An Investigation into the evolution of racial based diseases – Glioma using a drosophila model.

By

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Declaration

I declare that this thesis has been composed by me, and while registered as a candidate for the degree for which this submission is made, I have not been a registered candidate for any other research degree at this or any other university or institution. No material in this thesis has been used in any other submission for academic qualification.

Signature ……..

Abstract

With cancer representing 13% of all deaths worldwide, it is the second biggest cause of death. Cancers of the brain despite being a rare cancer has shown increased occurrence rates of 39% in the last 40 years. This increased rate is due to difficulty in treating brain cancers.

Variation of the genome makes some individuals more susceptible to cancer than others. Brain tumors form familial mutations that are passed down to offspring, this is evidently seen throughout the human population and can be linked to an individual's racial background. This research looked at a protein associated with glioma formation (merlin) and the inheritance of that protein to deduce whether cancer associated proteins follow Mendelian genetics or if epigenetics carries an important role. Merlin is encoded by the NF2 gene, and mutation causes the glioma predisposition, neurofibromatosis in humans. Merlin is seen homologous within both humans and *Drosophila melanogaster (D. melanogaster)* proving *D. melanogaster* to be a suitable model organism to study merlin. In this study three populations of *D. melanogaster* were bred, each with a distinct allele of merlin (Mer 3, Mer 4 and Wild-Type Mer), these alleles represented the variation within the human population; races. Populations were crossed to produce hybrid populations of the three variants of merlin. The mortality and survival rates were calculated and together with the expected phenotypic ratio showed that the drosophila were often surviving in higher rates than were expected and thus natural selection is suggested to be at work with the evolution of a population based on a gene for a cancerous phenotype.

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Acknowledgements

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Abbreviations

A.D. – Anno Domini
ASE – Allele specific expression
Cdc42 – Cell division control protein 42 homolog
CRL4 - Cullin-ring ligase 4
CT – Cycle Threshold
CUL4 – Cullin 4A
D. melanogaster – Drosophila melanogaster
DCAF1 - DDB1 and CUL4 associated factor homolog 1
DDB1 – DNA damage binding protein 1
Dmer – Drosophila Merlin
DNA - Deoxyribose Nucleic Acid
ERK – Extracellular signal-regulated kimases
ERM – Ezrin Radixin Moesin
FERM – 4.1 Protein Ezrin Radixin Moesin
GBM – Glioblastoma multiform
Gln - Glutamine
IDT – Integrated DNA Technologies
Ile - Isoleucine
JNK – c-Jun N-terminal kinases
kDa - Kilo Daltons
M3 – Merlin 3
M4 – Merlin 4
Mer - Merlin
Mer 3 – Merlin 3
Mer 4 - Merlin 4
Merlin – Moesin Ezrin Radixin like protein
Met - Methionine
mTOR – mechanistic Target of Rapamycin
mTORC1 - mechanistic Target of Rapamycin complex 1
NF1 - Neurofibromatosis Type 1
NF2 - Neurofibromatosis Type 2
NHEJ – Non-homologous end joining
NHERF - Na+ H+ exchange regulatory cofactor
Non-GBM – non-Glioblastoma multiform
Pak – Serine/Threonine protein kinase
PCR – Polymerase Chain reaction
PDGFR – Plate-derived growth factor receptor
PI3K – Phosphoinositide 3-kinase
PIKE-L - PI3K enhancer long form
POT1 – Protection of Telomeres protein 1
QoL – Quality of Life
Rac1 – Ras-related C3 botulinum toxin substrate 1
Raf – Rapidly accelerated fibrosarcoma
RNA – Ribose Nucleic Acid
RTK – Receptor tyrosine kinase
SEER - Surveillance, Epidemiology and End Results program
SNP – Single Nucleotide Polymorphism
TPP1 – Tripeptidyl peptidase 1
TS – Tumour Supressor

UK – United Kingdom
WHO - World Health Organisation
Wt – Wild-Type
Xch – X chromosome

CHAPTER 1

INTRODUCTION

1.1 The Origins of Cancer

The human body is a multi-cellular organism, with many types of cells that are often specialized to a specific role. These individual roles work as a collective, thus maintaining the body's natural ecosystem and homeostasis. As with all living things, the human body ages and the cells die by natural causes including apoptosis, necrosis and autophagy, which must be replaced. Cells undergo mitotic division by which they duplicate and then separate their genome and organelles, dividing into two identical daughter cells (See Figure 1.1).

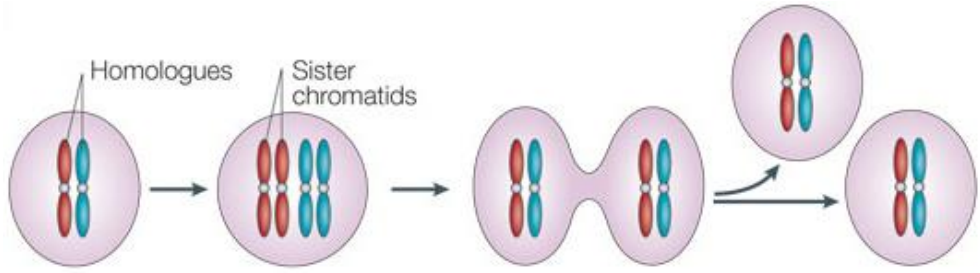


Figure 1. 1 – Adapted from Marston and Amon, 2004 shows a cell going through cell division. The cell duplicates its' genome/chromosome(s) and then divides into two identical daughter cells

As cells replicate their genome, mistakes may occur within the DNA code. These mistakes are mutations, where one nitrogenous base is replaced by another. Though minor, these mistakes may result in a change of the protein in which that genomic segment is encoded for. Similarly, other forces such as an environmental change can alter/mutate DNA. Insignificant to the origin, DNA mutations are often dealt with efficiently by the body's DNA repair mechanisms (Sudhakar, 2009). The mechanisms that repair DNA work both effectively and efficiently and repair most mutations that occur. Despite the sufficient repairs that cells can make to their DNA some cells cannot correct the genome, and this can result in the cell often over or under producing a protein or altering a mechanism or pathway (Iyama and Wilson, 2013).

The changes to proteins and pathways often add up, until a cell has several mutations in key areas, which will be discussed below. This cell with its' multitude of mutations can be deemed 'cancerous'. All is not lost, however, as again most cells that reach this stage would be swiftly removed by the bodies adept immune response. The cancerous cells would need further mutations to its' recognition to evade immune destruction (Sudhakar, 2009). Ultimately, this would require mutations to the proteins that are used for cells to recognize each other and recognize their condition as either viable or non-viable (Vinay, et. Al,. 2015). Cells that are non-viable would 'appear' this way to the immune response and be removed. However, a mutation in this recognition system would allow a non-viable cancer cell to appear functionally normal and the immune response would swiftly move on, leaving the cancer cell unharmed. From here cancer cells would rapidly begin to proliferate due to mutations in the control of their cell cycle, which would lead to tumour formation (Sudhakar, 2009).

Cancer as a disease is seen throughout the phylogenetic tree of multi-cellular organisms due to replication mutations causing tumors, both benign and cancerous (Ewald and Swain Ewald, 2015). The disease's origin would have occurred with the evolution of replication, a fundamental principle that began with the transition from chemistry to biology; life. The transition of single cell becoming multi-cellular and specializing would have therefore produced cancer as seen within plants and most of the animal kingdom. Potential fossil evidence of cancer and even early descriptions may show that cancer was described by our oldest of human ancestors (Odes *et al*., 2016).

Firstly described as an imbalance of fluids, the idea of cancers' origin has vastly shifted throughout the centuries. Theories such as cancer arising from trauma or a parasite have all led to modern times with

malignant cell theory to the introduction of genes and thus the process described above (Sudhakar, 2009).

Cancer as a disease is now defined by its' over-proliferative capabilities. This is a result of the aforementioned inability of a cell to repair its' mutated DNA and as mentioned, mutations in key areas of the cell's genome are what birth a cancerous cell.

In 2000 Hanahan and Weinberg deduced that most cancers, if not all, required mutations in various traits to be deemed cancerous. Similarly to above, key areas of a cell's genome must mutate for cancer to occur. Therefore, the authors assimilated that there were six 'Hallmarks of cancer' (see Figure 1.2.)

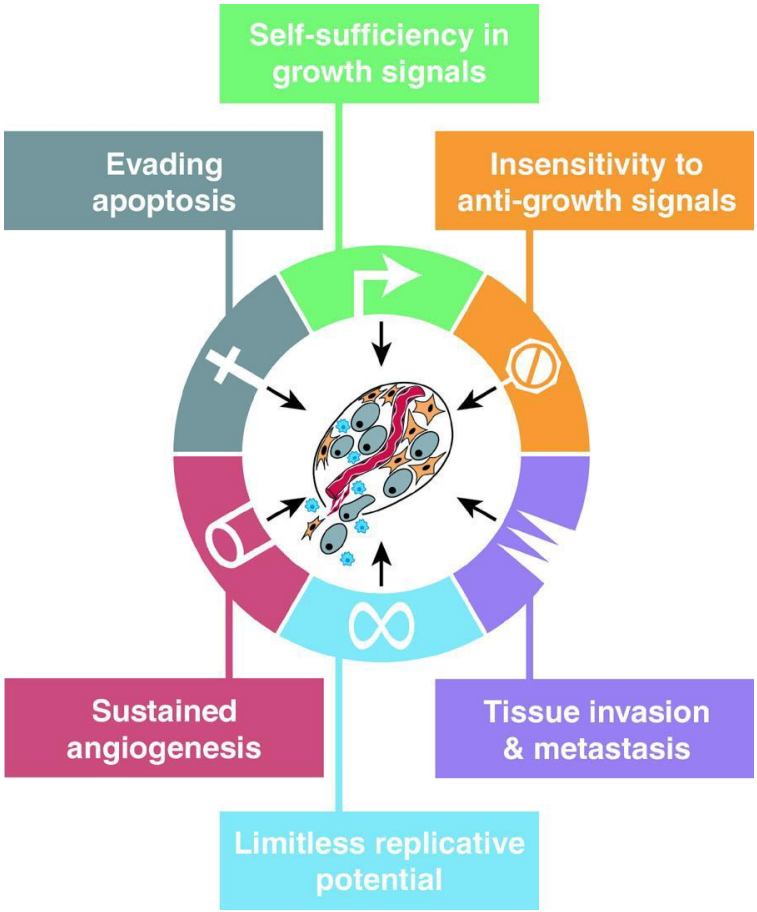


Figure 1. 2 - Taken from Hanahan and Wienberg, 2000. The figure shows the six hallmarks of cancer, the cell traits or processes that many cancers show a mutation in.

Hanahan and Weinberg further stated that these six hallmarks were essential for cancer formation. Self-sufficiency in growth signals and insensitivity to anti-growth signals work in unison, as a mutation to

the cell's growth-signal recognition allows it to ignore signals to stop growing and constantly activate signals to grow (Hanahan and Weinberg, 2000). These in turn allow the next pair to commence; evading apoptosis and limitless replicative potential (Hanahan and Weinberg, 2000). Evading apoptosis or programmed cell death results in the cell ignoring signals to begin to die at the same time following signals to both grow, and to continue replicating. The four hallmarks discussed, seen in figure 1.2, allow the cells to grow and replicate to begin forming a tumour. However, with rapid growth comes the need for high quantities of oxygen and energy in the form of glucose. It is therefore essential for the fifth hallmark to be present, sustained angiogenesis (Hanahan and Weinberg, 2000).

Angiogenesis is the formation of new blood vessels from currently existing ones. Cancerous tumours can reach a growth limit, restrained by their need for excessive oxygen and glucose. A tumour would remain in this restraint if it were not for another mutation. A mutation in angiogenesis-based genes allows the tumor to begin manipulating blood vessels, producing its own supply. This new supply of blood can unrestraint the tumor, increasing its growth capacity. This lastly brings in the sixth hallmark, tissue invasion and metastasis. Without angiogenesis or a blood vessel in close proximity, cancers will remain in the area they have initially formed. However, with a mutation in the cell's ability to stay connected to other cells, a single cancerous cell can become dislodged and enter the blood vessel. Further mutations to this dislodged cell allow it to take hold in a new location and continue its growth and replication causing secondary tumours.

In 2011 Hanahan and Weinberg produced a follow-up journal to their hallmarks and discussed the knowledge gained about them in the decade between that and their previous papers. In this paper they

discuss two further hallmarks, which they deem 'emerging hallmarks'. Both emerging hallmarks have been mentioned, avoiding immune destruction and deregulating cellular energetics (see Figure 1.3.)

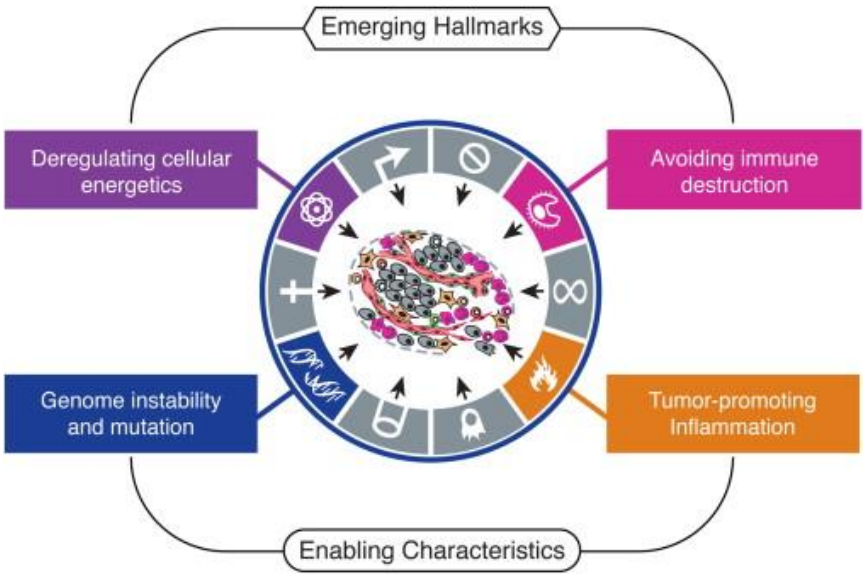


Figure 1. 3- Taken from Hanahan and Wienberg, 2011. The figure shows the two emerging hallmarks of cancer, and two enabling characteristics of cancer. These are an addition to the previous six hallmarks, the cell traits or processes that many cancers show a mutation in.

The ability to avoid the immune response by cancer cells has been discussed above and would be utilised the most during a cell's migration through the blood stream i.e metastasis, and the invasion of new areas of the body (Hanahan and Weinberg, 2011). The requirement for high levels of oxygen and glucose has been stated earlier in this chapter. However the emerging hallmark results in a cancerous cell having mutations in the structure of the energy pathways and thus a change in these may allow more glucose uptake. This potential increase of glucose would in turn fuel cancerous growth, replication and metastasis (Hanahan and Weinberg, 2011).

Enabling characteristics are those traits that appear at the beginning of cancer. Genomic instability and mutation is by far the most extensively researched of these as the mutations of the unstable genome are what produce the further hallmarks. The inflammatory response under taken by the innate immune system, can promote the other hallmarks,

producing a cancer positive environment, allowing cancer to take hold more easily (Hanahan and Weinberg, 2011).

1.2 Epidemiology/Cancer Statistics and Treatments

An estimated 8.2 million people die from cancer each year representing globally, 13% of worldwide deaths. Cancer is the second biggest cause of death, after cardiovascular diseases. Figure 1.4, shows the incidences of cancer worldwide in 2012. Note that genders have not been defined and age has been standardised (World Health Organisation, 2012).

Estimated age-standardized rates (World) of incidence cases, both sexes, all cancers excluding non-melanoma skin, worldwide in 2012

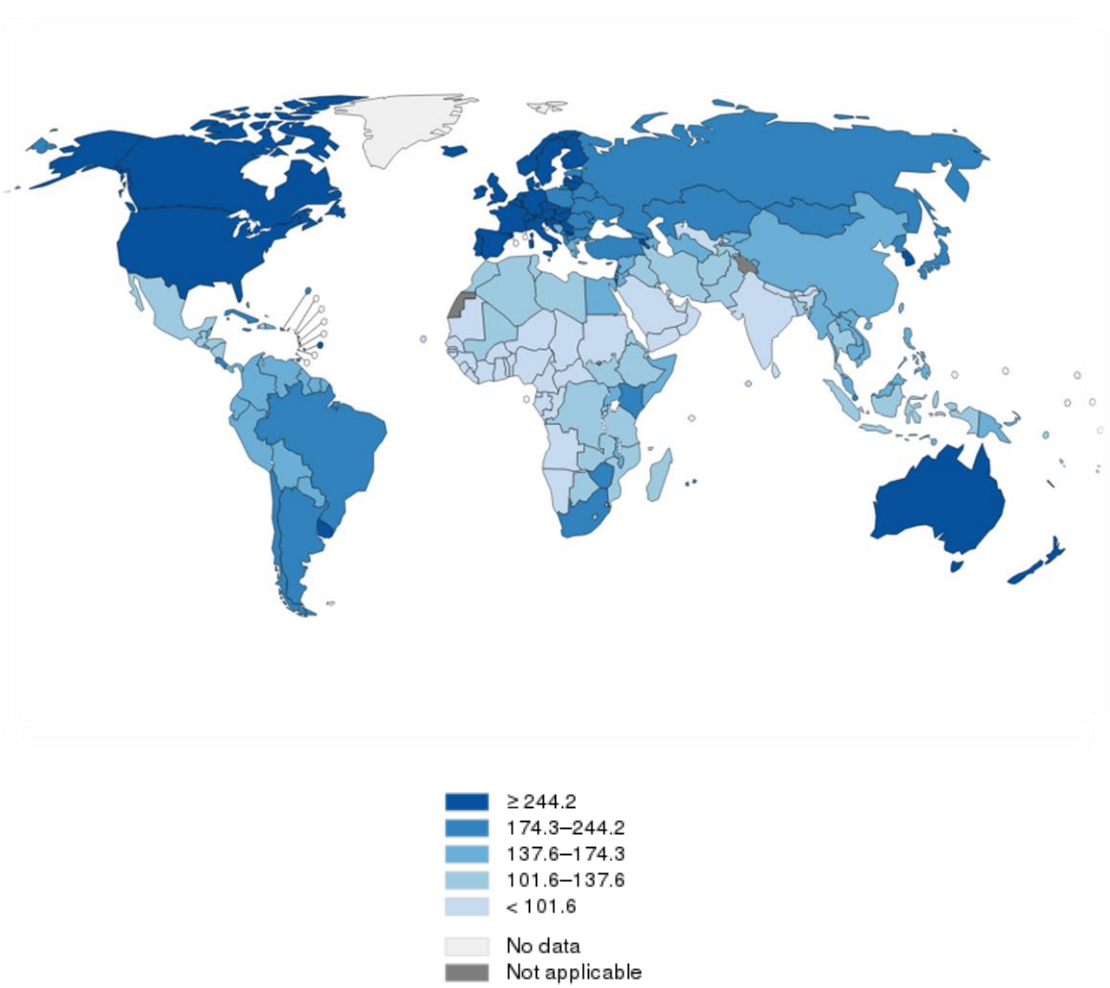


Figure 1. 4– Map showing the estimate cases of cancer worldwide in 2012 taken from World health Organisation. Key is the number of case per 100,000.

The cost of cancer is of major concern worldwide, but can be seen with specificity to the NHS in the UK (Department of Health GOV.UK, 2017). The government policy 2010-2015 on cancer research and treatment gives the following statistics (Department of Health GOV.UK, 2017). Annually, 250,000 people are diagnosed with cancer and, 130,000 people die as a result of cancer in the UK (Department of Health GOV.UK, 2017). The document produced by the Department of Health states that despite the UK's wealth, the survival rates are not improving as significantly as countries of a similar wealth. Lastly the Department of Health document discusses some monetary figures. The NHS annually spends £5 billion on cancer services. However, the cost overall to society, including further treatment and loss of production is approximated at £18.3 billion annually (Department of Health GOV.UK, 2017).

With statistics this high, research into cancer is required to allow for new concepts and potential treatments to be introduced as well as providing a sustainable cost to cancer services and treatment.

The current treatments of cancer fall into three main areas: radiotherapy, chemotherapy and surgery (World Health Organisation, 2017). Radiotherapy is treatment through radiation usually in the form of rays. These can include X-rays and electron beams. Chemotherapy is treatment through chemicals and substances. Chemotherapy often targets one of the hallmarks mentioned above. For example, temozolomide targets cancer cell DNA during its replication, vincristine targets the cells as they replicate, and bevacizumab is used to stop angiogenesis (National Cancer Institute, 2017). In the aforementioned Department of Health policy document 2010-2015, additional funds to treatment were discussed for the future. £23 million invested to improve radiotherapy and £250 million to develop

proton beam therapy. Increased funds have been available through 2010-2015, and further increases were available through 2016 and beyond that, as NHS England supply funds to the NHS sectors. From treatments and funding for medical care, comes a second major aspect to patients, their quality of life (QoL). Quality of life is significantly reliant on the cancer type and the amount of pain that the patient feels, with demographics such as age or income having little or no correlation to QoL (Heydarnejad *et al.* 2011). Last to discuss is some of the underlying factors to cancer. Highlighted in the above Department of Health policy were apparent changes to society and the population, which in turn are changing cancer statistics but are not being emphasized enough. The two, mainly focused on, are smoking and improving diet/reducing obesity (Department of Health GOV.UK, 2017).

Cancer treatments are often held back by the extensive studies that must be completed but more so by the research that leads to new potential treatment and therapeutic options.

1.3 The Inheritance of Cancer

Changes within a genome are often seen as the root of cancers because without the multitude of mutations to the genome, a cell cannot become cancerous (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). However, it is important to discuss that many cells will gain a mutation through mistakes in cell division, as mentioned above, and will not become cancerous. Without several mutations in the key areas of the genome i.e. those areas that encode proteins active or associated with the hallmarks of cancer, many cells do not become cancerous (Hanahan and Weinberg, 2000). The cells that do gain mutations, be it one or numerous, will usually be detected by the body and enter apoptosis or be subject to immune destruction. (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

The fundamental statement to the theory of evolution, proposed by Charles Darwin in 'On the Origin of Species' in 1859, which is now known to be the main factor for evolution is mutation of the genome and variation.

"It seems pretty clear that organic beings must be exposed during several generations to the new conditions of life to cause any appreciable amount of variation" – Charles Darwin, 1859.

Exposure to new conditions causes variation and this was apparent to Darwin and is still key to the entire central dogma of molecular biology. Cancer can only occur by variation, often this variation is causes by a new or changing condition.

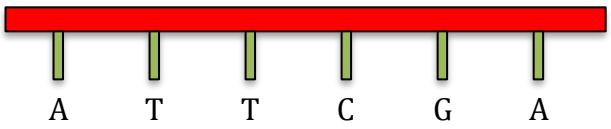
Cellular mutation is often anchored to the individual person/patient due to the mutated cell location i.e. non-sex cells. In contrast, a mutation within the sex cells can be passed down to an offspring. All cells within the body have the same genome within them, so if a mutation inherited increases a person's chance of cancer, this can in

turn be passed down and form a hereditary cancer. This can be explained by the schematics below.

Described below is three short sections of single-stranded DNA, they have no scientific relevance and are used only as an explanation.

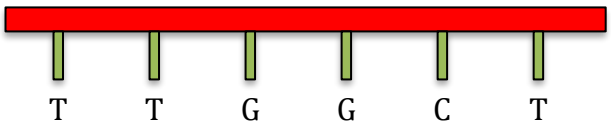
The first line is the DNA seen in many of the cancer cells, in which a key gene and therefore protein is a mutation that causes cancer.

A)



The second row is the DNA seen in non-cancerous cells, in which the gene and protein continues its normal function.

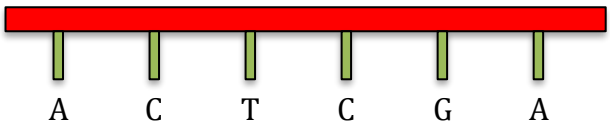
B)



Looking at the two lines of code (A and B), it is clear that the bases are dissimilar and would require many mutations to the one section of DNA to mutate this single protein. Of the six DNA bases shown, only one (the second base, thymine) is the same between both segment A and B. This means five point mutations would be required to healthy, non-cancerous segment B for it to become cancerous segment A.

The last segment of DNA (C) is a healthy, non-cancerous segment and still encodes the protein that functions normally. However, comparing this segment (C) to the first (A) it is clear that they are more similar and in fact only one change of base (the second base, cytosine to a thymine) is required to mutate this healthy, non-cancerous DNA segment into the cancerous one.

C)



From this, it is clear that throughout humanity many variations of the same DNA code exists and some individuals carry a variation that is more similar to the cancerous form and would therefore require less mutation to allow a cell to become cancerous.

In 1971, Alfred Knudson conceived the theory known as the two-hit hypothesis. Knudson's theory not only stated that cancer is an accumulation of many mutations, but Knudson explained how cancer can be inherited and develop later in life. It is important to note that a mutation of one chromosome would often result in the cell utilizing the second chromosome and thus cancer can often be avoided through this action. However, if the second chromosome then becomes mutated, the outcome is inevitable, and cancer will form. The variations of a chromosome, which produce different phenotypes, are called an allele.

Knudson stated that cancer has two categories in his hypothesis, namely familial and sporadic. Familial cancer is an inherited mutation in one chromosome, where the second becomes mutated during development. Sporadic cancer, on the other hand, is where no mutated alleles are inherited and instead both chromosomes mutate within development at different stages of cell differentiation. In 2012 Lee highlighted Knudson's theory and its uses in modern disciplines and principals. Lee discussed genotypic mutation, epigenetics and modification of gene expression; concepts that are now often discussed with cancer and that the concepts require research with regards to cancer inheritance (Lee *et al.* 2012).

A new study has highlighted that up to 10% of all cancers are non-sporadic and are due to a hereditary links (Romero and Castro, 2017). The authors continued by stating that high-throughput sequencing has revealed many new germ line cancers that were previously thought to be only somatic and anchored to the individual patient with no

possibility of being passed down a generation (Romero and Castro, 2017). Many genetic causes related to hereditary cancer are unknown this can be seen in figure 1.5, taken from the study by Romero and Castro, 2017.

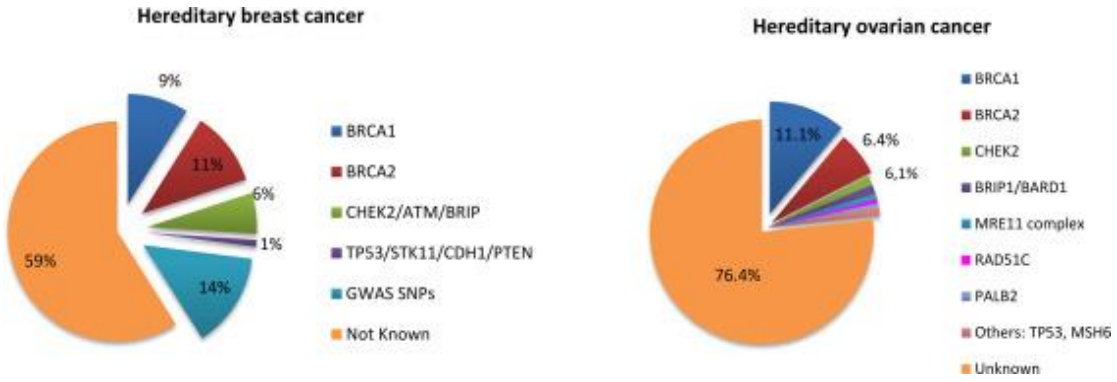


Figure 1. 5- Taken from Romero and Castro, 2017. Two pie charts to show the genetic causes of hereditary breast and ovarian cancer. Note the unknown genetic cause to hereditary breast and ovarian cancer to be 59% and 76.4% respectively.

The authors noted that it is expected for many more genes to be revealed to show a link to both breast and ovarian cancer (Romero and Castro, 2017). Transferring this concept to other cancer types would not be difficult with most other cancer types expected to have a similar hereditary disposition.

1.4 Cancers of the Brain – Glioma

The human brain is the feature that is always discussed in regards to human evolution and the differences in brain size to the rest of the animal kingdom. But despite this, most animals have similarly structured brains and entire nervous systems (Ledda *et al.,* 2004). The nervous system evolved to complete the role of various action potentials in eukaryotes as single-celled organisms became multi-cellular organisms. The brain was born from collections of neuronal cell bodies/ ganglia and has rarely changed throughout vertebrate evolution. This can be seen in the neuronal cell study on reptiles and mammals (Ledda *et al.,* 2004).

With the evolution of the brain, so followed brain tumours/ brain cancer. Brain cancers are classified into various types and recently the classification has been revised from the 2007 edition of the 'World Health Organisation (WHO) classification of tumours of the Central Nervous System' (Shown in the appendix). The new classification has particularly focused on molecular parameters in sequence with histology. The latter was the sole technique for classification prior to this (Louis *et al.*, 2016). The new addition of WHO classification shows similarity to the previous 2007 addition with major brain tumour groups including astrocytic tumours, oligodenroglial tumours and ependymal tumours, the former seen as the largest group (Louis *et al.*, 2007; Louis *et al.*, 2016).

Astrocytic tumours are again split into various sub-types including glioblastoma, astrocytoma, pilocytic astrocytoma etc. However, this is usually kept at two major factions of astrocytoma, the malignant form; glioblastoma multiforme (GBM), and non-GBM. It is important to note that astrocytomas are the largest group of brain tumours because astrocytes are the most abundant cell within the brain.

Neuronal cells are split into nerve cells and glial cells. Nerve cells send signals/action potentials, conversely, glial cells send no action potential. Glial cells make up much of the brains' mass and include the astrocyte mentioned, oligodendrocytes and microglia, amongst others. Astrocytes form the blood brain barrier and act as the mediator between the blood stream and other brain cells. Microglia are phagocytic, which allows them to perform the role of an immune cell and act as the brains personal immune system. Oligodendrocytes form various sheaths around the nerve cells, allowing for faster action potentials. When cancer occurs within these glial cells, it is deemed glioma

1.5 Brain Tumour Treatments and Statistics

The treatment of brain tumours is similar to the treatment of all cancers overall with radiotherapy, chemotherapy and surgery being the main routes of treatment (Department of Health GOV.UK, 2017).
The outcome for brain tumours in both treatment and survival often relies on a combination of factors: position within the brain, type of brain tumour, and the size and shape. The grade of the tumour is key, and all of the types and sub-types previously discussed are graded I-V. The table below shows the WHO grading in 2007 (Louis *et al.*, 2007).

Grade I	Low Proliferation	Potential cure following surgery	No infiltration
Grade II	Low Proliferation	Often recur following surgery	Can Infiltrate
Grade III	Fast proliferation	Often recur following chemotherapy and radiation	Malignant
Grade IV	Rapid proliferation	Rapid progression of disease with most treatments, often fatal outcomes	Highly malignant

Table 1.1 - The World health Organisation brain tumor grading system from 2007. Adapted from Louis *et al.*, 2007.

It is the difficulty in treatment that makes brain tumours so detrimental to patient lives. Moreover, successful treatment is often shrouded by the repetitive nature of recurrent tumours. Coupled with low survival, brain tumours are fundamentally a destructive and horrific disease.

Despite representing only 3% of all cancer types, tumours of the brain and nervous system, as mentioned earlier, can often be the most difficult. The difficulty around treatment and survival has been mentioned, and these difficulties have resulted in the rate of brain tumours increasing each year. Below is a figure from Cancer Research UK representing the rate of brain tumours per year from 1979 – 2013.

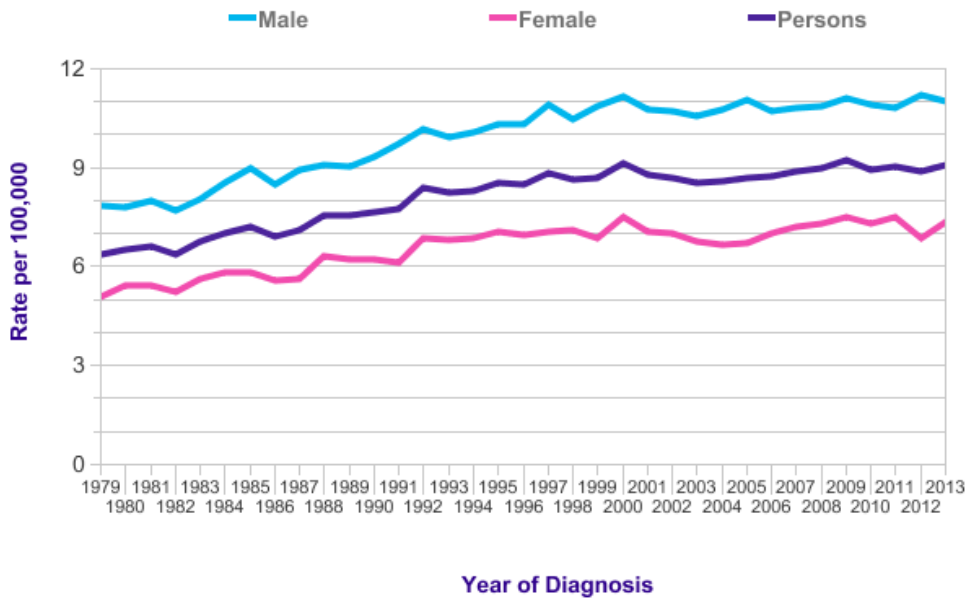


Figure 1. 6– A graph showing the rates of brain tumours per 100,000 people between the years 1979 and 2013. Sex is shown both separated and together (Cancer research UK, 2017).

Cancer Research UK estimates this increase at 39% since the first date shown. Despite a slight difference in the sexes, with males having a higher rate than females, both show a similar trend (Cancer Research UK, 2017). With brain tumour rates drastically increasing, and survival rates increasing at a much slower rate, brain cancers are rapidly becoming one of the most problematic diseases with treatments often showing little or no destruction of the tumour. Finally, more issues arise from the cancer's position within the body, the brain. Access to treatment and surgery is often problematic due to location and the need for treatment/ drugs to cross the blood-brain barrier. The blood-brain barrier hinders more than 98% of drugs from reaching and

treating the brain (Anand *et al*., 2015). This difficulty is therefore shown in survival.

A treatment into brain tumours that is advancing rapidly is immunotherapy, where treatment involves the use of the patients own immune system as a form of cancer treatment. Immunotherapy treatment is becoming increasingly popular in the treatment of GBM, and thus research has focused on its' current applications and future use in cancer treatment (McGranahan *et al*., 2017). McGranahan's research was focused on the history of immunotherpy's and its' current uses and applications. The article concluded that the history of immunotherapy in brain tumour treatment was a reminder of the treatment risks associated with immunotherapy. The authors finished with stating that the recent gain of knowledge and development into immunotherapy holds great hope for the future and all the possible advancements in glioma treatments (McGranahan *et al*., 2017).

1.6 Brain Tumours – Racial Bias Statistics

With an increase of occurrence and lack of an effective treatment, brain tumours are an area of research that is growing but needs more study.

In 2011 Dubrow and Darefsky began reviewing the statistical data of brain tumours. From their findings, they hypothesised that the subtypes glioblastoma multiforme (GBM) and non- glioblastoma multiforme (non-GBM) showed a difference due to demographics. Using age, sex and race, Dubrow and Darefsky studied the cases of GBM and non-GBM in the USA, utilising the SEER (Surveillance, Epidemiology and End Results program) data of 1992-2007. SEER provides online statistics of 'new cases of' and 'deaths by' specific cancers. In the case of Dubrow and Darefsky, the statistics for brain and nervous system cancers were used and in the study the authors state that a previous study showed race to have a major impact on brain tumours and the data they provided confirmed this. The authors confirmed that race played a key role in brain tumours by reviewing the SEER data, mentioned above. Dubrow and Darefsky concluded that non-hispanic whites numbered in over 20,000 new cases within the time period, whilst Native American new cases were approximating at 100. Dubrow and Darefsky (2011) continued that race had significance in the glioma subtypes. This can be closely linked to the succeeding data, the most recent SEER data from 2008-2012. These data correlated with the results and conclusions put forward by Dubrow and Darefsky and supported their initial hypothesis with regards to race having significance in glioma cases. Utilising the similar races, white and Native American, the number of new cases for a white individual was more than double that of a Native American. In males

8.4 per 100,000 people were of white ethnicity showing a new case of brain and/or nervous system cancer, on the other hand 3.7 per 100,000 were Native American. A similar trend is present with females of both white and Native American ethnicity with 5.9 and 2.7 people respectively per 100,000 showing new cases (Seer.cancer.gov, 2017). These data are shown in table 1.2. These data have been created from the above data (SEER 2008-2012).

Male		Female	
White	Native American	White	Native American
8.4	3.7	5.9	2.7

Table 1.2- The table shows the data from SEER 2008-2012 for new cases of brain and/or nervous system cancer. The table shows the sex, race and then the number of new cases per 100,000 people in USA.

This shows synergy with research by Crocetti *et al.* whom studied the case of glial and non-glial brain tumours across Europe in 2012. Their study indicated that different areas of Europe showed a different rate of occurrence and survival of brain tumours. The highest rates of brain tumours were in Western and Northern Europe i.e. UK, Iceland, and Norway. Central Europe showed intermediate rates of brain tumours. i.e. France, Germany and the Netherlands (see figure 1.7). Lastly, the lowest rates were found in Eastern, and Southern Europe i.e. Poland, Czech Republic, Spain, Portugal, and Italy. Coalescing all of this data on brain tumour incidences/ rates, it is obvious that race or racial background; a person's ancestry, may have a larger impact on cancer, more specifically brain cancer, than previously thought.

The schematic map in figure 1.7 shows Europe using Crocetti *et al.*'s research colouring the areas of high (red), intermediate (green) and low (blue) rates of brain tumours.

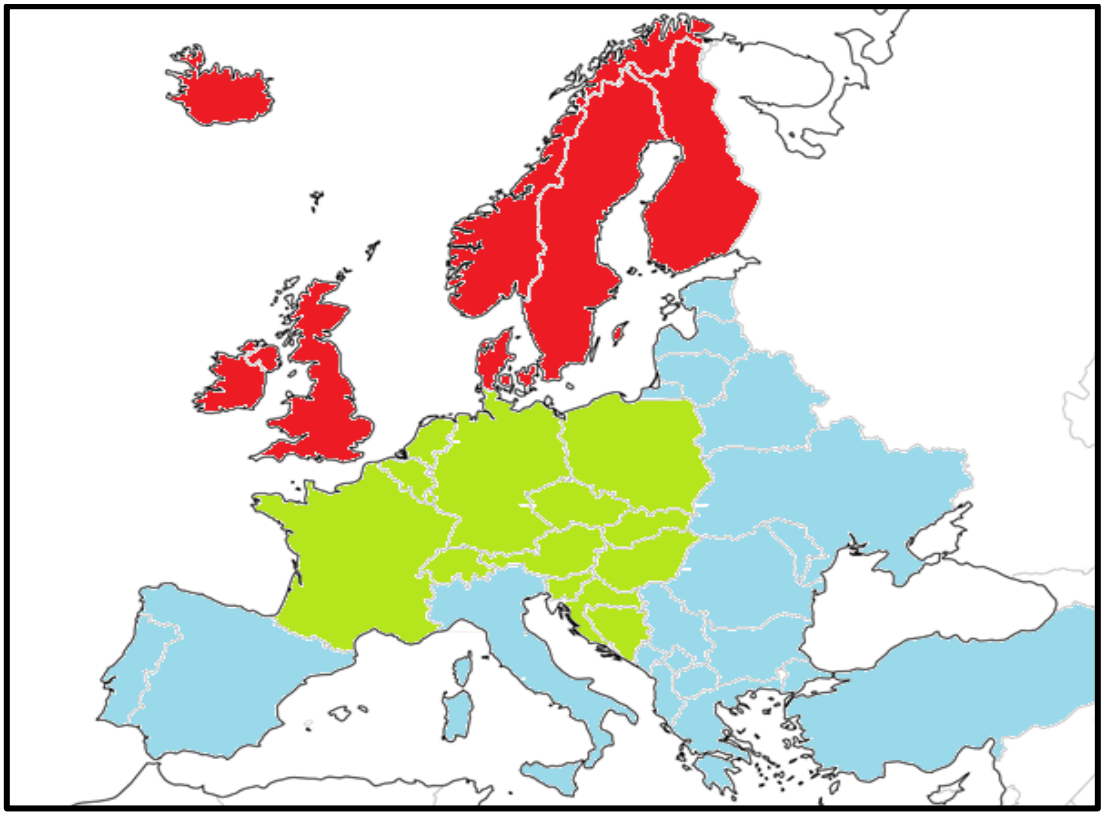


Figure 1. 7- A schematic map of Europe showing the glial tumour occurrence rates. The red indicates high rates in Northern and Western Europe. Green shows intermediate rates in Central Europe and blue shows low rates in Eastern and Southern Europe.

The human population in terms of genetics is extremely closely related, independent of your racial/ ancestral background. Statistically all humans are an extended family tree with close and distant cousins living all over the planet. The original populations of humans were small communities that were extremely inbred (Marshall, 2013).

These communities ultimately lead to the entirety of the current human population, which since has again been mixing and cross breeding over the millennia; forming a hyper inbred population. The simple fact that our genetics are so close to everyone else's should result in disease genotype, phenotype, and disease rates being almost identical throughout humanity. The above data shows this to not be the case (Dubrow and Darefsky, 2011; Crocetti *et al.* 2012).

Reviewing the work by Crocetti *et al.* (2012), it is clear that even in Europe, an area of the world where almost all inhabitants can claim descent from King Charlemagne in the eight-century A.D., moreover brain cancer rates differ greatly from country to country (Ralph and Coop, 2013).

In terms of genetics this is expected with a UK native genetically being a majority of western and northern European, with reference to England being conquered by the Anglo-Saxons, Vikings and French chronologically. These conquests have each added their genetics to the region, genetics where a majority has come from Western and Northern Europe, as shown in figure 1.5 in red. A sizable proportion of UK genetics would be central European, France and Germany, mentioned above. Lastly, very little of UK genetics would be southern and eastern European. This collection of genetics in specific proportions may be the root of this distinct difference in brain tumour rates. A central European is intermediate because they have genetic drift (the passing of genetics with no specific selection) with both of the surrounding areas of high and low brain tumour rates. An example can be seen in any European royal family tree. Often were the marriages of royals in France, England and Scotland, and France, Spain and Italy. However, the marriages between Italian and British royal families were much rarer. These examples hold true for the populations of the countries mentioned, some inter-country marriages were more common than others. These simple occurrences may have all resulted in the brain tumour rates that are present today. Finally, a native of Eastern Europe, would have little western and northern European ancestry, but would contain some genetics from Asia, the middle east and perhaps even eastern Asia; China, India.

This supports the racial bias described by Dubrow and Darefsky (2011) with Asian brain tumour rates being much lower than European. Therefore, an eastern European native with their mixed genome of little western/northern European and a proportion of continental Asian genetics would appear in between both European and Asian rates of brain tumours (Dubrow and Darefsky, 2011).

1.7 The Debate of Race

The close relation between humanity allows all races to be imagined as extended branches of the same family tree, and as such this is true. Figure 1.8 is a schematic taken from Campbell & Tishkoff, (2010) who did a review of the genetic diversity within Africa. The image shows a phylogenetic tree of human races, those whom are African and those who are not.

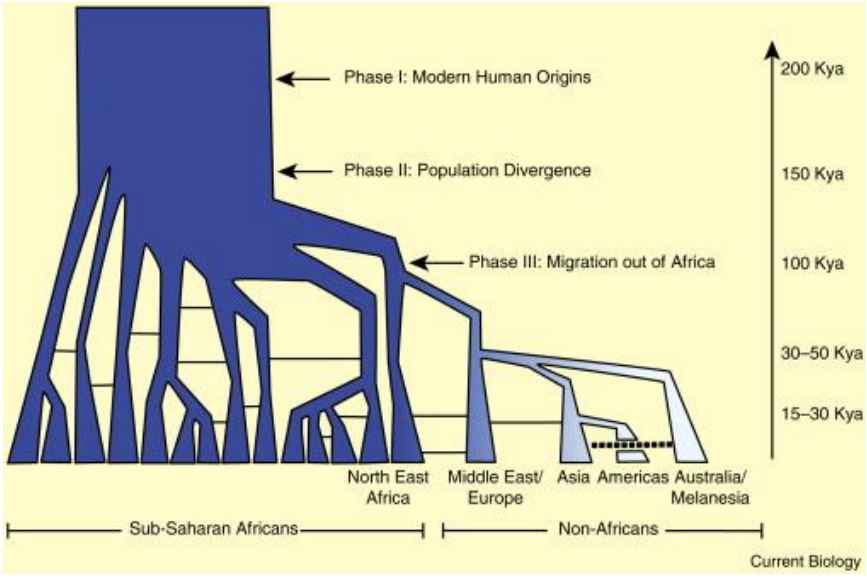


Figure 1. 8– Figure taken from Campbell and Tishkoff 2010, showing that all non-africans come from a single line of individuals who migrated out (Shown as Phase III). Whilst the figure shows that sub-Saharan Africans have the highest amount of genetic variation. Note 'Kya' stands for kilo (thousand) years ago.

Their study demonstrates the extreme closeness of all non-Africans, whilst indicating that Sub-Saharan Africans have the highest amount of genetic variation. This again proposes the question if the races are so closely related in terms of genetics, what genetic cause produces the difference in brain tumour rates?

This question is parallel to the 'race debate' this is a term coined from the debate regarding whether races truly exist. This debate has seen positive and negative inputs from research over time, and more recently was the focal point of research by Quayshawn Spencer in

2015. Spencer wanted to deduce whether the small difference in genetics warranted the term ‘race’. The outcome of the research was mostly philosophical about the terminology of race. However, despite this Spencer confirmed his predecessors by stating that the human population has various clusters of individuals with specific genomic differences, termed genetic clusters.

Avoiding the philosophy of race, biological evidences show that various clusters within humanity share closer genetics and similarly, have differences to those outside that specific cluster. These small differences within the genome of individuals are knows as single nucleotide polymorphisms (SNPs). These SNPs are single bases or small segments of a gene that differ to other individuals. It is important to note that whilst the two individuals’ genes would differ their genomes would both still encode the same protein, and both encode a functioning protein. The differences of genes between races can be assimilated to the description above (‘The inheritance of Cancer’ section), a schematic showing the inherited mutations that increase the chances of cancer. The ‘silent’ acting differences between individuals are what give the human populations its’ variation and ultimately produce these clusters of humans; race (Spencer, 2015).

1.8 The Inheritance and Genetics of Brain Tumours

With the human population being described above as an extended tree of the same family, familial/ germline mutations may then be prominent (Campbell and Tishkoff, 2010).

In 2014, Bainbridge *et al*. researched germline mutations associated with glioma that specifically show a familial link, focusing on *POT1*. Germline mutations are heritable mutations that are transmitted from parent to offspring as mentioned above. Protection of telomeres protein 1 or POT1 protein is a member of the telomere shelterin complex that binds to both DNA and tripeptidyl peptidase 1 (TPP1). The role of these proteins is the activation of telomeres and protection of telomeres from DNA repair mechanisms that require chromosomal merging such as non-homologous end joining (NHEJ). A news article in the Lancet Oncology produced from this journal research written by Bagcchi (2015) reiterates how key this concept of familial linked genetic mutation and its' research, may be. Bagcchi continues that the research by Bainbridge *et al*. found 2 POT1 mutants with an extremely strong association with familial inherited glioma. Both the journal and the news article stated that this genetic research would assist families and patients with these germline and familial mutations. The genetic 'passing down' of DNA and the way the alleles are selected for to produce a phenotype is known as Mendelian genetics. A phenotype is the physical characteristics that appear or occur due to the genotype; genetic characteristics. Like the rest of the body, cancer inheritance is often allele linked and thus a question can be formed from this. If most alleles follow Mendelian genetics when being transmitted from parent to offspring, then it can be assumed that the alleles/ mutations specific

to cancer would also follow the pattern of Mendelian genetics, specifically natural selection.

1.9 Mendelian Genetics

Mendelian genetics is a set of hereditary rules aptly named after its' founder, Gregor Mendel (1822-1884). Mendel studied pea plants, and how their flowers differed from parent to offspring. His experiments are shown below in a schematic of three generation of pea plant with two different version of the plant, one of white flowers and one of purple flowers (Mendel, 1866).

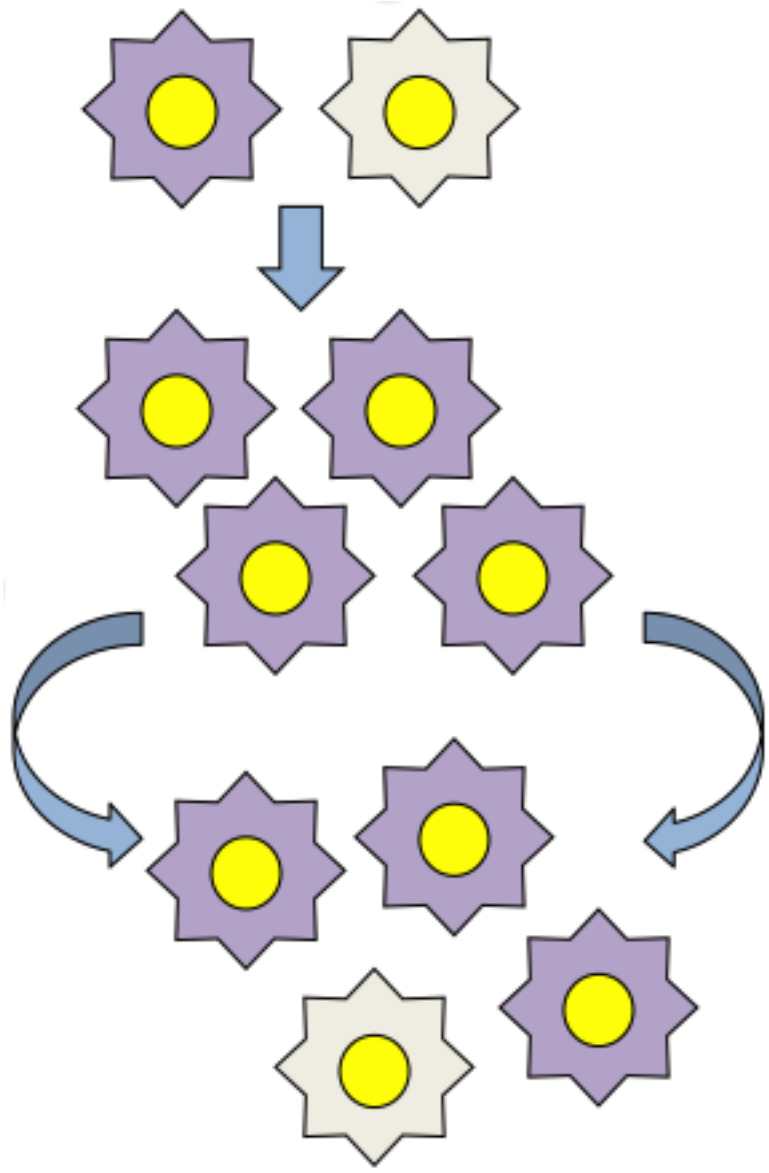


Figure 1. 9– A schematic showing the experiments performed by Mendel on pea plants. Note the purple and white flower and the interbreeding of the 4 purple flowers to produce the 3:1 ratio of purple flowers: white flowers.

Figure 1.9 shows that when Mendel crossed the purple and white flowers, the only outcome was purple flowered offspring. From this Mendel self-pollinated those offspring and discovered that the second generation of the pea plants always grew in a ratio of three purple flowering plants to one white flowering plant (Mendel 1866).

From this result, Mendel deduced that offspring inherit hereditary 'factors' and that these factors can have two forms. These forms are inherited one from each parent and together they produce a particular single characteristic. Unbeknownst, Mendel had discovered genes and that those genes have multiple forms or alleles.

This work has since been confirmed with the discovery of genes, and dominant and recessive alleles that Mendel himself coined. From this many studies have mapped inheritance patterns for various genes for example, the monogenic disease cystic fibrosis (Bowen and Hull, 2015).

Mendel published his work in 1866, which is now known to go perfectly alongside the 1859 work by Charles Darwin. Darwin proposed evolution by natural selection and dubbed two major factions of natural selection as fecundity and sexual selection. Natural selection is fundamentally a difference in survival/reproductive rates due to a different phenotype. A different phenotype can be seen in the pea plants Mendel had studied during his research. Darwin discusses fecundity as reproductive success due to phenotype, and sexual selection as choice of mate based on their potential reproductive success (the number of offspring produced that survive into the next generation).

Both mechanisms worked in synergy with Mendelian inheritance and thus became combined by Ronald Fisher during the first half of the twentieth century. Fisher had introduced the foundation for the

modern genetic theory of evolutionary biology and believed that natural selection was the major aspect of evolution with genetic drift playing a very minor role (Fisher, 1930). Genetic Drift is a change in the frequency of an allele and is due, in part to a lack of selective pressure, being completely due to random chance events favoring some individuals of a species over others. In 1968, Motoo Kimura introduced a sister branch within Mendelian genetics, promoting genetic drift as the process by which most variation is inherited. Kimura dubbed his theory as Neutral Selection, needing no selective pressure; due to the completely random nature of genetic drift. A branch in comparison to Mendelian genetics is Non-Mendelian Genetics, which includes various epigenetic patterns for inheritance including: genomic imprinting, X chromosome inactivation and various other expression patterns of alleles, RNA, and post-translational modifications.

All Mendelian inheritance patterns follow the three law of Mendel shown below.

Law of Segregation	**Law of Dominance**	**Law of Independent Assortment**
Each gamete gains one allele	One or more allele is dominant and is displayed as the dominant characteristic	Genes are independently assorted during gamete production

Table 1.3 – A table showing the three laws of Mendelian genetic/inheritance. Law of Segregation, Law of Dominance and Law of Independent Assortment.

These laws are adhered to by both branches of the Mendelian inheritance seen overleaf, but not by the Non-Mendelian inheritance of epigenetics mentioned above. Figure 1.10 is an image showing the branches of Mendelian inheritance that have been discussed above.

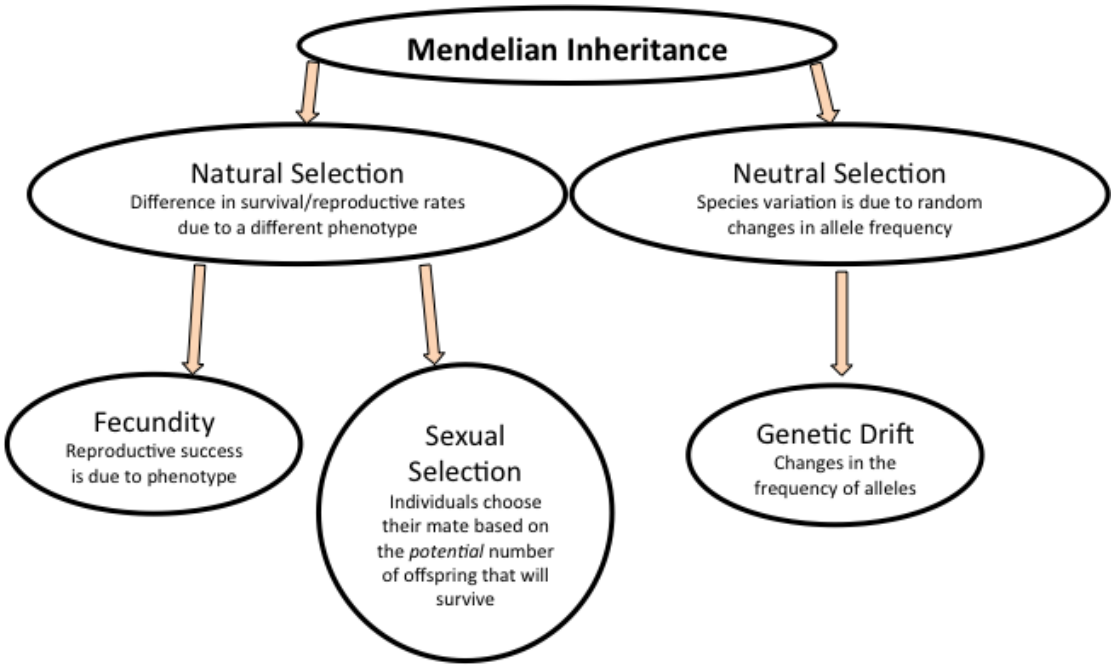


Figure 1. 10- A schematic tree of Mendelian inheritance, showing the two branches of natural and neutral selection, proposed by Darwin (1859) and Kimura (1968) respectively.

It has been concluded that approximately 50% of Mendelian phenotypes and conditions are unknown (Chong et al., 2015).

1.10 Epigenetics

It has been apparent for some time that genetics is a major aspect of cancer development. However more recent studies are beginning to focus on genetic mutation perhaps not being the cause for various differences in the human population as no genes have yet been found for many conditions. Epigenetics is the study of heritable changes in gene expression (Lee, 2012). Epigenetics does not include changes in the DNA sequence or genome Lee continued, focusing his research on two epigenetic phenomena of X chromosome inactivation and genomic imprinting.

X chromosome inactivation occurs when an X chromosome (xch) is silenced, for example in females, who carry two xch. Each cell of a female's body will change the gene expression of xch to silence one, which occurs randomly in all cells; producing an xch mosaic affect throughout their body. The silencing of xch includes modifications of histone proteins and DNA methylation, the latter of which is the main form of epigenetic modifications of gene expression (Lee, 2012).

Genomic imprinting is the expression of specific genes from the one un-silenced chromosome with silencing dependent on parent-of-origin. The parent-of-origin has a crucial role in inheritance of disease states and thus cancer. Similar to X chromosome inactivation, DNA methylation (and therefore inherited methylation) and histone modifications are the main epigenetic changes in genomic imprinting; however unlike X chromosome inactivation, genomic imprinting occurs with all chromosomes and in both males and females. Dependent on the chromosome being from either the mother or father, the epigenetic change i.e. the change in gene expression, can cause a different phenotype despite no change of the genomic sequence

occurring, this again links to the familial inheritance of genetic information. These epigenetic changes occur early during embryonic development with abnormal imprinting being the route problem of many human diseases. More recent research is showing epigenetic changes to also control other aspects of phenotype that have no genomic evidence such as addiction, metabolism, weight and sexuality (György, 2017). The change of expression in one allele is commonly found in the genome and termed mono-allelic expression (Lee, 2012). Further to this, the change in expression of two alleles and their differences has been termed allele specific expression (ASE) (Lee, 2012). ASE is largely affected by genetic polymorphism i.e. SNPs; which change the DNA base, but do not alter the amino acid, ultimately affecting the phenotype by manipulating the bodies 'decision' of which allele is used, changing expression (Lee, 2012). ASE opens studies up by allowing the research into the gene expression differences between different people whom have slight differences in their genotype. This links to the study of racial bias toward cancer with races differing only slightly in genetics, SNPs. Methylation of DNA, as mentioned, is the main modification identified in epigenetics and Lee (2012) mentions how allele specific methylation can be transmitted through the germ-line and hence through generations of descendants. This highlights the importance that the inheritance pattern of cancer may have. Further to this, it shows that epigenetics may also be subject to Mendelian genetics much like the genomic mutations discussed.

1.11 Neurofibromatosis – Predisposition to Glioma

Glioma has been discussed in chapter 1.4 as the major group of brain tumours, cancers of the glial cells. Various types have been shown and multiple sub-types from these. However, many tumours begin somewhere else and often are dubbed as a different disease. In a chapter of 'Glioma- Recent results in cancer research', by von Deimling (2009) discusses the importance of understanding hereditary tumour syndromes and their predisposition to gliomas. The main predispositions they discuss are neurofibromatosis type 1 (NF1) and 2 (NF2). Type 1 is known as the peripheral form, whereas type 2 is the central form. Type 2 involves tumour growth along the spine, brain stem and predisposes patients to develop brain tumour growths specifically in glial tissue and hence a predisposition to gliomas.

The chapter continues discussing the overlap of molecular pathogenesis between glioma and both types of neurofibromatosis amongst other predispositions and finds a likeness to the disruption to the likes of Ras and its downstream pathway. The authors conclude that gaining a greater understanding into hereditary predispositions of brain tumours will aid a better understanding of their formation and their inheritance (von Deimling, 2009).

NF2 is an inheritable disorder first described in the early 20th century, having since been greatly researched. Lloyd and Evans (2013) in the Handbook of Clinical Neurology, stated that 50-60% of NF2 cases are de novo with no previous familial link, however of these, 70% carry the mutated NF2 gene in all cells meaning that the individual can now transmit the disorder to an offspring, creating a familial link. The remaining 30% of de novo cases gain a mutant cell line and a non-mutant cell line producing unilateral and bilateral forms both having a

chance of transmitting the disease down the germ line; again, creating a familial link. Lloyd and Evans (2013) described the variation in NF2 phenotype including the age of disease onset, speed of the disease progression and the extent of the disease; with a family's phenotype often showing a similar severity. Therefore, if a family shows a similar phenotype, it can be hypothesized that the family's genotype would be similar. This tumorous disorder shows many characteristics to its' potential successor; glioma, in that both have a strong familial link and similar phenotypes. The change in phenotype and its variation, despite a similar and often linked genotype again may show evidence that epigenetics may have a key role in cancer inheritance and more so in NF2, and its' inheritance. The disorder NF2 is associated with a change in the protein Merlin, encoded by the *NF2* gene.

1.12 Merlin Protein Biology

1.12.1 Molecular Biology

Merlin stands for Moesin-Ezrin-Radixin-Like Protein and is part of the ERM (Ezrin, Radixin and Moesin) family of protein (Sun *et al*, 2002). Merlin was named so due to the similarity it has to Ezrin, Radixin and Moesin. Merlin is placed in its family mainly due to the FERM domain all family members have. The FERM domain is the module of the proteins that all of the family share; the domain allows all family members to bind to the cell membrane. FERM stands for Band 4.1 Ezrin Radixin Moesin homology domain (Sun *et al*, 2002). The ERM family is highly conserved throughout evolution due to the association and activity within the cell membrane and its interface to the cytoskeleton; often having roles in membrane localisation of proteins. Other roles of this protein family are the processing of extracellular signals and downstream signaling within cells, as described by Morrow and Shevde (2012) and Cooper and Giancotti (2014). All members of the family have a FERM domain, allowing ERM proteins to interact with cell membrane proteins. The merlin protein in humans is 68kDa with 17 exons comprising of 595 amino acids. The three domains are the tri-lobed FERM domain at the amino terminal, an alpha-helical domain, often referred to as a coil-coil region, and the carboxyl terminal domain. Cooper and Giancotti (2014) explain that Merlin shares much homology to other ERM proteins and it is currently assumed that they share a similar activity to suppress mitogenic signaling at the cell cortex to mediate contact inhibition. Despite this assumption, no evidence has yet confirmed merlin's similarity to ERM proteins; however, ERM proteins are shown to have roles as a tumour suppressor (TS) again implicating a homology to merlin. Lloyd and

Evans (2013) describe that there are 2 isoforms of Merlin; one is an active, dephosphorylated and closed form, and the other is an inactive, phosphorylated and thus open form. This is now thought to be correct as further research, and the review by Cooper and Giancotti (2014) corresponds with the concept described by Lloyd and Evans (2013). The image figure 1.11 shows merlin's similarity to the ERM family proteins, however the image also shows that the FERM domain is present in other proteins that are not in the same family and share no other homology to the ERM family other than the FERM domain (Sun *et al*, 2002).

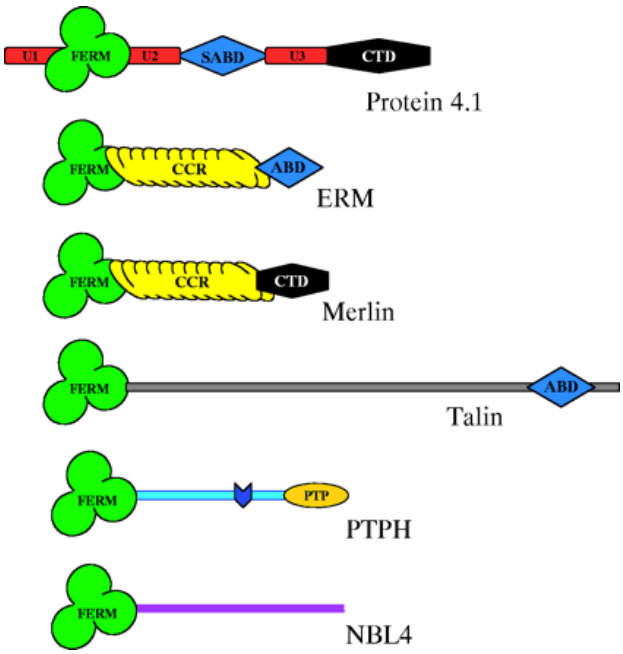


Figure 1. 11- Taken from Sun et al, 2002 shows the similarity that merlin has to other ERM family proteins

The image figure 1.12 shows merlin's two forms (active and inactive) and their speculated shape and conformation. Section C of the image below shows known molecules that merlin binds to, these include structural molecules and its' fellow proteins.

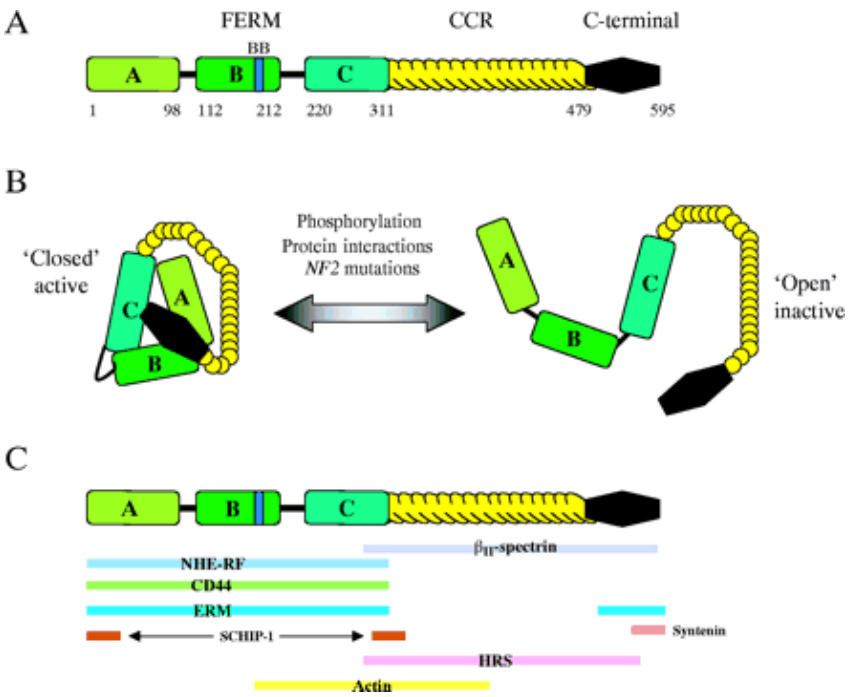


Figure 1. 12- Taken from Sun et al, 2002 shows the active and inactive forms of merlin and known molecules that merlin interacts with.

1.12.2 Activity of Merlin

1.12.2.1 General activity of Merlin in Humans and D. melanogaster

Despite the expected homology to other ERM proteins the true details of merlins' role is unknown requiring further study (Lloyd and Evans, 2012; Cooper and Giancotti, 2014). However, research shows links to many cancer-associated pathways at the cell membrane and within the nucleus. Morrow and Shevde (2012) reviewed merlin's protein stability as a tumor supressor stating that the main aspect that has been researched was the loss of function of merlin which results in the over proliferation of nerve cells. The three mechanisms of tumour suppression that merlin utilises are decreases in proliferation, increases in apoptosis and the disputed contact-dependent growth inhibition (CDGI). Cooper and Giancotti (2014) explain that merlin was suspected to utilise contact inhibition due to its homology to the ERM family. Morrow and Shevde (2012) in their review discuss merlin's ability to disrupt Ras and Rac signalling pathways, which can lead to

contact-dependant growth inhibition. They next focus on the homology between humans and *D. melanogaster* in terms of the merlin protein and how merlin's ability to decrease proliferation and induce apoptosis is highly conserved in both species. This highlights not only the similarity between merlin and its associated mechanisms and pathways in both *D. melanogaster* and humans, but the key role merlin has in terms of proliferation and apoptotic regulation in both organisms. Recent research into merlin has also shown new pathways and links to its role in decreasing or stopping downstream proliferation signals. (Yu and Guan, 2013) One of the more recent pathways researched is the Hippo pathway, present and highly homologous in both humans and *D. melanogaster* (Yu and Guan, 2013), in which the open state of merlin is now considered active in terms of tumor suppressor activity and the control of contact inhibition. In the hippo pathway, merlin activates Yap/Taz in mammals and the homologue in *D. melanogaster*, Yorkie. This reveals a role in regulating organ size, stem cell behaviour and cell proliferation. Morrow and Shevde (2012) state that much research has focused on merlin mutations in the genome, however epigenetics and protein regulation may in fact be the principle cause of NF2 and the associated brain cancers of glioma and meningioma, amongst others (Morrow and Shevde, 2012). The review continues stating that multiple mechanisms of genomic change are the likely cause of merlin inactivation; this again supports both the genomic change and epigenetic change that may influence the inheritance and therefore the activity of merlin. DNA methylation is likely to play the largest role in the epigenetics of merlin, however other studies have shown there to be some contradiction within research showing a decreased methylation in merlin inactivation. Morrow and Shevde (2012) highlight the need for

more research into the genetics of merlin, not only the inherited mutation, but also the epigenetics of merlin. The authors finish by stating that the expression level of merlin may have implications to its inactivation, however this again requires further research due to lack of conclusive evidence (Morrow and Shevde, 2012).

Figure 1.13 shows a simple schematic overview of merlin's activity throughout the cell. At the cell membrane, merlin interacts with integrins, RTK's, and catenin and cadherin that allows the regulation of many cancer aspects and the hallmarks previously mentioned. Merlin has a second major role within the nucleus where it interacts with an E3 ligase, regulating further genomic aspects of cancer (Utermark, Kaempchen, and Hanemann, 2003; Rong et al., 2004; Higa et al., 2006; Mitra and Schlaepfer, 2006; Curto and McClatchey, 2007; Curto et al., 2007; Lee and Zhou, 2007; Lallemand et al., 2008; Pan, Weinman, and Le Dai, 2008; Dummler et al., 2009; Streuli and Akhtar, 2009; Li et al., 2010; Soung, Clifford, and Chung, 2010; Li and Giancotti, 2010; Read *et* al., 2011; Li et al., 2012; Zhou and Hanemann, 2012).

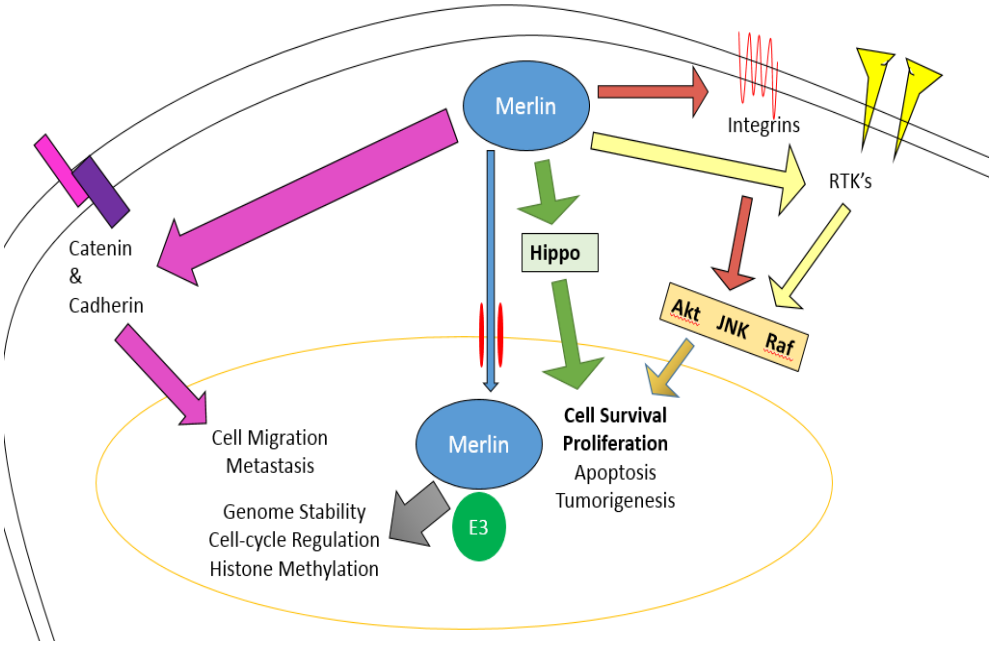


Figure 1. 13- Merlin's role at both the membrane and the nucleus. At the membrane merlin regulates a number of proteins that are involved in various oncogenic pathways (See text for details of references, such as page 51).

1.12.2.2 Merlin activity at the membrane

Merlin has various roles throughout the cell acting primarily at the membrane and within the nucleus. Merlin acts as a control, a regulator, and forms numerous complexes during its' role as a multi-suppressor (Zhou and Hanemann, 2012). Merlin has some control over integrins, the cell-cell bridges, with a loss of merlin producing an increase in integrins and in turn increase cell adhesion (Utermark *et. al*, 2003). Alterations in cell adhesion are associated with cancer metastasis. Once activated, integrins undergo various interactions that begin downstream affects and pathways. For instance, integrins recruit signalling proteins Rac1 and Cdc42, whom in turn interact. From this Pak is activated (Dummler et al., 2009), which creates a feedback loop by phosphorylating merlin at serine 518, producing the inactive form (Rong et al., 2004). Secondly Pak activates some major cellular pathways associated with cancer; ERK and JNK, both which promote the regulation of cell survival, apoptosis, and tumourigenesis. Integrins

also regulate: Fak and the associated src proteins (Mitra and Schlaepfer, 2006), which function upstream of more cancer associated pathways; Raf and PI3K which promote cell survival, proliferation and migration. Merlin has a second regulatory control of PI3K, by actively inhibiting it. Merlin inhibits PI3K by competing with PI3K enhancer long form (PIKE-L) (Rong et al., 2004). Therefore, by inhibiting PI3K, merlin can regulate cell proliferation and survival. Integrins have been shown to work with regulatory tyrosine kinases (RTK's) in recent studies (Streuli and Akhtar, 2009; Soung et al., 2010). The synergy between integrins and RTK's, including VEGFR, IGF1R, ErB receptors and PDGFR mediates signalling pathways. Merlin has control over both RTK's and Integrins. Merlin's process of regulation of RTK's is unknown however it has been suggested that it regulates endocytotic trafficking of RTK's (Lallemand et al., 2008). Merlin forms a complex with many proteins, for example, the complex with PDGFR through Na^+ H^+ exchange regulatory cofactor (NHERF), a complex which was suggested by Pan *et al.* (2008) and confirmed by Lallemand *et al.* (Pan, Weinman, and Le Dai, 2008; Lallemand et al., 2008). Na^+ H^+ exchange regulatory cofactor has recently been described as a potential new cancer marker due to its gradual increase and cofactor role in many cancer types, including glioma (Dell'Endice et al., 2014). Lastly integrins have been shown to activate mTORC1, a complex of mTOR. mTOR pathway regulates protein synthesis, and due to this, many cancer-associated affects arise from this including cell proliferation and survival. Cadherins and Catenins are proteins that allow cell-cell adhesion, anchoring cells to one another. Merlin has been shown to form complexes with the catenin and cadherin complex. Other studies have shown merlin not only forms complexes with E/N-cadherin and B-catenin, but through contact-dependent inhibition of proliferation

may control all downstream affects by regulating catenins-cadherin complexes, integrins and RTK's (Curto and McClatchey, 2007; Curto et al., 2007). By regulating downstream pathways, merlin may ultimately control the development of the major hallmarks of cancer specifically proliferation, cell survival, apoptosis and metastasis (Hanahan and Weinberg, 2000).

1.12.2.3 Merlin activity within the nucleus

In its dephosphorylated/closed form, merlin enters the nucleus due to a strong affinity to DNA damage-binding protein 1 (DDB1) and, DDB1 and CUL4 associated factor homolog 1 (DCAF1) (Lee and Zhou, 2007; Li and Giancotti, 2010; Li et al., 2012). DCAF1 is a substrate receptor for Cullin-ring ligase 4 (CRL4), E3 ligase. Merlin only has said affinity when it is the wild-type allele. Knock-out or null model organisms for merlin have shown no affinity to enter the nucleus (Lee and Zhou, 2007). It is also unlikely that mutant merlin would have the same affinity if any at all. The CRL4 complex was shown to have many roles in cell cycle regulation, histone methylation and genomic stability (Higa et al., 2006). By binding to DCAF1, merlin can inhibit DCAF1 binding to CRL4 and therefore inhibit CRL4 activity. From this merlin can broadly regulate gene expression by inhibiting CRL4 (Li et al., 2010). By entering the nucleus, merlin ultimately inhibits CRL4. In merlin deficient tumours, CRL4 must have a role in tumourigenesis. A change in merlin or DCAF1 levels alters the production of integrins and RTK by altering protein degradation (Li et al., 2010). This allows the hypothesis that merlin can create its' own feedback loop. By entering the nucleus merlin interacts with DCAF1 and CRL4, altering the production of integrins and RTKs. Merlin, as aforementioned, interacts with these and by affecting their production affects its' own activity with integrins and RTKs at the membrane.

1.12.2.4 Dysfunction of Merlin

A dysfunctional or lack of merlin is known to cause NF2, merlin is however dysfunctional in many cancer forms and the reason for this is stated above. Merlin produces its' own feedback loop by regulating CRL4 within the cell's nucleus. This in turn regulates many of the proteins in production, majority being RTKs and integrins. Therefore, a change or loss of merlin alters the regulation of integrins and RTKs by altering their production. Secondly to this, merlin has a variety of interactions at the cell membrane with integrins and RTKs, and a change in the production of these changes merlin's ability to interact. However, if a cell is lacking merlin, or it is dysfunctional, the interaction that would normally occur at the membrane would not occur or would not produce the same outcome. It is therefore important for merlin to be present and more importantly, functional (Zhou and Hanemann, 2012).

1.13 Drosophila melanogaster – Model Organism

With a simple eukaryotic genome; *Drosophila melanogaster* (*D. melanogaster*) have become a common model organism, being utilised in many areas of scientific research. The genome consists of 4 chromosomal pairs with homology to human disease states. *D. melanogaster* live for approximately a month, from egg through to adult. After hatching from an egg, *D. melanogaster* feed as a larva. This larva grows through three stages of larval growth called instars; each instar increasing the larva's size. The larva then pupate before they eclose as adult *D. melanogaster*. The process from egg to adult *D. melanogaster* takes approximately 14 days.

Figure 1.14 shows *D. melanogaster* as seen down the lens of a dissecting microscope at x10 magnification. Adult flies can be seen surrounding several pupa cases. The blue substance is the media in which they grow and feed. Note that these are Wild-Type *D. melanogaster* of both sexes.

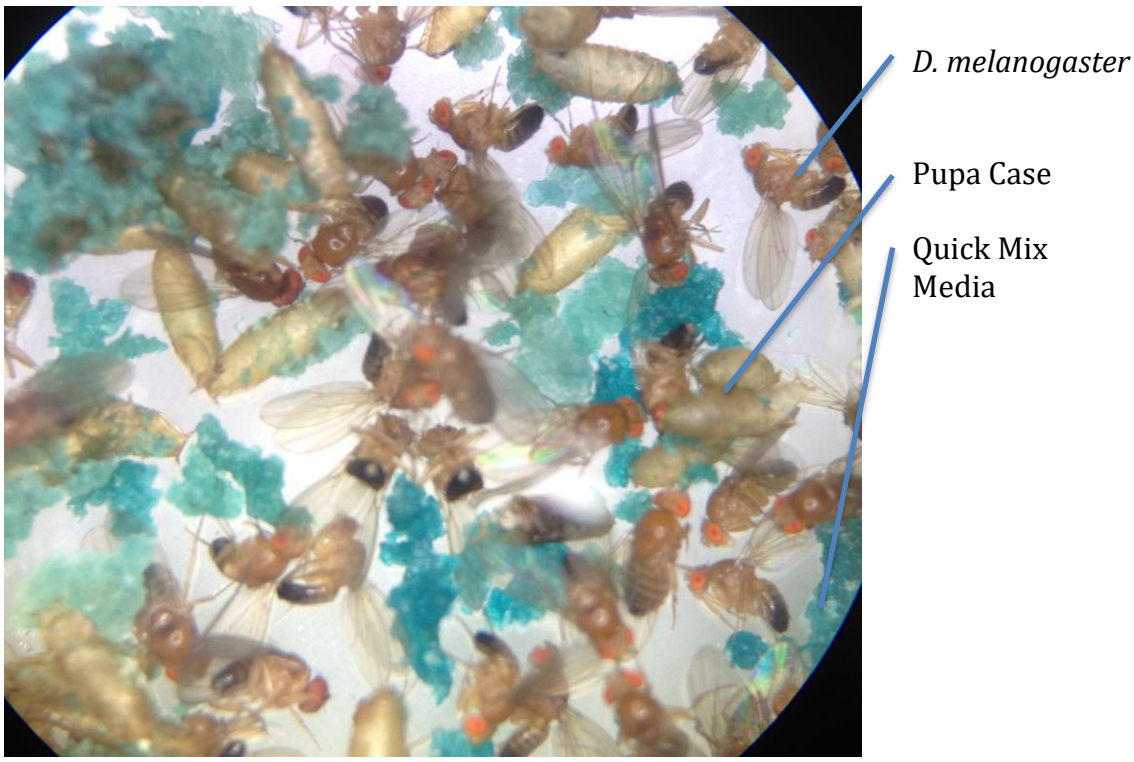


Figure 1. 14– Wild-Type D. melanogaster shown using x10 magnification

D. melanogaster, being insects, means their development is impacted by temperature, as temperature increase, so does *D. melanogaster* development, shortening the time from larval to adult *D. melanogaster* growth. The opposite is said for cold temperature, lengthening the *D. melanogaster* development (Oliphant *et al*., 2014).

For many years the use of *D. melanogaster* as a model organism specifically in cancer has been researched because of their genetic homology to the human genome. This has been the main advantage for use of *D. melanogaster*. Many of the proteins seen in human cancers and those associated with human cancers are highly conserved in *D. melanogaster* as stated by Polesello *et al.* (2011) in 'modelling cancers in *D. melanogaster*' an article regarding animal models for human disease. The chapter continues discussing the use of *D. melanogaster* and their often-identical oncogenic pathways to a mammalian model, coupled with many homologous proteins, which control and suppress said pathways. This is supported by Read *et al.* (2009) who explain the homology of glial cells and thus the tumours associated with them in relation to both *D. melanogaster* and mammals. From this, a great homology between *D. melanogaster* and mammalian glial tissue and hence glioma can be seen; *D. melanogaster* can be utilised as a practical model organism for glioma study. Read *et al.* discussed the Ras and PI3K pathways in *D. melanogaster*, showing a great similarity to humans (Read *et al.,* 2009). Read (2011) further researched *D. melanogaster*, studying pathways for the development of glioma, confirming their evident use as a model organism for modeling GBM and non-GBM glioma subtypes. *D. melanogaster* have been used for genetic experiments for over a century because of the easy breeding, fast life cycle and simple eukaryotic genome. The knowledge gained from *D. melanogaster* experiments has shaped the molecular world

today, with genetics, evolution and inheritance playing major roles. However, it is key to note that they do have various advantages, the major factor being the temperature dependency, not seen in mammalian models. Similarly to mammal models is the simple fact that *D. melanogaster* are not human and therefore despite a similar genome it is not an exact replica.

1.14 Merlin in *D. melanogaster* and Humans

D. melanogaster and humans both have a similar merlin, *D. melanogaster* merlin dubbed 'dmer' (McCartney, et al., 1996).

Found on the X Chromosome positioned 19,689,697 to 19,693,500. Dmer is bigger than its' human counterpart being 74kDa, whereas human merlin is 69kDa. However, both still appear to perform a similar role in studies that have been done. One such study used human merlin to rescue merlin-lethal *D. melanogaster* with success; this shows the highly conserved nature of merlin in both organisms (LaJeunesse, et al., 1998; Golovnina, et al., 2005). Despite this similarity, no alleles of merlin have been researched in humans, opposing to this, many alleles have been researched in *D. melanogaster* such as Mer 3 and Mer 4 amongst many others.

Dmer has been shown to function upstream and regulate regulatory proteins for cell growth and division. Dmer itself showing the function of cell survival, and cell proliferation regulation. With a loss of Dmer showing a compromise to normal apoptosis functioning and spermatogenesis (Pellock et al., 2006; Dorogova et al, 2008; Bolobolova et al., 2011).

This shows much similarity to figure 1.15, showing the function of merlin in human studies with reference to cell-cycle, cell proliferation and apoptosis (Figure 1.15).

Studies have focused on the hippo pathway, a pathway mentioned above in human models. The image below (Figure 1.15) shows the pathway in both *D. melanogaster* and humans parallel (Zhao *et al*, 2011).

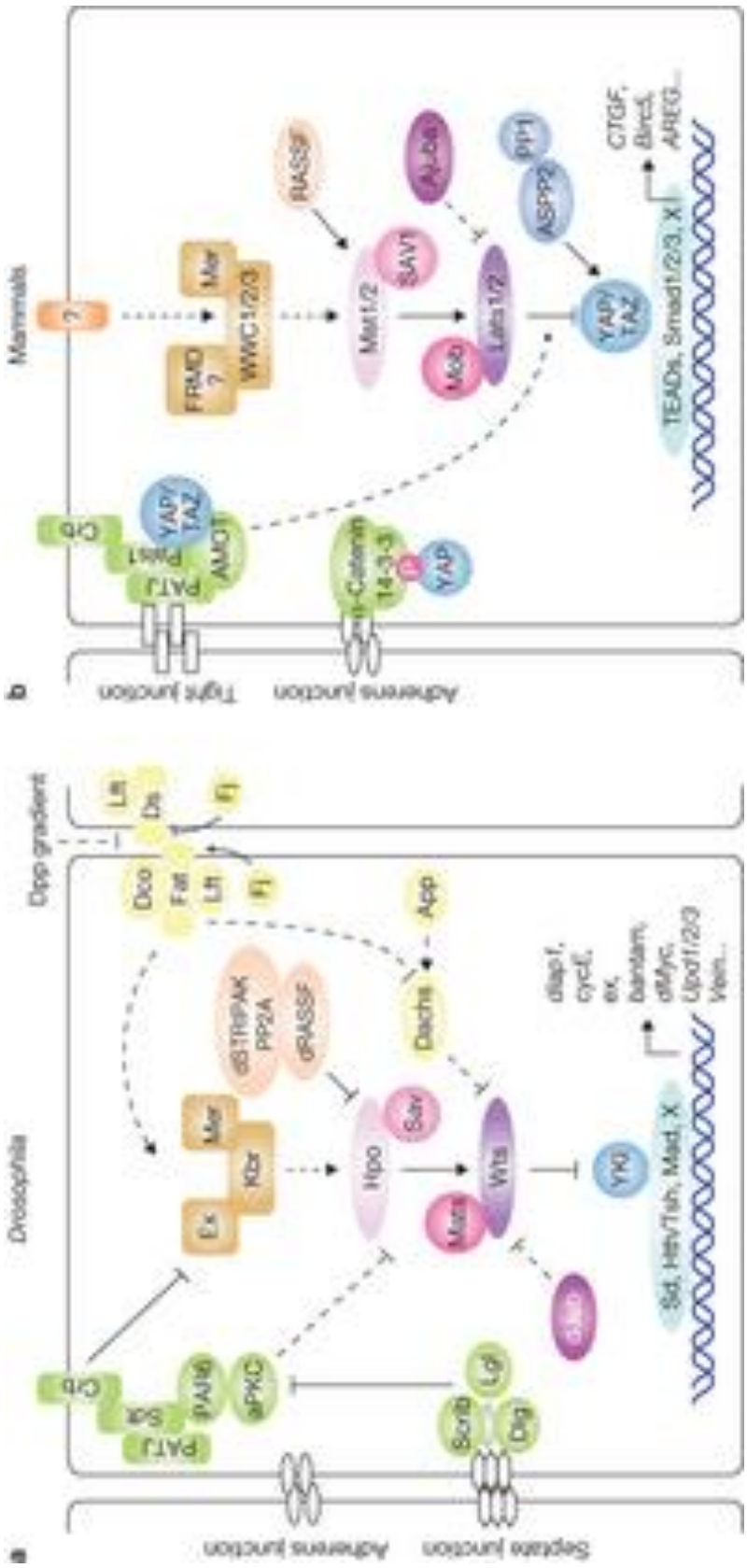


Figure 1. 15- Taken from Zhao et al, 2011 an image showing the Hippo pathway in both mammals and drosophila, showing the similarity between the two and the homologous proteins (Mer).

The image is evidently similar; both models look almost exact, with merlin playing the same roles in both. Despite this similarity, little research has investigated the other roles of merlin, aforementioned in humans, such as the role in the nucleus or interactions with integrins and RTKs.

1.15 Introduction to the Methodology

Given that each race of humans differs mostly by SNPs, it is key that the *D. melanogaster* model has groups that differ also by a single point mutation. The wild type *D. melanogaster* became the control 'normal' merlin allele. The two remaining *D. melanogaster* populations had different variants of merlin. The variants are both a single point mutation, a substitution of one DNA base for another. No studies have mapped the merlin alleles within the human population, however due to our close relatedness as a species a single point mutation seems the most applicable variation that would be present.

D. melanogaster with different versions of merlin gene represents the different races of the human population. Therefore, by isolative reproduction, a model of human race evolution has been produced. This is shown represented by the three distinct populations of *D. melanogaster* with their allele/variant of merlin. From this, one can then assimilate the recent mixing of human races and discover the inheritance pattern of merlin in *D. melanogaster* by allowing the different populations to mix and breed. These mapping strategies, called linkage mapping have been shown to be useful techniques. Linkage mapping is mapping a particular locus across generations and progeny and has been shown to be 'fruitful' in many organisms when studying gene variants that contribute to a phenotypic variation (Schacherer, 2016).

The three variants of *D. melanogaster* merlin (Mer) were Mer^3, Mer^4 and Wild type (Wt) Mer. Mer^3 and Mer^4 produce a different phenotype with increased mortality and would thus act as 'recessive' traits in comparison to the wild type variant. Mutant alleles are a loss of function. A loss of function causes over proliferation, often seen in a change of phenotype, specifically the head and eyes, also throughout the body of the *D. melanogaster*. This leads to major physical deformities and an increased mortality. The three merlin variants were chosen mainly due to their viability, many other merlin mutants did not produce offspring and those that did had sterile or undeveloped offspring. Both Mer 3 and Mer 4 have been used in various studies, with authors often claiming various classes of phenotype from viable and increased cell number, through to sterile and lethal. An example is Mer 3 being claimed as viable in 1998, but a decade later in 2008 the male Mer 3 are sterile (LaJeunesse, et al., 1998; Dorogova et al., 2008).
This would not be viable in this type of methodology, where inheritance pattern is a main concept, therefore non-viable and non-breeding offspring would be unsuitable. Both mer 3 and 4 are point mutations in the promoter region. This is similar to humans who too have a point mutation of merlin to cause the NF2 phenotype. Mer 3 is a missense mutation (Met^{177} – Ile) and Mer 4 is a nonsense mutation (Gln^{170} – Stop Codon) (Dorogova et al., 2008).
Multiple populations of all variants will be required to allow for mortality of *D. melanogaster* and to ensure genetic variation is withheld and not lost due to excessive interbreeding. The number within a population can vary from 15-50 to begin with and as they continue breeding a population can reach up to 500 individuals. It is therefore the number of individuals that is particular instead of the population numbers. This cannot be predicted due to the

unpredictable breeding of *D. melanogaster*. Mutant merlin in humans is unstable, thus producing the cancerous phenotype. This instability is mirrored in *D. melanogaster* and is seen as increased mortality rates.

1.16 Working Hypothesis

The working hypothesis states that merlin will produce an inheritance pattern dictated by Mendelian genetics (either by natural or neutral selection), and/or provide probability of epigenetic manipulation.

1.16.1 Main Aim

Identify if merlin follows Mendelian genetics in regards to inheritance and/or that epigenetic manipulation may be apparent in the proteins inheritance

1.16.2 Specific Aims

The first aim is to deduce whether proteins such as merlin, which have major effects on cells and are encoded by a single gene (Monogenic), are inherited in a specific pattern. A pattern dictated by Mendelian genetics, either dominant or recessive by natural selection or by genetic drift/ neutral selection.

Secondly, the research aims to discover whether many proteins like merlin have a genetic dependency, or in fact whether epigenetics plays any role in their dysfunction and therefore cancerous phenotype produced.

CHAPTER TWO

MATERIALS

&

METHOD

2.1 Materials

2.1.1 Flies

Wild-Type *D. melanogaster* Melanogaster from Blades Biological Ltd

Mer ^{3} *D. melanogaster* Melanogaster from Bloomington *D. melanogaster* Stock Center at Indiana University (9103)

Mer^{4} *D. melanogaster* Melanogaster from Bloomington *D. melanogaster* Stock Center at Indiana University (9104)

2.1.2 Fluids, Chemicals and Reagents

D. melanogaster Quick Mix Media from Blades Biological Ltd

Flynap Kit from Blades Biological Ltd

Distilled H_2O

2.1.3 Equipment

D. melanogaster Vial

Plastic 'ladder'

Paint Brush

Petri Dish

Foam Bung

Gilson Pipette

Eppendorf Tubes

PCR Tubes

Water Bath

Vortex

Microcentrifuge

2.2 Method – *D. melanogaster* Breeding

Three populations of *D. melanogaster* were set up initially and crossed throughout the experiment. Each population required a plastic vial with a bung. In this vial 5 g of Quick Mix medium and 15 ml of distilled water create a blue medium base for the vial, in which the larva live. A small amount of yeast was added atop the medium, providing the *D. melanogaster* with food. A plastic mesh ladder was placed on top of the medium (curved into a 'U' shape), providing a support for the *D. melanogaster* pupa. The protocol for this is provided on the packaging of the Quick Mix medium.

The first stage of the experiment was establishing the three distinct *D. melanogaster* populations. This is shown in figure 2.1 below.

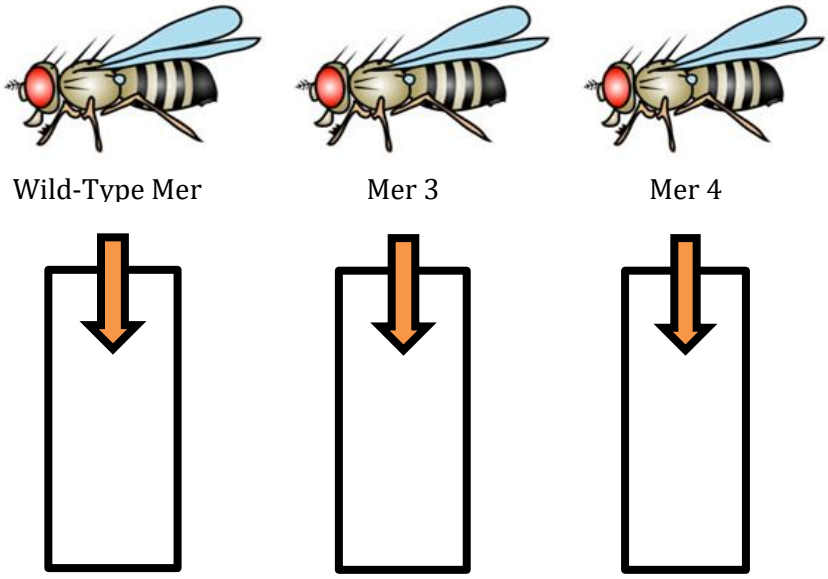


Figure 2.1- Diagram showing the three variants of Mer, each established separately from each other in individual vials.

The *D. melanogaster* populations, once established, were crossed. These new mixed populations interbreed, producing a hybrid generation of offspring. Figure 2.2 shows the primary crossing of two of the three variants.

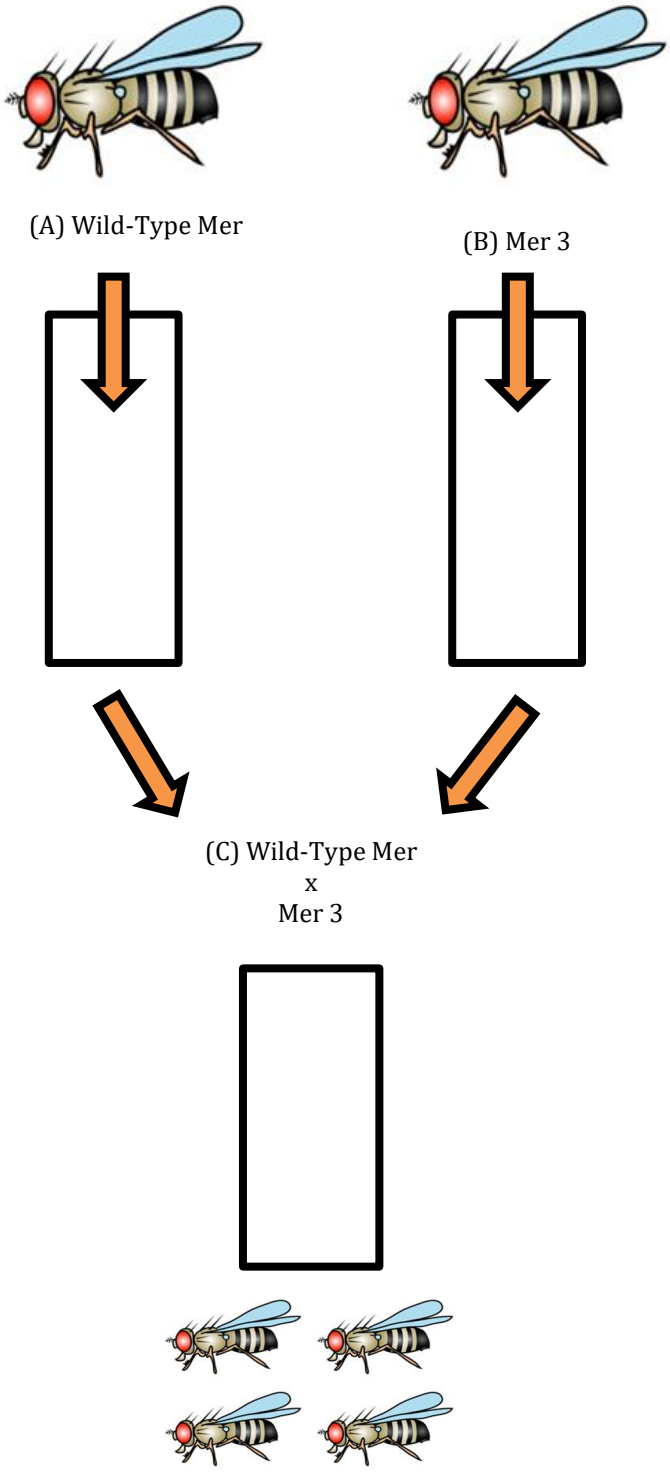


Figure 2.2- Diagram showing the crossing of two Mer variants, Wild-Type Mer and Mer 3. With their offspring being a hybrid population (Wild-Type Mer x Mer 3).

This hybrid population was then established and allowed to reproduce to the next generation of *D. melanogaster*. These offspring of the hybrid *D. melanogaster* were bred for a further generation to fix the genes within the population. The remaining Mer mutation variant was

crossed with the Wild-Type, producing two hybrid populations and this again was allowed to continue breeding to fix the genes within this population. This can be illustrated by figure 2.4, which shows the populations breeding for a further generation to which genes became fixed within the population. The figure (2.4) also shows those population that were mixed with an additional population.

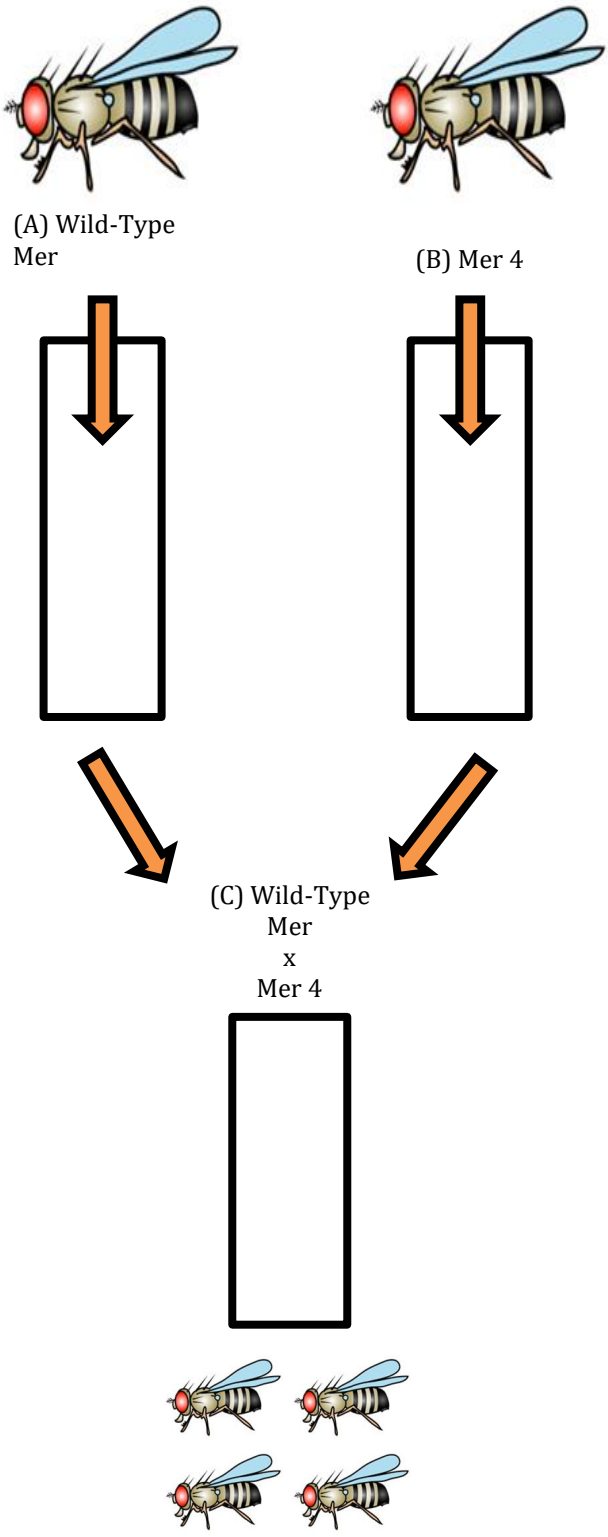


Figure 2.3- Diagram showing the crossing of two Mer variants, Wild-Type Mer and Mer 4. With their offspring being a hybrid population (Wild-Type Mer x Mer 4).

As stated above the two hybrid populations were in turn allowed to breed, fixing the genes within the population; producing generation two of each individual hybrid population. Individuals from both hybrid population were then manipulated producing a 'double hybrid' population with all three merlin variants (Wild-Type, Mer 3 and Mer 4) as their ancestral populations. Lastly the stock population of Wild-Type *D. melanogaster*, was used as a control descendant with no merlin mutants present in the lineage at any point. The sample of this population was taken at approximately generation ten.

The schematic of the population family tree below shows the populations relatedness to one another. It is important to note that despite the simplistic schematic, care was taken to ensure the populations kept natural genetic drift and variation, and such multiple sub-populations of each stated population were kept ensuring little interbreeding occurred.

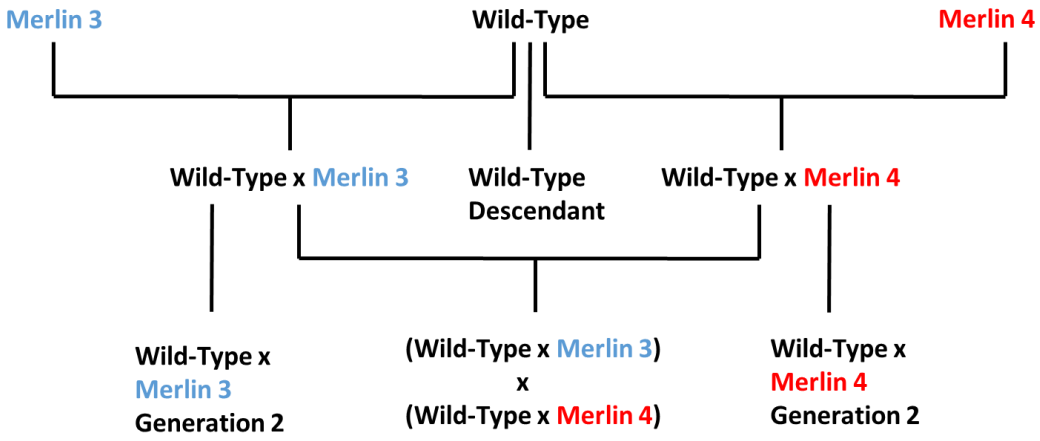


Figure 2.4 – A schematic family tree of the populations researched within this study. The tree shows the relationships between the various populations

The survival rate and mortality rate were calculated for all populations to determine the viability of the three Mer variants. These rates and the phenotypic ratios of potential offspring from the possible combination of parents were also calculated from this.

CHAPTER THREE

RESULTS

3.1 Qualitative Results – *D. melanogaster* Breeding

D. melanogaster breeding can be a simple, productive method due to their quick succession in population size and reproductive capabilities. Despite this, *D. melanogaster* with the mutations have been more challenging to breed.

Breeding the *D. melanogaster* with merlin variant Mer 3 and Mer 4 proved difficult as many of the *D. melanogaster* would die as young/ larva or die during pupation. The outcome of this was that fewer numbers of these mutant *D. melanogaster* did not survive to adult and therefore many did not reach the stage where reproduction was possible. Therefore, multiple populations of merlin mutant *D. melanogaster* were required to allow this method to succeed. The *D. melanogaster* mutants Mer 3 and Mer 4 had already been selected by myself from a vast array of merlin mutant *D. melanogaster* due to their ability to breed. Many *D. melanogaster* with mutations in merlin showcased an inability to breed due to merlin's activity in gamete production. Despite these apparent issues, a sustainable population of both merlin mutants; Mer 3 and Mer 4, was produced and could be manipulated per this methodology.

Those Mer 3 and Mer 4 mutant *D. melanogaster* that did survive were not as easy to maintain as their wild-type counterparts, resulting in the high mortality mentioned. This high mortality continued down the generations with the offspring population of Mer 3/ Wild-Type hybrids and Mer4 /Wild-Type hybrids, often dying early in development. Due to this many populations of *D. melanogaster* perished before experimentation was able to take place. The main factor to consider in the maintenance of the *D. melanogaster* was and is temperature control. As with all insects, *D. melanogaster* require a near constant temperature to breed, however increased temperature can in turn

increase their breeding capabilities and speed up their embryonic and metamorphic development. The opposite, however can be said for *D. melanogaster* populations maintained in decreased temperature. This decreased temperature in turn decreases embryonic and metamorphic development, and breeding capabilities.

Once populations were established, and hybrids themselves had survived, the rate of mortality drastically decreased to that which can be considered normal levels, those levels of natural mortality seen by a Wild-Type population. The images below show the initial population's phenotype and the outcome that becomes individuals with a mutant merlin allele. Wild-Type have an unaffected merlin gene/protein and so have normal growth and reproduction (see figure 3.1). Mer 3 *D. melanogaster* have less coloration on their abdomen and no coloration in their eyes, this is apparent when compared the red eye pigment of the Wild-Types. The Mer 4 *D. melanogaster* similarly to the Mer 3, have the lighter body coloration and again have a phenotype seen by their eyes with the Mer 4 individuals having 'slit eyes' with only a small section of pigmentation. It is also important to note the lack of uniformity in Mer 4 *D. melanogaster* sizes, the two individuals shown in figure 3.2 are two individuals of the same population and the size is not a result of sexual dimorphism, just a characteristic of the mutant merlin phenotype.

Figure 3.1 – Wild-Type *D. melanogaster* showing various body colours, normal levels of growth and replication and red pigment in the eye. Sizes are uniform (x10 magnification)

Figure 3.2 – Mer 3 *D. melanogaster* showing a single colouration on their body and lack of red pigment in their eyes. Sizes are uniform (x10 magnification)

Figure 3.3 - Mer 4 *D. melanogaster* showing a single colouration on their body and lack of red pigment in their eyes. The eyes are only pigmented in a small area of the eye producing 'slit eyes' Sizes are not uniform (x10 magnification).

3.2 Quantitative Results

3.2.1 Mortality Rates and Survival Rates

The *D. melanogaster* that survived breeding as mentioned above were crossed to produce hybrid populations of Mer 3 and Wild-Type, and Mer 4 and Wild-Type. These were then bred using the breeding method outlined in Chapter 2. Note the control population was the Wild-Type population.

D. melanogaster Population	**Survival rate of D. melanogaster (Percentage/%)**	**Mortality rate of D. melanogaster (Percentage/%)**
Wild-Type	97%	3%
Mer 3	34%	66%
Mer 4	27%	73%
Wild-Type x Mer 3	55%	45%
Wild-Type x Mer 4	56%	44%
Mer 3 x Mer 4	0%	100%
Wild-Type x Mer 3 Generation 2	61%	39%
Wild-Type x Mer 4 Generation 2	81%	19%
(Wild-Type x Mer 3) x (Wild-Type x Mer 4)	87%	13%
Wild-Type Descendant	93%	7%

Table 3.1 – A table showing the survival rates and mortality rates for each population of *D. melanogaster*.

Table 3.1 shows the survival rate and mortality rate of the various populations of *D. melanogaster*. The mortality rate is the number of

individuals that died divided by the number of individuals that were produced/eclose, multiplied by 100. This is seen in the equation below.

$$\frac{Number\ of\ Individuals\ that\ died}{Number\ of\ Individuals\ that\ were\ produced/eclose}\ x\ 100$$

Once the mortality rate was calculated, the survival rate could also be calculated as this was 100% minus the mortality rate. Table 3.1 shows that the highest survival rate and therefore the lowest mortality rate was seen in the wild-type *D. melanogaster* this only decreased slightly to 93% in their descendants, generations later. In contrast to this, the lowest survival rate and therefore highest mortality rate was seen in the Mer 3 and Mer 4 population where all offspring died/ never reached adulthood.

The next highest mortality rates were seen in the Mer 3 and Mer 4 populations with mortality at 66% and 73% respectively. The hybrid populations of wild-type with Mer 3 and Mer 4 showed an increased survival and therefore lower mortality rate than the Mer 3 and Mer 4 populations. The rate however, was still resulting in 44% and 45% of *D. melanogaster* dying.

The wild-type/Mer 3 hybrid population showed a small decrease in mortality rate in the second generation, with the rate being 39%. On the other hand, the hybrid population of wild-type/Mer 4 *D. melanogaster* showed a more drastic decrease in mortality rate in the second generation, with the mortality rate decreasing to 19%.

Lastly the population that contained all three Mer variants had a mortality rate of 13% which was approaching the rates seen in the wild-type populations of *D. melanogaster*.

3.2.2 Phenotypic Ratios

In the Wild-Type population, only wild-type individuals are available and therefore the parents of all offspring are wild-type *D. melanogaster*

Parent Phenotype	Phenotypic ratio of offspring expected
Wild-Type x Wild-Type	1:0 of Wild-Type: Other variants Or 100% of offspring would be Wild-Type.

Table 3.2 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of wild-type *D. melanogaster*.

In the hybrid population of wild-type *D. melanogaster* and Mer 3 *D. melanogaster* there would be three possible parental combination as shown in the table below, each with a specific phenotypic ratio.

Parent Phenotype	Phenotypic ratio of offspring expected
Wild-Type x Wild-Type	1:0 of Wild-Type: Other variants Or 100% of offspring would be Wild-Type.
Wild-Type x Mer 3	1:0 of Wild-Type/Mer 3 Hybrids: Other variants Or 100% of offspring would be heterozygous Wild-Type/Mer 3.
Mer 3 x Mer 3	1:0 of Mer 3: Other variants Or 100% of offspring would be Mer 3.

Table 3.3 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of wild-type and Mer 3 *D. melanogaster*.

In the hybrid population of wild-type *D. melanogaster* and Mer 4 *D. melanogaster* there would be three possible parental combination as shown in the table below, each with a specific phenotypic ratio.

Parent Phenotype	Phenotypic ratio of offspring expected
Wild-Type x Wild-Type	1:0 of Wild-Type: Other variants Or 100% of offspring would be Wild-Type.
Wild-Type x Mer 4	1:0 of Wild-Type/Mer 4 Hybrids: Other variants Or 100% of offspring would be heterozygous Wild-Type/Mer 4.
Mer 4 x Mer 4	1:0 of Mer 4: Other variants Or 100% of offspring would be Mer 4.

Table 3.4 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of wild-type and Mer 4 *D. melanogaster*.

The hybrid populations of wild-type and Mer 3 *D. melanogaster*, and wild-type and Mer 4 *D. melanogaster* bred to produce the second generation of these hybrid populations. The offspring from tables 3.3 and 3.4 above reproduced, becoming the possible parental genotypes for generation 2.

Parent Phenotype	Phenotypic ratio of offspring expected
Wild-Type x Wild-Type/Mer 3 Hybrid	1:1 of Wild-Type: Wild-Type/Mer 3 Hybrids
Wild-Type x Mer 3	1:0 of Wild-Type/Mer 3 Hybrids: Other variants Or 100% of offspring would be heterozygous Wild-Type/Mer 3.
Wild-Type/Mer 3 Hybrid x Mer 3	1:1 of Mer 3: Wild-Type/Mer 3 Hybrids

Table 3.5 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of wild-type and Mer 3 *D. melanogaster* bred to a second generation.

Parent Phenotype	Phenotypic ratio of offspring expected
Wild-Type x Wild-Type/Mer 4 Hybrid	1:1 of Wild-Type: Wild-Type/Mer 4 Hybrids
Wild-Type x Mer 4	1:0 of Wild-Type/Mer 4 Hybrids: Other variants Or 100% of offspring would be heterozygous Wild-Type/Mer 4.
Wild-Type/Mer 4 Hybrid x Mer 4	1:1 of Mer 4: Wild-Type/Mer 4 Hybrids

Table 3.6 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of wild-type and Mer 4 *D. melanogaster* bred to a second generation.

Mer 3 and Mer 4 *D. melanogaster* populations both had lethal, detrimental effects to phenotype and survival. Breeding of the two was difficult and any offspring that did get produced died during development. However, potential offspring of this mixed population is shown in table 3.7 below.

Parent Phenotype	Phenotypic ratio of offspring expected
Mer 3 x Mer 4	1:0 of Mer 3/Mer 4 Hybrids: Other variants Or 100% of offspring would be heterozygous Mer 3/Mer 4.

Table 3.7 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of Mer 3 and Mer 4 *D. melanogaster*.

Lastly the initial hybrid populations of wild-type and Mer 3 *D. melanogaster*, and wild-type and Mer 4 *D. melanogaster* were mixed. This population contained all three Mer variants. The population had 9 possible parental phenotypes, taken from the offspring of the previous population, seen in table 3.3 and 3.4.

Parent Phenotype	Phenotypic ratio of offspring expected
Wild-Type x Wild-Type	1:0 of Wild-Type: Other variants Or 100% of offspring would be Wild-Type.
Wild-Type x Wild-Type/Mer 4 Hybrid	1:1 of Wild-Type: Wild-Type/Mer 4 Hybrids
Wild-Type x Mer 4	1:0 of Wild-Type/Mer 4 Hybrids: Other variants Or 100% of offspring would be heterozygous Wild-Type/Mer 4.
Wild-Type x Wild-Type/Mer 3 Hybrid	1:1 of Wild-Type: Wild-Type/Mer 3 Hybrids
Wild-Type/Mer 3 Hybrid x Wild-Type/Mer 4 Hybrid	1:1:1:1 of Wild-Type: Wild-Type/Mer 3 Hybrids: Wild-Type/Mer 4 Hybrids: Mer3/Mer4 Hybrids
Wild-Type/Mer 3 Hybrid x Mer 4	1:1 of Wild-Type/Mer 4 Hybrids: Mer 3/Mer 4 Hybrids
Wild-Type x Mer 3	1:0 of Wild-Type/Mer 3 Hybrids: Other variants Or 100% of offspring would be heterozygous Wild-Type/Mer 3.
Wild-Type/Mer 4 Hybrid	1:1 of Wild-Type/Mer 3 Hybrids: Mer

x Mer 3	3/Mer 4 Hybrids
Mer 3 x Mer 4	1:0 of Mer 3/Mer 4 Hybrids: Other variants Or 100% of offspring would be heterozygous Mer 3/Mer 4.

Table 3.8 – A table showing the parent phenotype and the phenotypic ratio of their offspring in a population of wild-type and Mer 4 *D. melanogaster* bred with wild-type and Mer 3 *D. melanogaster*.

CHAPTER FOUR

DISCUSSION

4.1 General Discussion

This study aimed to investigate the inheritance of genes associated with brain tumors in humans using a *D. melanogaster* model. Merlin has been shown to be an active protein with an extensive role within various aspects of the cells' microenvironment (Zhou and Hanemann, 2012). It has been stated that a proteins' functionality and role within a cell has much control over the rate of evolution, and the rate of change to that protein (Chakroborty *et. al.,* 2015).

The work by Chakroborty *et. al.,* indicates that a protein, such as merlin, with its' multitude of functions related to core biological process of division and growth should therefore be highly conserved with few mutations occurring over the generations. Disease related genes and non-disease genes were shown to have a variety of factors that affect evolutionary rate (Podder *et. al.,* 2009). The study showed that non-disease genes were the most highly conserved and of those the 'housekeeping' genes were genes with the lowest rate for evolutionary change. Housekeeping genes are the genes involved the cells' basic maintenance, and can be thought of as the minimal set of genes required to sustain life (Eisenberg and Levanon, 2013).

Polygenic and Monogenic disease genes were both less conserved and of those two, polygenic was shown to be the least conserved and therefore most likely to be subject to mutation and evolutionary change (Podder *et al.,* 2009). As a monogenic disease gene, merlin falls in the middle of the two showing some conservation but not a high amount, whilst also showing some evolutionary change and mutation. Lastly Podder *et. al.,* ranked the factors that would produce a change with the proteins functionality being a restraint to oppose evolutionary change, as expected with example to the highly conserved

housekeeping proteins. However the mRNA levels were shown to have little restraint on the protein/genes' conservation and that the change in expression level would therefore be the most likely factor to introduce evolutionary change to a protein (Podder et al., 2009).

The initial three populations have been discussed above to show difficulty in breeding due to their mortality rate. Despite Mer 3 and Mer 4 being selected from a vast range of *D. melanogaster* mutants due to their relative success in survival and breeding. This study has shown it to be apparent that in *D. melanogaster* Merlin plays a crucial role to overall survival, shown by vast amounts of research indicating the multiple roles merlin has.

4.2 Mendelian Genetics in the hybrid populations

Two hybrid population of Wild-Type and Mer 3, and Wild-type and Mer 4 were produced from the initial three populations. The offspring of these two populations would contain individuals shown by their genotype in the table below.

Potential Phenotypes of an individual in the hybrid population	**Two gene variants, one from each parent in hybrid population**
Wild-Type Homozygous	Wild-Type Gene, Wild-Type Gene
Heterozygous	Wild-Type Gene, Mer 3 Gene
Mer 3 Homozygous	Mer 3 Gene, Mer 3 Gene

Table 4.1 - Table showing the possible phenotypes of the offspring of the hybrid population and the two alleles they would have obtained from their parents.

An offspring of the hybrid population had either: two Mer 3 parents, two Wild-Type parents or one of each. Therefore, the offspring would be Mer 3 Homozygous, Wild-Type Homozygous or Heterozygous. This can be seen by the family trees shown below.

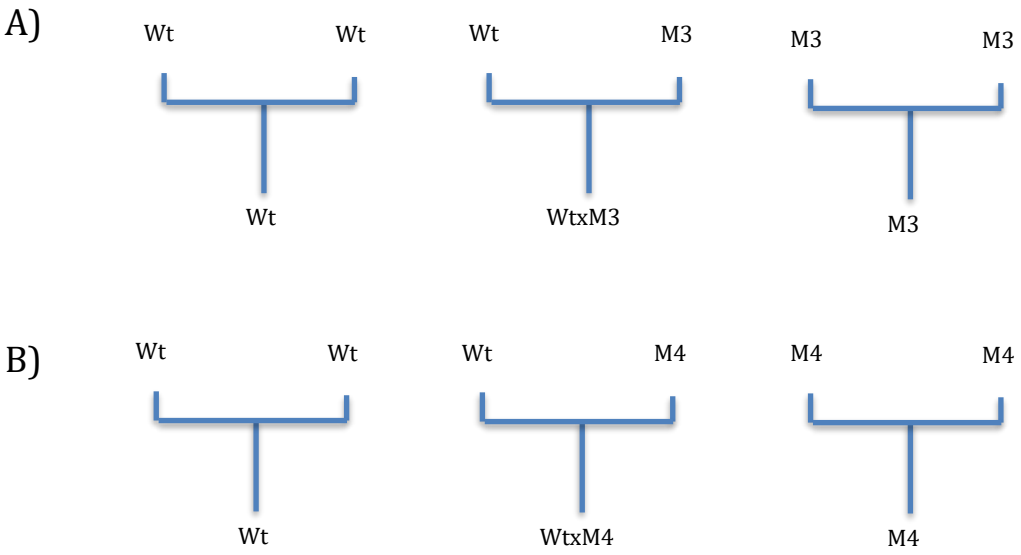


Figure 4.1 – Schematic family trees showing the potential pairing in the hybrid populations

These family trees show an individual's parentage and the potential alleles they have. Both Mer 3 and Mer 4 are represented as (A) and (B) respectively, in this image.

This could also be represented as a punnetts square as shown below (Table 4.2-4.6).

	Wt	Wt
Wt	Wt Wt	Wt Wt
Wt	Wt Wt	Wt Wt

Table 4.2 – A punnetts square showing the potential genotypes of the offspring produced when two wild-type *D. melanogaster* reproduce

	Wt	Wt
M3	Wt M3	Wt M3
M3	Wt M3	Wt M3

Table 4.3– A punnetts square showing the potential genotypes of the offspring produced when a wild-type *D. melanogaster* and a Mer 3 *D. melanogaster* reproduce.

	M3	M3
M3	M3 M3	M3 M3
M3	M3 M3	M3 M3

Table 4.4 - A punnetts square showing the potential genotypes of the offspring produced when two Mer 3 *D. melanogaster* reproduce.

	Wt	Wt
M4	Wt M4	Wt M4
M4	Wt M4	Wt M4

Table 4.5 - A punnetts square showing the potential genotypes of the offspring produced when a wild-type *D. melanogaster* and a Mer 4 *D. melanogaster* reproduce.

	M4	M4
M4	M4 M4	M4 M4
M4	M4 M4	M4 M4

Table 4.6 - A punnetts square showing the potential genotypes of the offspring produced when two Mer 4 *D. melanogaster* reproduce.

Loss of function alleles of Mer 3 and Mer 4 have been shown to be recessive traits (McCartney, et al., 2000).

If one assumes that the no-mortality allele, Wild-Type is dominant over Mer 3 then the hybrid population would be 50% Wild-Type and 50% Mer 3 genotypically. The table below shows the outcome of three Punnett's squares. A Punnett's square shows two breeding individuals (Parents) and their 4 alleles, it then shows the genetic ratios of their offspring.

The first generation of a breeding population has been shown to be the most susceptible to genomic erosion (Matusse et al, 2016). This research focused on stocks of wreckfish, to determine the first-generation genetic drift. The results showed that the stocks lost 26% of the genetic variation; despite the authors ensuring interbreeding was

kept minimal. This reduction was seen in 80% of the wreckfish populations at the first-generation. This research highlights the 'founder effect', where the initial founders are the only individuals to provide genetic information and with no access to individuals outside the stock population, genomic erosion is difficult to avoid (Matusse et al, 2016).

4.3 Wild-Type Population and Wild-Type Descendants

The wild-type *D. melanogaster* populations would only ever produce wild-type offspring therefore 100% of the offspring would be wild-type and of those 97% would survive to reproduce. With the wild-type allele being the allele that is the most viable this is expected that the highest survival rate and lowest mortality rate is seen in these individuals. Generation later the wild-type descendants showed an increase in mortality from 3% to 7%. This population was again only wild-type *D. melanogaster* and thus this change in survival could be due to random chance or genetic drift. In 1932 Sewell Wright produced his model of adaptive landscapes stating that genetic drift allowed as small population to have a large genetic variation and that natural selection would 'take over' from genetic drift to evolve individuals or populations through an adaptive selective pressure. The image below shows Wright's landscape model with valleys and peaks.

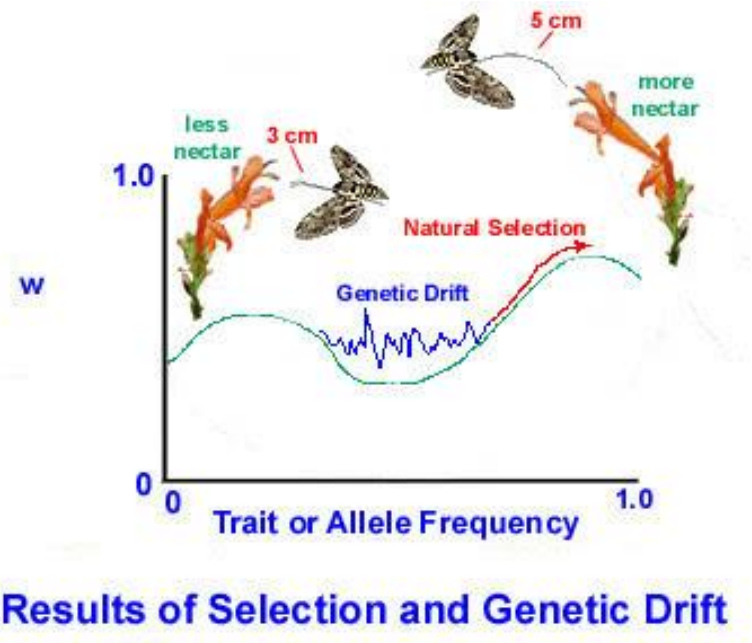


Figure 4.2 – An image showing the adaptive landscape model, showing valleys and peaks. Genetic drift is shown to fluctuate throughout the valley, whilst natural section can be seen as directional toward a peak. An example is shown within the image of the two peaks of natural selection showing butterflies with differing proboscis sizes and how this produces one more adapted than the other to feeding on the particular plant. Taken from MacNeill, 2017.

Wright modeled that a valley was where a small population had much variation and genetic drift allowed movement throughout the valley, but once selective pressure had begun then natural selection began to move traits and individuals up towards a peak. Fixing them in a population and resulting in that population being unable to re-enter the valley again. This was shown in research of phenotypic evolution, where the authors discussed genetic drift allowing population on a peak to migrate from one peak to another through the uneven line of genetic drift (Engen and Saether, 2016). Genetic drift is the most likely of Mendelian genetics to have occurred in this wild-type population to result in the increased mortality rates in the descendant population showing once more the affect genetic drift can have on a small isolated population (Matusse et al., 2016).

Genetic differentiation can be produced through a population shifting from on peak to another by genetic drift or an epigenetic interaction

(Frank, 2011; Engen and Saether, 2016). The initial population of wild-type *D. melanogaster* was used as the control and could be seen as the survival/mortality rate that is considered normal/average for *D. melanogaster*.

4.4 Mer 3 and Mer 4 Populations

The Mer 3 and Mer 4 populations showed high rates of mortality with the Mer 4 population showing the highest rate observed during this research with mortality rates at 73%

4.5 Wild-Type and Mer 3 Hybrid Populations – Generation 1 & 2

In the initial hybrid population of wild-type and Mer 3 there are three possible parent combinations (shown is table 3.3). From this there are potentially three outcomes for offspring. If one presumes that the parental couples are equally random to occur then each of the three potential offspring would encompass 33% of the population each. Each of these three population have their own mortality and survival rate. The 33% of offspring that are homozygous wild-types, the mortality rate is 3% this results in almost all of the 33% surviving and very little mortality. This is due to these individuals being phenotypically wild-type with a high survival rate of 97%. The second 33% are homozygous Mer 3 individuals and their mortality rate is much higher as seen in table 3.1. The mortality rate of the Mer 3 population is 66%, meaning that only 34% or one third survive. If one third of the 33% survive, then only 11% of the individuals survive to adult. Therefore of the 66% discussed 44% of those individuals survived. The true mortality rate as a shown in table 3.1 for the wild-type and Mer 3

crossed population is 45% resulting in 55% surviving. This means the difference between the 44% calculated and the 55% total is the survival rate of heterozygous wild-type and Mer 3 individuals. Therefore, the survival rate of heterozygotes is 11% out of a possible 33%, resulting in approximately one third of heterozygotes surviving and two thirds dying. The mortality rate of the heterozygotes is 66%. This has shown that heterozygote *D. melanogaster* have the same survival rate as the *D. melanogaster* which are homozygous for Mer 3, as both have a decreased survival rate.

Generation two of this Mer 3 and wild-type hybrid population had a mortality rate of 39% as shown in table 3.1. The potential phenotypic ratios can be seen in table 3.5. If the three possible ratios are equally likely to randomly occur then similarly to above each represents 33% of the entire population. The first 33% would result in a 1:1 ratio of a homozygous wild-type individual and a heterozygous individual whom are both equally likely to occur, representing 16.5% of the entire population (100%) each. The homozygous wild-type would again have a low mortality rate of 3%, where 97% survive. Whereas, the heterozygote would have 66% mortality rate and thus only 5.5% of a possible 16.5% would survive. The second 33% results in a 1:1 ratio of a homozygote Mer 3 individual and a heterozygote. Whom both have a mortality rate of 66%, resulting in only 11% of the 33% surviving. Lastly the third 33% would result in all individuals being heterozygotes wild-type and Mer 3 individuals. These again have a mortality rate of 66%, resulting in 11% of this 33% surviving. Ultimately the expected survival rate of this second generation is 44%. However, table 3.1 shows that the survival rate is in fact 61%. This may be the result of the three potential parental couplings not being

equally distributed and that the *D. melanogaster* are selectively breeding, choosing mates based on their fecundity and the potential offspring they could produce, following natural selection as opposed to random chance by neutral selection and genetic drift. This suggests that the *D. melanogaster* are selecting mates that are most likely to produce offspring that will survive, therefore naturally selecting the wild-type *D. melanogaster*.

4.6 Wild-Type and Mer 4 Hybrid Populations – Generation 1 & 2

In the initial hybrid population of wild-type and Mer 4 there are three possible parent combinations (shown is table 3.3). From this there are potentially three outcomes for offspring. If one presumes that the parental couples are equally random to occur then each of the three potential offspring would encompass 33% of the population each. Each of these three population have their own mortality and survival rate. The 33% of offspring that are homozygous wild-types, the mortality rate is 3% this results in almost all of the 33% surviving and very little mortality. This is due to these individuals being phenotypically wild-type with a high survival rate of 97%. The second 33% are homozygous Mer 4 individuals and their mortality rate is much higher as seen in table 3.1. The mortality rate of the Mer 4 population is 73%, meaning that only 27% or approximately one quarter survive. If one quarter of the 33% survive, then only 8.3% of the individuals survive to adult. Therefore of the 66% discussed 41.3% of those individuals survived. The true mortality rate as a shown in table 3.1 for the wild-type and Mer 4 crossed population is 44% resulting in 56% surviving. This means the difference between the 41.3% calculated and the 56% total is the survival rate of heterozygous wild-type and Mer 3 individuals. Therefore, the survival rate of heterozygotes is 15% out of

a possible 33%, resulting in approximately half of heterozygotes surviving and half dying. The mortality rate of the heterozygotes is approximately 50%.

This has shown that heterozygote *D. melanogaster* have a lower mortality rate than the *D. melanogaster* which are homozygous for Mer 4.

Generation two of this Mer 4 and wild-type hybrid population had a mortality rate of 19% as shown in table 3.1. The potential phenotypic ratios can be seen in table 3.6. If the three possible ratios are equally likely to randomly occur then similarly to above each represents 33% of the entire population. The first 33% would result in a 1:1 ratio of a homozygous wild-type individual and a heterozygous individual whom are both equally likely to occur, representing 16.5% of the entire population (100%) each. The homozygous wild-type would again have a low mortality rate of 3%, where 97% survive. Whereas, the heterozygote would have 50% mortality rate and thus only 8.3% of a possible 16.5% would survive. The second 33% results in a 1:1 ratio of a homozygote Mer 4 individual and a heterozygote. The homozoygous Mer 4 individual has a quarter survival rate meaning that 4% of the potential 16% survive. The heterozygote has a 50% mortality rate meaning 8.3% would survive. Lastly the third 33% would result in all individuals being heterozygotes wild-type and Mer 4 individuals. These have a mortality rate of 50%, resulting in 16.5% of this 33% surviving. Ultimately the expected survival rate of this second generation is 53.6%. However, table 3.1 shows that the survival rate is in fact 81%. This may be the result of the three potential parental couplings not being equally distributed and that the *D. melanogaster* are selectively breeding, choosing mates based on their fecundity and

the potential offspring they could produce, following natural selection as opposed to random chance by neutral selection and genetic drift. This suggests that the *D. melanogaster* are selecting mates that are most likely to produce offspring that will survive, therefore naturally selecting the wild-type *D. melanogaster*.

4.7 The population containing all three variants (Wild-Type, Mer 3 and Mer 4)

This last population, which was a hybrid population of the previous two hybrid populations, a double hybrid, contained all three merlin alleles (Wild-Type, Mer 3 and Mer 4) within the gene pool of the population. An individual of this population may have had two of its' grandparents as pure Wild-Type alleles, one grandparent of pure Mer 3 and the fourth grandparent as pure Mer 4. The potential genotypes of the individuals in this double hybrid are shown in the table below with their 4 grandparent's phenotypes.

(Potential) Alleles of an individual of the double hybrid population (Wt = Wild-Type, M3 = Mer 3, M4 = Mer 4)	**Four grandparents of the individual**
Wt Wt	Four Wild-Type
Wt M4	Two Wild-Type and Two Mer 4
Wt (Wt or M4)	Three Wild-Type and One Mer 4
Wt (Wt or M3)	Three Wild-Type and One Mer 3
M4 (Wt or M3)	One Wild-Type, One Mer 3and Two Mer 4

(Wt or M3) (Wt or M4)	Two Wild-Type, One Mer 3 and One Mer 4
Wt M3	Two Wild-Type and Two Mer 3
M3 M4	Two Mer 3 and Two Mer 4
M3 (Wt or M4)	One Wild-Type, One Mer 4and Two Mer 3

Table 4.7 – This table shows the potential or known alleles of an individual depending on their grandparent's phenotype.

These nine potential offspring are also shown in table 3.8. If the nine phenotypic ratios are equally likely to occur then they each represent approximately 11% of the overall population. The first 11% would be 100% homozygous wild-type. The second would result in a 1:1 ratio of homozygous wild-type to heterozygous Mer 4 and wild-type *D. melanogaster*. The third would be 100% heterozygous Mer 4 and wild-type *D. melanogaster*. The fourth would result in a 1:1 ratio of homozygous wild-type to heterozygous Mer 3 and wild-type *D. melanogaster*. The fifth would result in a 1:1:1:1 ratio of homozygous wild-type: heterozygous Mer 3 and wild-type: heterozygous Mer 4 and wild-type: heterozygous Mer 3 and Mer 4. The sixth would result in a 1:1 ratio of heterozygous Mer 4 and wild-type, and heterozygous Mer 3 and Mer 4. The seventh would be 100% heterozygous Mer 3 and wild-type *D. melanogaster*. The eight 11% would be a 1:1 ratio of heterozygous Mer 4 and wild-type, and a heterozygous Mer 3 and Mer 4. The last 11% would result in 100% heterozygous Mer 3 and Mer 4 Individuals. Note that heterozygous individuals with Mer 3 and Mer 4 genes were not viable and did not develop into adults. Using the mortality rates and survival rates from table 3.1 and Section 4.5 and 4.6. The overall mortality rate of this population is calculated to be approximately 56% with the survival rate at 44%. However, table 3.1

shows that the true mortality rate is 13% and the survival rate is 87%. This is drastically higher than expected and this is likely due to the suggestion made in the previous two section (4.5 and 4.6). This suggestion inferred that the *D. melanogaster* are selecting mates based on natural selection and their ability to produce many viable offspring. This seem to also be the case see here with the survival rate double that which is expected.

4.6 Conclusion

In conclusion, this methodology has highlighted the lethal nature of mutants for merlin, particularly those previously addressed as recessive and detrimental to *D. melanogaster*. All populations showed evidence that natural selection was occurring within the populations and that genes associated with cancer phenotypes such as merlin in this research are subject to natural selection and are indicated to be lost via breeding within a population.

4.7 Future Work

Research into cancer genes is increasing often, with more data and theories available. It is important that the model organisms used is viable and can produce increased reliability and usable data. Using other models than *D. melanogaster* would be useful for this type of research. Using mouse models would improve experimentation due to the mouse being a closer model to humans. Eventually using a human model itself, mapping the merlin variation throughout the population, within genetic clusters (races). Mapping the variation within the human population would be the next step to this research, specifically if individuals of varying demographic ancestry e.g. mixed race individuals, would allow an inheritance pattern to be mapped for the human population, confirming the genetic drift and natural selection discussed in this thesis. A similar model using *D. melanogaster* could be used for other proteins. This research focused on monogenic diseases, but many diseases like cancer are the result of polygenic interactions, many proteins becoming non-functional, again further research into more proteins and the interactions they create would benefit the literature, possibly even producing a prevention or treatment in the future. A useful form of treatment for lethal alleles within the population would be gene therapy, introducing the normal/ functioning allele into an individual with a mutant or non-functioning allele. Further to this immunotherapeutic avenues could be considered, with the use of antibodies toward mutant merlin (McGranahan *et al*., 2017).

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Appendix

WHO classification of tumours of the central nervous system

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	9400/3
Gemistocytic astrocytoma, IDH-mutant	9411/3
Diffuse astrocytoma, IDH-wildtype	*9400/3*
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH-mutant	9401/3
Anaplastic astrocytoma, IDH-wildtype	*9401/3*
Anaplastic astrocytoma, NOS	9401/3
Glioblastoma, IDH-wildtype	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epithelioid glioblastoma	*9440/3*
Glioblastoma, IDH-mutant	9445/3*
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M–mutant	9385/3*
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9451/3
Anaplastic oligodendroglioma, NOS	*9451/3*
Oligoastrocytoma, NOS	*9382/3*
Anaplastic oligoastrocytoma, NOS	*9382/3*
Other astrocytic tumours	
Pilocytic astrocytoma	9421/1
Pilomyxoid astrocytoma	9425/3
Subependymal giant cell astrocytoma	9384/1
Pleomorphic xanthoastrocytoma	9424/3
Anaplastic pleomorphic xanthoastrocytoma	9424/3
Ependymal tumours	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Papillary ependymoma	9393/3
Clear cell ependymoma	9391/3
Tanycytic ependymoma	9391/3
Ependymoma, *RELA* fusion–positive	9396/3*
Anaplastic ependymoma	9392/3
Other gliomas	
Chordoid glioma of the third ventricle	9444/1
Angiocentric glioma	9431/1
Astroblastoma	9430/3
Choroid plexus tumours	
Choroid plexus papilloma	9390/0
Atypical choroid plexus papilloma	9390/1
Choroid plexus carcinoma	9390/3
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	9413/0
Gangliocytoma	9492/0
Ganglioglioma	9505/1
Anaplastic ganglioglioma	9505/3
Dysplastic cerebellar gangliocytoma (Lhermitte–Duclos disease)	9493/0
Desmoplastic infantile astrocytoma and ganglioglioma	9412/1
Papillary glioneuronal tumour	9509/1
Rosette-forming glioneuronal tumour	9509/1
Diffuse leptomeningeal glioneuronal tumour	
Central neurocytoma	9506/1
Extraventricular neurocytoma	9506/1
Cerebellar liponeurocytoma	9506/1
Paraganglioma	8693/1
Tumours of the pineal region	
Pineocytoma	9361/1
Pineal parenchymal tumour of intermediate differentiation	9362/3
Pineoblastoma	9362/3
Papillary tumour of the pineal region	9395/3
Embryonal tumours	
Medulloblastomas, genetically defined	
Medulloblastoma, WNT-activated	9475/3*
Medulloblastoma, SHH-activated and *TP53*-mutant	9476/3*
Medulloblastoma, SHH-activated and *TP53*-wildtype	9471/3
Medulloblastoma, non-WNT/non-SHH	9477/3*
Medulloblastoma, group 3	
Medulloblastoma, group 4	
Medulloblastomas, histologically defined	
Medulloblastoma, classic	9470/3
Medulloblastoma, desmoplastic/nodular	9471/3
Medulloblastoma with extensive nodularity	9471/3
Medulloblastoma, large cell / anaplastic	9474/3
Medulloblastoma, NOS	9470/3
Embryonal tumour with multilayered rosettes, C19MC-altered	9478/3*
Embryonal tumour with multilayered rosettes, NOS	*9478/3*
Medulloepithelioma	9501/3
CNS neuroblastoma	9500/3
CNS ganglioneuroblastoma	9490/3
CNS embryonal tumour, NOS	9473/3
Atypical teratoid/rhabdoid tumour	9508/3
CNS embryonal tumour with rhabdoid features	*9508/3*
Tumours of the cranial and paraspinal nerves	
Schwannoma	9560/0
Cellular schwannoma	9560/0
Plexiform schwannoma	9560/0

Melanotic schwannoma	9560/1
Neurofibroma	9540/0
Atypical neurofibroma	9540/0
Plexiform neurofibroma	9550/0
Perineurioma	9571/0
Hybrid nerve sheath tumours	
Malignant peripheral nerve sheath tumour	9540/3
Epithelioid MPNST	9540/3
MPNST with perineurial differentiation	9540/3
Meningiomas	
Meningioma	9530/0
Meningothelial meningioma	9531/0
Fibrous meningioma	9532/0
Transitional meningioma	9537/0
Psammomatous meningioma	9533/0
Angiomatous meningioma	9534/0
Microcystic meningioma	9530/0
Secretory meningioma	9530/0
Lymphoplasmacyte-rich meningioma	9530/0
Metaplastic meningioma	9530/0
Chordoid meningioma	9538/1
Clear cell meningioma	9538/1
Atypical meningioma	9539/1
Papillary meningioma	9538/3
Rhabdoid meningioma	9538/3
Anaplastic (malignant) meningioma	9530/3
Mesenchymal, non-meningothelial tumours	
Solitary fibrous tumour / haemangiopericytoma**	
Grade 1	8815/0
Grade 2	8815/1
Grade 3	8815/3
Haemangioblastoma	9161/1
Haemangioma	9120/0
Epithelioid haemangioendothelioma	9133/3
Angiosarcoma	9120/3
Kaposi sarcoma	9140/3
Ewing sarcoma / PNET	9364/3
Lipoma	8850/0
Angiolipoma	8861/0
Hibernoma	8880/0
Liposarcoma	8850/3
Desmoid-type fibromatosis	8821/1
Myofibroblastoma	8825/0
Inflammatory myofibroblastic tumour	8825/1
Benign fibrous histiocytoma	8830/0
Fibrosarcoma	8810/3
Undifferentiated pleomorphic sarcoma / malignant fibrous histiocytoma	8802/3
Leiomyoma	8890/0
Leiomyosarcoma	8890/3
Rhabdomyoma	8900/0
Rhabdomyosarcoma	8900/3
Chondroma	9220/0
Chondrosarcoma	9220/3
Osteoma	9180/0
Osteochondroma	9210/0
Osteosarcoma	9180/3
Melanocytic tumours	
Meningeal melanocytosis	8728/0
Meningeal melanocytoma	8728/1
Meningeal melanoma	8720/3
Meningeal melanomatosis	8728/3
Lymphomas	
Diffuse large B-cell lymphoma of the CNS	9680/3
Immunodeficiency-associated CNS lymphomas	
AIDS-related diffuse large B-cell lymphoma	
EBV-positive diffuse large B-cell lymphoma, NOS	
Lymphomatoid granulomatosis	9766/1
Intravascular large B-cell lymphoma	9712/3
Low-grade B-cell lymphomas of the CNS	
T-cell and NK/T-cell lymphomas of the CNS	
Anaplastic large cell lymphoma, ALK-positive	9714/3
Anaplastic large cell lymphoma, ALK-negative	9702/3
MALT lymphoma of the dura	9699/3
Histiocytic tumours	
Langerhans cell histiocytosis	9751/3
Erdheim–Chester disease	9750/1
Rosai–Dorfman disease	
Juvenile xanthogranuloma	
Histiocytic sarcoma	9755/3
Germ cell tumours	
Germinoma	9064/3
Embryonal carcinoma	9070/3
Yolk sac tumour	9071/3
Choriocarcinoma	9100/3
Teratoma	9080/1
Mature teratoma	9080/0
Immature teratoma	9080/3
Teratoma with malignant transformation	9084/3
Mixed germ cell tumour	9085/3
Tumours of the sellar region	
Craniopharyngioma	9350/1
Adamantinomatous craniopharyngioma	9351/1
Papillary craniopharyngioma	9352/1
Granular cell tumour of the sellar region	9582/0
Pituicytoma	9432/1
Spindle cell oncocytoma	8290/0
Metastatic tumours	

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) [742A]. Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification, taking into account changes in our understanding of these lesions.
*These new codes were approved by the IARC/WHO Committee for ICD-O. *Italics*: Provisional tumour entities. **Grading according to the 2013 *WHO Classification of Tumours of Soft Tissue and Bone*.