relocated Prokarium to London."

"What are the 3 main things you do in your job?"

"Design and introduce new technologies, manage our patent portfolio and assist my colleagues with grant applications."

"What does a typical day look like for you?"

"I don't have a typical day, but in a typical week I travel to our office in London on Monday morning, spend Monday and Tuesday there for meetings with my colleagues, then travel back on Tuesday evening and spend the rest of the week working from home in rural Cheshire. Occasionally I visit academic collaborators and go to conferences."

"What are the skills & attributes valued most by employers in your field?"

"An ability to think for yourself and come up with solutions to problems, to research the literature and to be well organised."

"What are the top 3 things you love about your job?"

"1. Inventing new technologies, 2. Learning about the latest developments in molecular biology and immunology, 3. Working with a great team."

"What advice would you give to a current student?"

"There's never been a better time to enter the world of biotechnology, with lots of new and exciting companies to choose from."

"Is there anything else you want to tell our students?"

"The most important thing is to choose a career that interests you and that you enjoy."

Considering Diagnostics?

Find out more from *Dr Stuart Liddle*

"What is your job title?"

"Head of Genetics & Molecular Pathology"

"Describe your field in 3 words"

"Clinical molecular diagnostics"

"What 3 - 5 words best describe you?"

"Hard working & conscientious"

"How did you get to your current position?"

"I wasn't quite sure what job I wanted to do so I went on to do a PhD in Molecular Genetics, and through contacts I made I got a job in London in a new private clinical genetics laboratory. Here I helped develop a new prenatal diagnostic assay that helps revolutionise foetal diagnostics. My developing company was bought by a larger pathology provider and expanding our testing portfolio and providing high quality, fast bespoke service helped the lab grow. Eventually, we formed a new company collaborating with large NHS hospitals, consolidating many molecular pathology labs together in a new building in central London, where I was made the head of the department."

"What does a typical day look like for you?"

"The days are varied and not necessarily typical. A day may include staff organisation and management, emails & phone calls to clients, some authorisation of patient reports, developing new assays and testing pathways, attending meetings, quality assurance/compliance, staff training and problem-solving."

"What is one thing that most surprised you about your company/career?"

"My family consists of many generations of coal miners in Northumberland (including my dad and brother), but when the mines closed in the 1980's I had to find something else to do! I was lucky enough to make it to university and while I was never ambitious, I was always hard working and wanted to do the best job I was capable of. On occasional introspective moments, I wonder how I got here and how life could have been different!"

"What are the skills & attributes valued most by employers in your field?"

"Attention to detail, organisation, hard work and genuinely caring about the work you do."

"What are the top 3 things you love about your job?"

"Providing a fast, quality service for patients. Successfully developing and introducing new tests required by consultants. Helping people is very rewarding."

"What advice would you give to a current student?"

"It can be very rewarding providing a health service to the public, but please make sure it's something you really want to do."


Insider
Imprint