AN INVESTIGATION OF VARIOUS INTRINSIC AND EXTERNAL FACTORS THAT INFLUENCE IN VITRO CELL SURVIVAL OUTCOMES DURING RADIATION-INDUCED BYSTANDER EFFECT EXPERIMENTS

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Lay Abstract

The radiation-induced bystander effect is an important phenomenon in the field of radiation biology and radiation safety. Irradiated cells are able to communicate radiation damage to other, potentially healthy, cells in the surrounding area. Over the past 30 years, many studies have been performed to better understand how this bystander effect works. This thesis aims to determine what variables have a significant influence on the results of these bystander effect experiments. This will allow future research in this area to become more standardized and consistent, thus making comparisons between studies easier and results more powerful.

Abstract

The radiation-induced bystander effect is an important phenomenon in the field of radiation biology. It has been shown that cells, after exposure to radiation, can communicate with surrounding cells and affect their physiology. Otherwise-healthy recipient cells can be influenced to undergo cellular senescence or apoptosis through this process. This has potential utilizations for radiation oncology and as well as our understanding of radiation safety. The radiation-induced bystander effect has been extensively investigated since the 1990s, but the scientific community struggles to come to a unanimous decision on how strongly these signals impact the survival of bystander cells. Results show various degrees of impact on cell survival whereas certain studies refute the existence of a radiation-induced bystander effect. This may be due to the fact that there is a great deal of study heterogeneity within the radiation-induced bystander effect community. Most experiments follow a similar general bystander protocol but often use different donor and reporter cell lines that vary in sex, organ of origin, and p53 status. The type of radiation and dose rate also typically differ between experimental designs. In this analysis, 67 *in vitro*, medium-transfer, radiation-induced, bystander effect studies were retrospectively graphed and analyzed to determine which intrinsic and external factors contributed significantly to the overall survival percentage change observed in reporter cells. A Two-Way ANOVA was conducted on each variable and showed that the reporter cell line, p53 status, and radiation type had a statistically significant effect on survival percentage change. These findings may explain the variation in results seen in past experiments and may help standardize future research allowing for more direct comparisons.

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Chapter 1: Introduction

1.1 Early Days of Radiobiology

The field of radiobiology explores how ionizing radiation affects living things. Before discussing the physics responsible for these radiation effects, it is important to understand the beginnings of this area of research. In 1895, Wilhelm Röntgen announced a novel kind of ray known as the “x-ray”. He used the x-ray to demonstrate how one can produce a radiograph by exposing an object in front of photographic film (Podgorsak, 2018). The diagnostic and therapeutic potential were expanded upon one year later, when Leopold Freund showed that a hairy mole could be treated with exposure to x-rays (Kogelnik, 1997). That same year, Antoine Becquerel discovered radioactivity emanating from uranium compounds. In 1898, the “mother of radioactivity”, Marie Curie, and her husband, Pierre Curie, announced the discovery of two new radioactive elements, polonium and radium (Reed, 2011). The first radium "burn" was reported by Pierre Curie in 1901 (after having intentionally attempted to self-induce skin erythema), and thus began the field of radiobiology as we know it (Reed, 2011).

1.2 The Physics of Radiation

Radiation in general refers to energy in the form of waves or particles, which is transferred from one site to another. Radiation that is described as “ionizing” has sufficient energy to eject electrons from the shell of the atom, thus creating an ion (Podgorsak, 2018). Ionizing radiation may be classified as either electromagnetic (which consists of photons such as x-rays and gamma rays) or particulate (which consists of particles such as alpha particles,

beta particles, etc.). Photon energy is dictated by the formula, E = hf, where h is Planck's constant and f is the frequency. Both x-rays and gamma rays have relatively short wavelengths, meaning a higher frequency and, by extension, increased energy per photon (Steiner, 2012). Photons and particles deposit their energy into cells, which may break chemical bonds present in the biological tissue and lead to physiological change (Podgorsak, 2018). Importantly, total energy is less biologically significant than the form of the delivered energy (for example, heat is less detrimental to biological systems than equivalent doses of radiation). In order to induce ionization, electromagnetic radiations require a photon energy of more than 124 ev, or a wavelength shorter than 10^{-6} cm (Hall & Giaccia, 2012).

Many different types of particulate radiation are used during radiation biology experiments. These include alpha particles, beta particles, protons, neutrons, and heavy charged particles. Alpha particles are nuclei of helium atoms and are positively charged. They are emitted during the decay of many radioactive elements such as uranium. Beta particles are fast-moving, high-energy electrons and are negatively charged. Protons are positively charged particles that make up the nucleus of an atom and are 2000 times more massive than electrons. Neutrons are another particle that make up the nucleus in an atom, however, they carry no electrical charge and have a mass just slightly greater than protons. Neutrons can be produced when a charged particle is accelerated into a certain target material, or they can be produced as a by-product of nuclear fission; when an atom splits into two smaller atoms. Lastly, heavy charged particles are nuclei of elements that have had some or all of their orbital electrons stripped away, resulting in a positively charged ion. Elements such as carbon and iron are commonly used during radiobiology

experimentation. All of these particles are considered directly ionizing, meaning that, with sufficient energy, they can interact with and change the atomic structure of the tissue they pass through, leading to biological damage (Bolus, 2017).

Electromagnetic radiation is considered indirectly ionizing, meaning that when the photons deposit their energy into the target tissue, the photons themselves do not cause any biological damage. However, the energy transfer produces charged particles that can cause biological damage. Two main types of radiation absorption occur when looking at photons with relatively low energy. The first method of absorption is through the Compton process, whereby the incident photon interacts with an orbital electron. The electron is liberated from the atom and proceeds at a high speed, while the photon loses energy and is scattered in a different direction (Bolus, 2017)). The second method of absorption is through the photoelectric process. During this process, a photon transfers all of its energy to the atom which then loses the energy by ejecting an inner-shell electron. This causes the electron to eject from the atom, leaving a vacancy in the electron shell. Due to this, outer shell electrons or free electrons fill the unoccupied space. As this requires electrons to change energy levels, the excess energy is released as a photon that can initiate further interactions (Bolus, 2017).

Another quality of radiation is linear energy transfer (LET). This characteristic helps to indicate how much energy is lost by a photon/charged particle per unit length of a given material. LET is inversely proportional to distance, meaning that as radiation travels further and deposits energy less frequently, LET decreases. Alpha particles and

secondary electrons released by x-rays are examples of radiation with a high and low LET, respectively (Paganetti, 2014).

It is understood that the critical target of radiation damage is deoxyribonucleic acid (DNA). DNA can be damaged through direct or indirect action of radiation. Direct action occurs when the ionizing photon or particle interacts with an atom in the target, creating a fast-moving electron that causes damage and leads to biological change. Conversely, indirect action occurs when radiation interacts with other atoms or molecules in the cell and produces free radicals that travel further and damage DNA. Free radicals describe atoms with an unpaired electron in their outer shell, making them extremely reactive. These free radicals can cause biological changes if they hit the critical target, causing chemical changes and breaking bonds (Heeran *et al*., 2019). It is important to note that, although these interactions happen within an extremely short period of time at the atomic level, significant changes might not occur for days, weeks, months, or even years. Noticeable changes, such as cell progression to cancer, occur over a longer period of time. In affected germ cells, heritable effects may not be seen for generations (Hanahan & Weinberg, 2000).

1.3 The Bystander Effect

It was originally thought that direct damage to the DNA was required for biological effects to occur. However, a paper published in 1992 by Nagasawa and Little uncovered a phenomenon later named the “bystander effect”. They found that cells that were not directly traversed by a photon or particle may exhibit biological effects when in close proximity to cells that have been irradiated (Heeran *et al*., 2019). In their experiment,

Nagasawa and Little exposed Chinese hamster ovary cells to alpha particles. They found that 30% of the cells showed an increase in sister chromatid exchanges, but only about 1% of the cells were actually traversed by an alpha particle (Nagasawa & Little, 1992). This showed that cells communicate radiation damage and can induce changes in neighbouring cells.

There are two methods by which bystander effects can occur. The first is through intercellular gap-junction communication. Gap-junctions exist between two cells and allow for molecular movement to occur through passive diffusion (Kumar & Gilula, 1996). This type of bystander effect is usually studied by using a microbeam, which allows researchers to deliver a specific number of particles to a specific number of cells. The effects can then be observed in the non-irradiated cells in close proximity to those traversed by a particle (Hall & Giaccia, 2012). The second method is when cells secrete a molecule into the intracellular matrix capable of inducing apoptosis and other biological effects in neighbouring cells. This is commonly studied by medium-transfer experiments where the medium from irradiated cells is filtered and used to culture new non-irradiated cells, known as “reporter cells” (Mothersill & Seymour, 1997).

1.4 The History of the Bystander Effect

Early 1900s

As discussed previously, the bystander effect is not just one phenomenon. It is a description of many chemical and biological processes that culminate in an observed change in cells that have not been directly exposed to radiation. As such, the bystander effect has been reported under many different names throughout the 1900s. Advances in

science and technology have allowed humanity to look back and better understand publications that describe these indirect radiation effects, all of which are important to the current understanding of the bystander effect (Mothersill *et al*., 2018).

The first noted reference of an indirect radiation effect was a paper by Heineke *et al*. in 1905. In this study, irradiation experiments were performed on tumours in mice, effectively stimulating the secretion of “lymphoid elements” (Heineke *et al*., 1905). This was expanded upon by Murphy and Morton in 1915. These subsequent experiments implemented tumour-bearing mice that had their tumour removed in the treatment group, or had their tumour left untouched in the control group. Afterwards, the mice were irradiated, and the tumours were implanted back into the mice in the treatment group. The tumours and mice were then observed. It was noted that, in the treatment group, unirradiated tumour grafts shrank by up to 50% and the animals lived for up to 5 weeks or more. The control group, on the other hand, saw their tumours continue to grow after a short period of time and resulted in the death of the animals after approximately one week (Murphy & Morton, 1915). Retrospectively, this was identified as a finding of radiation damage being communicated to normal tissues. (Mothersill *et al*., 2018).

Furthermore, Strangeway and Fell (1927) exposed chicken eggs to two different doses of radiation and then observed changes in the embryos after different incubation periods ranging from 20 hours to 17 days. Cells were explanted for culture *in vitro* after the incubation period and the cells were observed to determine if they were able to recover after irradiation. It was noted that, “with the exception of the relatively small number of cells destroyed by the direct action of radiation the death of the tissues in the

6-day embryos was the result of some indirect action" (T. S. P. Strangeways & Honor Bridget Fell, 1927). Two different modes of action were identified: Firstly, an indirect, local action where, "the rays irradiate the general tissues which supply nourishment, and death of the cancer cells is the result of the increase in local defensive processes: phagocytosis, changes in circulation, sclerosis, etc." (T. S. P. Strangeways & Honor Bridget Fell, 1927). In addition to an indirect, general action where, "the radiations liberate into the circulation, from the tissues, a substance which acts either as a toxin on certain cells, or as a stimulant to certain general organic chemical reactions" (T. S. P. Strangeways & Honor Bridget Fell, 1927).

The understanding of the bystander effect is inextricably linked to the discovery of DNA and its importance in oncogenesis. In 1914, Dr. Theodore Boveri suggested that cancer cells were, "formed through alteration of normal cells" and that, "tiny, microscopic bodies called chromosomes might be abnormally distributed in tumor cells" (Huang & Zhou, 2021). Aiming to explore this theory, Hermann Muller decided to expose common fruit flies to ionizing radiation in the form of x-rays. He reported that the radiation was able to contribute to aberrations in chromosomes in the form of "transmutation" of a gene (Muller, 1928).

Mid 1900s

The middle of the 20th century was characterized by advances in the understanding of DNA and how chromosomes store the genetic material required for cellular function. In 1930, a paper by McNaughton reviewed previous experiments performed by the author and his colleagues. In this review, it was stated that radiation can cause small changes

to the DNA, which he called “point mutations”, and larger changes such as heritable mutations in the chromosomes (McNaughton, 1930). Although most of his studies were focussed primarily on the direct effects of radiation exposure, indirect action of radiation was identified. In the review, it was noted that changes in a gene could be caused, “indirectly through the intermediary agency of injurious chemical substances or physical conditions that become diffused through the cell as a result of the irradiation of the latter” (McNaughton, 1930).

Over the following years, further discoveries challenged common beliefs on how radiation interacted with tissues (Mothersill *et al*., 2018). A study by Hevesy (1945) found that after irradiating rats with sarcomas, tumours only shrank under a specific condition: when a different part of the body was irradiated, especially areas that did not have any tumours. These findings implied that healthy cells were able to transmit bystander signals to other areas that were not exposed to radiation which was suggested to be due to free radicals (G. Hevesy, 1945).

Following this, a study by Benjamin Jolles (1949) queried the existence of a diffusible substance that was produced in irradiated tissue. In his experiment, volunteers were given a dose of x-rays to two different areas of skin separated by varying width. The reaction of the skin was compared with a control group where a single area of skin was exposed to the same total dose of radiation. It was found that the reactions produced by the two separated areas were much larger than the control group’s reaction. Backscatter/additional dosage was ruled out and a diffusible substance was suggested as an explanation for this result (Jolles, 1950).

This topic was developed further by Mole in 1953, who coined the term “abscopal effects” to differentiate from indirect effects. The term “abscopal” was made to describe changes that were, “at a distance from the irradiated volume but within the same organism” (Mole, 1953). This paper referenced an experiment conducted earlier that same year in which rats experienced an impaired function of the thyroid gland when a sufficiently large volume of the abdomen was irradiated, demonstrating this abscopal effect.

Building on this concept, Parsons *et al*. (1954) published a study that supported the concept of abscopal effects and challenged the scientific consensus. In their study, patients with chronic granulocytic leukemia had their spleens exposed to therapeutic doses of x-rays. Subsequently, a reduction of cellularity in the sternal bone marrow of each patient was observed. This effect on the sternal bone marrow was attributed to the spleen irradiation and strongly suggested an indirect effect of the radiation (Parsons *et al*., 1954).

Late 1900s

By the 1960s, there were numerous studies investigating DNA damage and how it related to the detrimental effects seen in atomic bomb and Chernobyl survivors (Mothersill *et al*., 2018). A new term, “clastogenic factors”, was used throughout the late 1900s. It was used to describe factors, present in the plasma, that irradiated cells can release to induce chromosome damage *in vivo* or *in vitro* (Mothersill *et al*., 2018). General indirect effects were still mentioned in the literature, although, to a lesser degree. The exploration of

abscopal effects seemed to take precedence, however, there is a great deal of overlap between the two terms (Averbeck, 2023).

Souto (1962) was the first to mention a clastogenic effect. In his experiments, gamma rays from cobalt-60 were used to irradiate Sprague-Dawley rats of both sexes and Hampshire sheep of both sexes. The blood plasma from the lethally irradiated rats and sheep were injected into non-irradiated counterparts and it was found that the blood plasma was able to increase the frequency of breast tumours in the recipient female rats. This was hypothesized to be due to, “tumorogenic substances or factors potentiating the onset of [the] mammary tumours” (Souto, 1962).

In the later years of the 1960s, Joseph G. Hollowell and L. Gayle Littlefield published important papers that supported the theory that clastogenic factors were able to induce chromosomal damage in non-irradiated cells, a major steppingstone for the radiation-induced bystander effect field. Six patients, who were being treated for various cancers and had received large doses of x-rays, were selected for the studies. Hollowell and Littlefield added blood plasma from these patients to normal non-irradiated lymphocytes in a short-term culture. Chromosomal abnormalities were noted and classified such as chromatid breaks, dicentric chromosomes, chromatid exchanges, translocations, and chromosomal clumping. They showed that the blood plasma from irradiated patients was able to cause significant damage to the chromosomes of otherwise healthy lymphocytes and that the communication of radiation-induced damage was not restricted to the same cell type or organism. Their papers mentioned a “chromosome-breakage factor” present in the blood plasma that was able to “cause a

shift in DNA polymerase equilibrium", which clearly fits the description of clastogenic factors (Hollowell & L. Gayle Littlefield, 1968).

Chromosomal aberrations in lymphocytes continued to be explored throughout the 1970s. Demoise and Conard (1972) conducted experiments using the blood plasma from residents of the Marshall Islands. The population was exposed to high levels of radioactive fallout after a nearby nuclear device detonation in 1954. Similarly, to the method used by Hollowell and Littlefield, blood plasma from the exposed population was transferred to cultures of normal lymphocytes. A population of unexposed people were used as a control group. The results showed that the exposed population induced double the amount of chromosome aberrations in recipient cells compared with the control group (Demoise & Conard, 1972). In 1975, Goh published a study that showed that blood plasma, from men who were exposed to high levels of radiation, induced chromosomal aberrations 10.5 years after the original exposure. This study showed that clastogenic factors were able to persist *in vivo* for much longer than originally thought (Goh, 1975).

Furthering this revelation, a study done in 1977 by Pant and Kamada used the blood plasma from atomic bomb survivors, who had been exposed more than 30 years earlier, to study the effect of the plasma on leukocytes. They also observed the effects of blood plasma from x-rayed patients on leukocytes. The groups had varying amounts of chromosome break percentages. Only 3.7% of the control culture experienced chromosome breaks, while 12.5% of the culture with plasma from x-rayed patients had chromosomal breaks. Interestingly, 10.9% of the culture from atomic bomb survivors had induced chromosomal breaks in the recipient leukocytes. It was concluded that the

longevity of clastogenic effects could last for a very long time, even up to 31 years after radiation exposure, as was the case with the atomic bomb survivors (Pant & Kamada, 1977).

Another important paper, expanding the knowledge of clastogenic factors, was published in the 1980s by Faguet *et al*. Whole-body irradiation of rats resulted in the immediate emergence of clastogenic factors in the circulation. Furthermore, these factors, capable of inducing chromosomal damage, persisted for up to 10 weeks post-irradiation (Faguet *et al*., 1984). Additionally, blood plasma was isolated from nonirradiated rats, and subsequently exposed the plasma to radiation *in vitro*. However, clastogenic factors were not found after the sole irradiation of the plasma. This confirmed that the factors were intrinsic to the cells of the organism, and not a byproduct of a biological process from within the plasma itself (Faguet *et al*., 1984).

One of the most important findings in the late 1980s was that cells were able to pass on heritable defects to progeny cells, even after multiple successful divisions. Previously, it was presumed that if a cell successfully divided after irradiation, all the DNA damage that occurred must have been repaired. This followed the thought that lethal mutations would result in the cell failing to reproduce and only non-lethal mutations would be passed on to progeny cells (Mothersill *et al*., 2018). A paper published in 1986 by Seymour *et al.* challenged this understanding. In their experiment, CHO-K1 cells were irradiated using a cobalt-60 teletherapy unit. Cells from the surviving colonies were used to establish confluent cultures, multiple times. This allowed for survivor cells to proliferate and undergo many cellular divisions up to approximately the 16^{th} generation. It was found

that plating efficiencies were considerably lower for the descendants compared with those from unirradiated cultures (Seymour *et al*., 1986). This initiated a change in the understanding of radiation-induced heritable effects.

1.5 The Modern Bystander Effect

In the early 1990s, the modern understanding of the radiation-induced bystander effect became more prominent. Many studies were published that changed the way the scientific community viewed the effects of radiation. The relatively simplistic view that cells would either recover, mutate, or die, when exposed to radiation evolved into a more complex and nuanced interpretation. Cells have been found to communicate radiation damage to one another through multiple different methods, and the observed effects aren't limited to cells in close proximity at the time of irradiation. Genomic instability can cause lethal heritable effects in unirradiated descendant cells (Averbeck, 2023). Although many of the underlying conclusions were similar to those reported years earlier, the results were more widely accepted and compelling after years of research in the area. This modern paradigm is variously considered to have begun with the 1986 paper of Seymour *et al.* that was referred to in the previous section, a paper in 1992 by Kadhim *et al.* which coined the term genomic instability to describe the finding of non-clonal lethal chromosome aberrations in clonal descendants of irradiated stem cells, or the paper published in 1992 by Nagasawa and Little, who, as mentioned previously, were able to show the induction of sister chromatid exchanges by low doses of alpha particles. In the study conducted by Nagasawa and Little (1992), Chinese hamster cells were irradiated during mitosis with radiation from a plutonium-238 source. The original expectation was

that only cells that had been traversed by an alpha particle would exhibit damage to the chromosomes. However, up to 30% of the cells showed, an “increased frequency of [sister chromatid exchanges]” even though, “less than 1% of cell nuclei were actually traversed by an [alpha] particle” (Nagasawa & Little, 1992). The exact mechanism was unclear but it was noted that further research was needed to update the radiation risk evaluations of occupationally exposed individuals, as well as exposure standards for the general population (Nagasawa & Little, 1992).

Carmel Mothersill and Colin Seymour introduced a potential mechanism with their paper published in 1997. They irradiated three epithelial cell lines (HaCaT, PC-3, and SW-48) and one fibroblast cell line (MSU-1). The culture medium from those cell lines was used to culture fresh, nonirradiated cells. The results showed a “highly significant fall in cloning efficiency in unirradiated normal and malignant epithelial cell lines receiving medium from irradiated cultures” (Mothersill & Seymour, 1997). The authors ruled out the possibility of a toxic substance present in the medium by irradiating the medium in the absence of cells which showed no effect on the survival of recipient cells. This confirmed the involvement of extracellular soluble factors as a mechanism by which cells communicate radiation damage.

Another mechanism of cellular communication was discovered in 1998 by Azzam *et al.* Their group sought to understand the role of cell-to-cell communication and the signal transduction pathways involved in the bystander effect. The group exposed human diploid fibroblasts to alpha particles released from a plutonium-238 source. Western blot analysis showed that nearby bystander cells had increased levels of genetic damage

markers (TP53, CDKN1A, CDC2, CCNB1, and RAD51) despite only 2% of the cells having been traversed by an alpha particle. In a follow up study, the gap-junction inhibitor, lindane, was used and the experiment was repeated. The results showed that, "the extent of modulation of TP53 and CDKN1A [was] significantly reduced" (Azzam *et al*., 1998). As such, it was clear that these specific signal pathways relied on gap-junction communication to induce a bystander effect in neighbouring cells. The authors made note of the many different forms of the radiation-induced bystander effect that were being reported at the time and suggested that, "reactive oxygen species, extra-nuclear originating signaling pathways, and secreted diffusible factors may also be involved and/or linked with intercellular communication in inducing the bystander response of cells exposed to low-dose [alpha] particle radiation" (Azzam *et al*., 1998).

A different "branch" of the bystander effect "tree" was presented in a study by Mothersill *et al*. (2006). Previously, little work had been done to translate the effects seen *in vitro* to an *in vivo* model. In their experiment, rainbow trout were irradiated and allowed to swim with an unexposed partner fish for two hours. A third fish was added to the tank after the removal of the previous two and allowed to swim for another two hours. Explants of five organs (skin, fin, gill, spleen, and kidney) were taken from each fish and subsequently cultured for two days. The outgrowth was then examined, and culture medium was added to an unirradiated clonogenic cell line to determine the surviving fraction. Notably, recipient cells grown with the medium from explants of partner fish had reduced survival percentage outcomes. This effect was slightly magnified when using a medium from the third fish that was allowed to swim in the water after the removal of the other fish. These results suggested that radiation-induced damage could be passed from

one fish to another in water by means of a chemical messenger released into the water and was one of the first instances to show inter-animal signalling (Mothersill *et al*., 2006).

More recently, it was shown that biophotons were able to induce a bystander effect in nonirradiated cells. Le *et al*. (2015) irradiated HaCaT human keratinocyte cells with tritium. Unirradiated recipient HaCaT cell cultures were placed above the beta-irradiated cells. The beta particles released from the tritium were able to cause secondary UV photons to be emitted from the irradiated cells. The clonogenic survival of the reporter cells was then assessed. This experiment resulted in a 23.2% reduction in reporter cell survival compared with controls. To confirm their hypothesis, a polyethylene terephthalate filter, was added into the space between the irradiated and nonirradiated cultures and the experiment was repeated. The filter was able to block the majority of the UV wavelengths, as was expected, and resulted in the treatment groups having similar survival outcomes to the control groups. These findings suggested that, “secondary photons in the UV spectral range induced by beta irradiation play a role in inducing a response in neighboring non-beta-irradiated reporter cells” and essentially expanded the number of possible mechanism responsible for the bystander effect (Le *et al*., 2015).

1.6 The Complexity of the Literature

It is evident that the bystander effect is not just one simple phenomenon, but a collection of biological processes that occur in recipient cells when exposed to previously irradiated cells or signals originating from them. According to the literature, the bystander effect manifests itself as clastogenic factors, abscopal effects, genomic instability, and inter-animal signalling (Averbeck, 2023). Furthermore, these effects are transferred through

different methods such as gap-junction intercellular communication, extracellular soluble factors, and UV biophotons (Averbeck, 2023). Each of these methods can be induced through different means, such as exposing a single cell using an electron microbeam for gap-junction experiments or using a medium-transfer technique to research extracellular soluble factors. As such, the radiation-induced bystander effect literature is a heterogeneous collection of publications that use various experimental setups to further the understanding of this field. Even when exploring identical endpoints, it is common for authors or groups to change parameters between experiments. This thesis aims to explore how these differences impact the survival fraction outcomes of radiation-induced bystander effect experiments. The following sections will go over the current understanding of how each of the chosen factors affects the outcomes observed during bystander effect experiments. The chosen factors are the reporter cell line, organ of origin of the reporter cell line, sex of the reporter cell line, p53 status of the reporter cell line, radiation source, radiation type, dose rate, and dose.

1.7 The Chosen Factors: Reporter Cell Line

A decade after Nagasawa and Little published their landmark paper, Mothersill *et al*. (2002) sought to further the scientific understanding of the relationship between radiation-induced low-dose hypersensitivity and the bystander effect. The group irradiated 13 different human cell lines and performed medium-transfer experiments and clonogenic assays to see how each cell line differed from the control groups. The results showed that responses to radiation varied greatly. Cell lines such as GS3 and SW48 had greatly reduced cloning efficiency after exposure to 0.5 Gy irradiated cell-conditioned medium.

Others, such as TG98, HT29, and MSU1.1, had recipient cloning efficiencies that were higher than the controls. It was shown that cell lines that are malignant, anaerobic and rapidly proliferating have the smallest bystander effect since these cell lines, "have poor cell–cell communication and poor mitochondrial function, both of which are important in [the] transmission of bystander signals" (Mothersill *et al*., 2002).

A prevalent theory within the scientific community was that bystander effects act as a protective system within an organism in the sense that they terminate cellular division before unrepaired DNA damage is able to be passed on. As such, Mothersill *et al*. (2004) hypothesized that repair-deficient cell lines would have a larger-than-normal bystander effect. AT Br1, 180Br, Raji 9 and 10, SW48, XRS-5, and XR-1 were the repair deficient cell lines that were chosen for their experiment to test their hypothesis. Cloning efficiencies of reporter cells were investigated after irradiation and subsequent medium transfer. Interestingly, repair-deficient and repair-proficient cells were used as both donors and reporters in an allogenic donor-reporter system. It was found that, "the signal produced by the repair-deficient cells [that were] tested produce[d] more clonogenic cell death in autologous and HPV reporter recipients" (Mothersill *et al*., 2004). The authors noted that the repair capabilities of the reporter cell line were important with regard to the bystander effect seen and reference an observation that, "HPV-G cell medium transferred to HPV-G recipient cells ha[d] less [of an] effect than the same medium transferred to SW48 repair-deficient cells" (Mothersill *et al*., 2004).

In 2008, Ryan *et al*. attempted to show a radiation-induced adaptive response in fish cell lines. At the time, the scientific community was interested in determining how low-

dose radiation affects non-human species (Ryan *et al*., 2008). Three cell lines, CHSE-214, ZEB-2J, and RTG-2 were used for this experiment. These cells were derived from embryos of the Chinook salmon, zebrafish embryonic stem cells, and rainbow trout gonads, respectively. Clonogenic assays showed that ZEB-2J cells were much more sensitive to low-dose radiation compared to mammalian cell lines and that overall, fish cell lines observed a "protective" bystander response. After exposure to the irradiated cell-conditioned medium, the fish cell lines had increased cloning efficiencies compared to control groups (Ryan *et al*., 2008).

It is evident that a large variety of cell lines have been used as reporter cells during radiation-induced bystander effect experiments over the years. It is understood that these cell lines react to donor signals in different ways and can manifest as either a reduction or an increase in cloning efficiency and survival. These effects are not limited to human cell lines, as experiments with non-human cells show significant differences in how they respond to radiation-induced bystander signals.

1.8 The Chosen Factors: Organ of Origin

There are few publications that compare radiation-induced bystander effect data at a tissue-specific level. Most papers focus on individual cell lines that are derived from different organs or observe the effects in an *in vivo* model. However, there are inferences to be made from these experiments on how the organ of origin may affect the results in an *in vitro* model.

In 1997, Seymour and Mothersill conducted an experiment using different human epithelial cell lines. HaCaT cells, which originate from the skin, MSU-1 cells, which also

originate from the skin, PC-3 cells, which originate from the prostate, and SW48 cells, which originate from the colon were irradiated and the survival percentages of reporter cells were calculated. Although all cell types were treated the same way, results varied amongst the cell lines. Only normal skin cells saw a reduction in plating efficiency, whereas the progeny of the other cell lines, such as prostate and colon, showed no lethal mutation in progeny groups (Seymour & Mothersill, 1997).

A review of the literature performed in 2004 provided evidence that bystander effects occur *in vivo* at the level of the tissue or even at the level of an entire organism. It was suggested that *in vitro* models don't accurately predict risks at a larger scale and that, "bystander effects make the tissue respond as a whole and further demonstrate that radiation effects may be related to organized tissue responses rather than to alterations induced in single cells" (Brooks, 2004). The review stated that the organ of origin, when related to individual cells, may not be relevant in the context of the radiation-induced bystander effect.

An experiment by Ilnytskyy *et al*. (2009) exposed rats to whole-body or partial radiation and conducted an analysis of the molecular changes in bystander skin and spleen. It was described as the "first study to compare epigenetic *in vivo* bystander effects in different organs of the same organism that were located the same distance from the exposure field" (Ilnytskyy *et al*., 2009). After treatment, spleen tissue had a greater amount of DNA damage compared with the skin tissue. The authors concluded that soluble bystander factors were able to induce a tissue-specific response within the same organism and hypothesized that it was controlled by the "inherent epigenetic parameters

of each tissue and may determine tissue responses to direct and indirect IR effects" (Ilnytskyy *et al*., 2009).

A few years later, mice were used in an *in vivo* model to see the effects of fractionated low-dose radiation on the physiological function of normal tissues. It was noted that tissue-specific stem cells were responsible for replenishing the functional, differentiated cells of an organism throughout its lifetime. The study showed that stem cells from slowly renewing tissues, such as the brain may not respond to bystander signals as quickly or as strongly as stem cells from more quickly renewing tissues, such as the mucosa of the small intestine or of the epidermis of the skin (Schanz *et al*., 2012).

In 2013, Chai *et al.* exposed mice during an *in vivo* study aiming to compare Cyclooxygenase 2 (COX-2) expression and mutagenesis in different organs. COX-2 is an enzyme that is upregulated in many cancers and inhibition of this enzyme shows a clear reduction in mutagenesis. As such, it is a useful marker for DNA damage in bystander cells. Interestingly, they found that the induction of COX-2 was tissue-specific. Only the lungs of the mice exhibited elevated levels of COX-2 while the same animals showed no induction of COX-2 expression in liver tissues, even after 72 hours (Chai *et al*., 2012).

Cell lines from different organs react to bystander signals in different ways, although whether this is inherent to epigenetic parameters of the tissue or some other cause, is yet to be confirmed. Cells from organs that require cells to proliferate and replenish quickly are likely to have a more pronounced bystander effect compared with cells from organs that replenish slowly. Several studies concluded that bystander effects *in vivo* are moderated on a tissue-specific or whole organism level. More research is

required to ascertain the different responses of organs to bystander stimuli at the cellular level.

1.9 The Chosen Factors: Sex of the Cell Line

There are a limited number of studies that specifically investigated how the sex of a cell line influenced the radiation-induced bystander effect. Most of these studies used *in vivo* models to compare the sexes or looked at a small number of patients, recorded their demographics, and made comparisons between the sexes afterwards. Many indicated a difference between the sexes, although firm conclusions have yet to be made with regard to an *in vitro* model.

Nevertheless, there are studies that have confirmed a difference between the soluble factors released by male and female patients. In an attempt to bridge the gap between results observed during *in vitro* experiments and practical *in vivo* effects, Mothersill *et al*. (2001) used tissues from over 100 patients with a history of smoking to assess the bystander signal produced from normal human urothelium. Upon analysis, it was found that the irradiated cell-conditioned medium from female patients resulted in a greater reduction in cloning efficiency than the medium from male patients (Mothersill *et al*., 2001). Although HPV-G cells were used as reporters, this paper supported that bystander processes could be sex-specific.

MiRNAs, also known as microRNAs, are, “evolutionally conserved, small, single-stranded, non-protein-coding RNA molecules which are presently recognized as major regulators of gene expression” (Koturbash *et al*., 2008). It has been shown that these molecules can impact, “cellular differentiation, proliferation, apoptosis, and even

predisposition to cancer" (Koturbash *et al*., 2008). Abnormal levels of MiRNAs have been reported in many different human cancers. As such, Koturbash *et al.* set out to see whether there were differences between males and females in the expression of miRNAs in bystander exposed tissue. The main findings of this study were threefold:

> (i) male and female spleen tissues are characterized by very distinct microRNA patterns; (ii) radiation exposure alters the microRNAome of male and female animals and induces sex-specific changes of microRNA levels in the exposed spleen tissue; (iii) cranial-only exposure also influences the spleen microRNAome and causes distinct changes in the male and female shielded bystander spleen. (Koturbash *et al*., 2008, p. 1663)

The authors believed that sex hormones could be involved in the stress response of a cell and, although it is not well-understood, pointed to the fact that radiation-induced cancers seem to present more often in exposed males compared to females (Noshchenko *et al*., 2001).

A few months later, a further study was done to expand on their findings reported in their previous paper. An *in vivo* mouse model was used to test the radiation-induced bystander effect in male and female mice. DNA damage levels, DNA methylation (altered levels are known as a cancer hallmark), cell proliferation, and apoptosis levels were assessed in the bystander spleen tissue of the selected mice. The results confirmed their hypothesis. Male spleen tissue was more sensitive to bystander signals which resulted in increased DNA damage. Furthermore, there was, "significant and persistent DNA hypomethylation [in the male spleen tissue], while no such changes were seen in the

female spleen [tissue]" (Koturbash, Kutanzi, *et al*., 2008). Lastly, cranial irradiation led to increased levels of apoptosis in bystander-exposed female spleen. The authors concluded that males are, "more susceptible to the radiation-induced induction of bystander effects" and that further research is required to elucidate the differences between sexes.

The following year, Howe *et al*. (2009) tested blood samples from healthy controls and from colorectal carcinoma patients. The *in vitro* radiation-induced bystander effects were assayed through viability testing. Although cancer patients had decreased levels of cellular viability, there was not a statistically significant correlation found between induction of radiation-induced bystander effects and sex (Howe *et al*., 2009). This is a contrasting result when compared with the *in vivo* conclusions discussed previously but highlights the importance of further testing and review.

1.10 The Chosen Factors: p53 Status of the Cell Line

The role of p53 in cellular mitosis and cancer prevention has been extensively studied. It is considered a critical part of the cellular response to ionizing radiation and is an "important modulator of oncogene-induced apoptosis" (Hall & Giaccia, 2012). This protein acts as a tumour suppressor which prevents cells from proliferating too quickly or in an uncontrolled manner. It is active during the G1 checkpoint of mitosis and, if DNA damage is detected, recruits other proteins to prevent the cell from further dividing. One of these recruited proteins is p21, which is considered a cyclin-dependent kinase inhibitor. Elevated levels of p21 prevent the cell from entering into the S stage of mitosis by causing a G1 arrest and helps begin the process of apoptosis. p53 is able to bind to the DNA itself

and act as a transcription factor. TP53 is the gene found on chromosome 17 that contains instructions for making the tumour protein p53. As such, mutations of this gene are found in a wide variety of human cancers (Hanahan & Weinberg, 2000).

Under normal conditions, cells keep levels of p53 to a minimum. This is achieved through binding to a protein known as murine double minute 2 (Mdm2). Subsequently, the complex is transported to the cytoplasm where it is degraded by the proteasome. However, if the cell is exposed to a stressor, such as ionizing radiation, several mechanisms prevent the binding of Mdm2 to p53. This causes levels of p53 in the cell to rise, stabilize, and fulfill its role as a, “powerful proapoptotic molecule capable of transcriptionally activating gene expression by sequence-specific DNA binding to regulatory sequences” (Hall & Giaccia, 2012). The importance of p53 in tumour suppression has led it to be known as the “guardian of the genome” (Ho *et al*., 2019).

Due to its newfound role in the radiation-induced bystander effect, further studies on p53 were initiated. A study conducted in 1998, identified genes that responded to p53 activation, measured their expression in normal tissues, and compared it to the gene expression after exposure to a cesium-137 source. The group also performed bystander assays by comparing the growth of target cells after being introduced to irradiated cell-conditioned medium from cells that had different p53 statuses. Interestingly, medium from p53 null cells and medium from untreated p53 wild-type cells did not affect the growth of the target cells. Only medium from irradiated p53 wild-type cells caused growth inhibition, although the magnitude of the effect varied among different cells (Komarova *et al*., 1998). It was speculated that the role of p53 was not only to restricted to tumour suppression,

but also as a “stress-dependent cellular export of growth inhibitory stimuli” due to the p53-dependent secretion of growth inhibitors within the conditioned medium (Komarova *et al*., 1998). It was noted that, “the observed growth suppression effect of p53-dependent secreted factors does not depend on the origin (mouse or human) and p53 status of the target cells” (Komarova *et al*., 1998).

B. Little *et al*. (2002) conducted a bystander experiment where confluent cultures of fibroblasts and epithelial cells were exposed to very low doses of alpha radiation. Only 1-2% of the cells were traversed by an alpha particle, so bystander signals were assumed to be passed through gap-junction intracellular communication. To better understand the signalling pathways, gene expression was assessed in bystander cells. As predicted, levels of p53 were significantly elevated compared with controls. The authors also concluded that the effect was mediated by oxidative stress and involved the production of reactive oxygen species (B. Little *et al*., 2002).

Many publications had confirmed the involvement of p53 in the cellular radiation-response pathway and, as such, research groups began to focus on more direct comparisons between cells with differing p53 statuses. A study by Tomita *et al.* used x-ray microbeams to irradiate a small number of cells with either a mutant or wild-type p53 status. These cells were then allowed to pass on their bystander signals in a confluent culture. It was found that wild-type p53 cells, “were not only restrained in releasing bystander signals, but were also resistant to the signals released by the mutated p53 cells” (Tomita *et al*., 2013). This provided a clear indication that the p53 status of bystander cells affected the overall cell killing that occurred.

Two years later, different forms of HCT-116, a colorectal carcinoma cell line commonly used in radiation experiments, were tested by Widel *et al*. Wild-type HCT116 and p53 null HCT116 cells were exposed to x-ray doses ranging from 2-8 Gy and the resulting viability was recorded in directly irradiated cells and bystander cells. Both p53 statuses showed bystander effects and viability was slightly lower in the p53 null cell line. Significantly greater levels of apoptosis were recorded in p53 null cell cultures compared with those with p53 wild-type. Remarkably, both types of HCT116 cells were able to induce bystander effects in reporter cells, but only p53 wild-type cells, "responded to signals from either cell line in a cross/match protocol using the medium transfer technique" (Widel *et al*., 2015). Bystander-induced senescence was also explored. Cells with wild-type p53 secreted stronger senescence-inducing signals into the medium than p53 knockout cells. Additionally, only cells with wild-type p53 genes were affected by irradiation-induced premature senescence (Widel *et al*., 2015).

After the discovery of the involvement of biophotons in the radiation-induced bystander effect, Le *et al*. (2017) set out to elucidate whether p53 status had an effect on the production of photon emission. Five cell lines with different p53 statuses were tested during their study. HaCaT (mutant p53), HCT116 (wild-type p53), HCT116 (p53 null), SW48 (wild-type p53), and HT29 (mutant p53) were cultured and irradiated through exposure to tritium. The photon emission of bystander cell cultures was recorded after being incubated within the UV-field generated by directly irradiated cultures. The results supported the current understanding of how p53 status affects the response in bystander cells. All of the cell lines with wild-type p53 status responded to the UV signals that they received, whereas the p53 null cell lines were unable to generate any significant

response, even after a photosensitizer was introduced. The authors reported different results between the two mutant cell lines. HT29 did not exhibit any reduction in survival percentage after exposure to UV signals, whereas HaCaT expressed a significant reduction in survival percentage. This was suggested to be due to different point mutation in p53 mutant cell lines eliciting a different response to radiation (Le *et al*., 2017).

A great deal of time and effort has been dedicated to understanding how p53 affects the radiation-induced bystander effect. There is substantial research to show that the p53 status of the reporter cell line influences a cell's response to radiation. The differences between wild-type cells and p53 null cells are well understood, but there is uncertainty with regard to the contrasting results of p53 mutant cells.

1.11 The Chosen Factors: Radiation Source

Most of the radiation-induced bystander effect literature focuses on how different types of radiation affect the cellular stress response. Differences between incident particles or photons used for irradiation are explored and compared. However, the idea to specifically look at the sources of radiation, regardless of their decay products, stems from the fact that different results were produced from two different sources that emit the same quality of radiation. One of the sources in question is a cobalt-60 source that emits gamma rays with energies of 1.17 and 1.33 MeV (Parsons, 2012). The other is a cesium-137 source that decays into barium-137m which further decays to ground state by releasing gamma rays with an energy of 0.66 MeV (Parsons, 2012). Although both emit gamma rays, and the same doses were used, different survival percentages have been recorded during experiments.

Seymour and Mothersill (1997) conducted an experiment that tested the bystander effect in HaCaT cells. Cells that were selected to donate medium were plated and allowed to incubate. After, the donor flasks were irradiated with a cobalt-60 source that delivered approximately 0.5 Gy/min. The flasks were placed 80cm from the source. The medium was poured from the donor flasks and filtered to ensure that no cells were present in the medium. The irradiated, cell-conditioned medium was then transferred to recipient flasks and the survival percentage was recorded in reporter cells. Bystander HaCaT cells saw an approximate 27% decrease in survival at 0.5 Gy and a 23% decrease in survival at 2 Gy (Seymour & Mothersill, 1997).

Additionally, the same authors published a paper with the purpose elucidating the relationship between low-dose hyper-radiosensitivity and radiation-induced bystander effects. Two cell lines were chosen, one of them being the epithelial HaCaT cell line. Cesium-137 was the source used in this experiment and flasks were placed 27cm from the source while being irradiated at a dose rate of 0.3 Gy/min. An identical medium transfer technique was used when compared with the study done in 1997. Survival fractions of bystander cells exposed to the irradiated cell-conditioned medium were recorded and graphed. For comparison, HaCaT cells had about a 7% decrease in survival at 0.5 Gy and only a 2% decrease in survival at 2 Gy (Fernandez-Palomo *et al*., 2016).

Although the reduction in survival was significant in each of these studies at the doses measured, one can see that the induced changes were more drastic when observing the effects produced by the cobalt-60 source. Even at the same dose and a similar dose rate, the cesium-137 source failed to replicate the magnitude of cellular

changes that occurred in the experiment years prior. Seeing as this was the main difference between experiments, the authors hypothesized that the source itself was the cause of this phenomenon.

1.12 The Chosen Factors: Radiation Type

The literature contains a multitude of experiments that used a wide variety of different types of radiation to irradiate donor cells. The quality and type of photon or particle affect how they interact with the molecules within the cells themselves and can lead to different degrees of DNA damage or bystander signal induction. Shao *et al*. (2001) showed that high LET radiation was able to elicit a stronger bystander response in reporter cells than low LET radiation. Human salivary gland cells were irradiated with either x-rays or a carbon ion beam during a co-culture bystander effect experiment. Reporter cells had lower survival and higher micronucleus formation when co-cultured with cells that were irradiated with the high LET carbon ion beam. These reporter cells also released higher amounts of nitic oxide molecules, causing further damage compared with those co-cultured with cells that were irradiated with low LET x-rays (Shao *et al*., 2001).

A study conducted in 2006 by Kadhim *et al*. sought to further the understanding of genomic instability in bystander cells and tested the influence of radiation quality on the mean number of aberrations per cell. X-rays from aluminum and alpha particles from plutonium-238 were used for this experiment. The authors believed that the highest dose of alpha particles used (1 Gy) would cause the highest number of cellular aberrations. However, x-rays at the same dose showed very similar results. At higher doses, of

approximately 3 Gy, x-rays did not induce genetic instability that was significantly different from controls (Kadhim *et al*., 2006).

Many research groups tried to determine if the radiation-induced bystander effect could be dependent on the LET of the radiation used. A study by Anzenberg *et al*. in 2008 tested this on prostate carcinoma cells. X-rays and alpha particles were again used to demonstrate the difference between low LET and high LET radiation. A co-culture technique was used with AG01511 fibroblasts or DU-145 cells as the reporter cell lines. There was no reported decrease in the survival fraction of DU-145 cells after co-culture with DU-145 cells that had been irradiated with x-rays or alpha particles. However, bystander AG01522 cells showed a significant reduction in survival fraction after co-culture with DU-145 cells that were irradiated by x-rays but no change was seen when alpha particles were used (Anzenberg *et al*., 2008). The authors concluded that LET-dependent differences were observed, however, they suggested that more research be done to gain a more succinct understanding.

A further paper by Sowa *et al*. (2011) reported that no adaptive response was seen in bystander RKO36 cells exposed to low LET radiation. X-rays were used to first deliver a priming dose of 1 or 10 cGy and then to deliver a challenging dose ranging from 1.5 to 6 Gy. There was no significant difference in surviving fraction or micronuclei frequency in bystander cells compared with controls. The authors pointed to an overwhelming number of publications confirming that alpha particles are able to produce a bystander effect, “while electrons, gamma rays, X-rays and Fe ions do not” (Sowa *et al*., 2011). They concluded the paper by mentioning the inter-laboratory variability that was present in the

literature, at the time, and questioned the existence of a bystander effect at all (Sowa *et al*., 2011).

Another study published by Buonanno *et al.* came to a similar conclusion. After exposure to iron ions, silicon ions, and protons, irradiated cells were co-cultured with non-irradiated reporter cells. It was shown that high LET radiation (iron and silicon ions) was, "[more] effective at inducing stressful effects in the progeny of bystander cells that were detectable at 20 population doublings after co-culture" (Buonanno *et al*., 2011). Cells cultured with low LET irradiated cells were able to proliferate as well as controls and had similar levels of micronuclei formation. The authors stated that, "the high-LET radiation-induced effects are dose-dependent" (Buonanno *et al*., 2011).

Moreover, Autsavapromporn *et al*. (2014) published a study that showed the persistence of oxidative stress in the progeny of bystander cells after irradiation with x-rays, protons, or carbon ions. The results showed that all types of radiation induced significant chromosomal damage and oxidative stress in bystander cells relative to control groups. However, the low LET radiation proved more effective at causing DNA damage in the progeny of bystander cells. Interestingly, when gap-junction communication was inhibited, the proton and carbon ion group saw a reduction in micronuclei formation whereas an increase was observed in the x-ray group. The authors stated that the data, "indicate that radiation-induced delayed stressful effects in the progeny of bystander cells is dependent on radiation quality and that GJIC may play a role in the observed effects" (Autsavapromporn *et al*., 2014).

Recently, Suzuki *et al.* irradiated normal human skin fibroblasts with ultra-low-fluences of different radiation types. Gamma rays, neutrons, helium, carbon, and iron ions were used as priming doses before a challenging dose with x-rays. There were no differences in survival fraction between the different radiation types, but higher mutation frequency was seen with cells pretreated with helium and carbon ions. Iron ions and gamma rays had similar mutation frequencies as controls, while neutron exposure resulted in fewer mutations. It was concluded that neutrons may induce a radio-adaptive response, heavy ions are able to trigger genomic instability in bystander cells, and low LET radiation has a negligible impact on cellular function (Suzuki *et al*., 2020).

Since the start of the modern bystander effect era in 1992, the scientific community has struggled to come to a consensus on whether or not radiation quality has a significant impact on the radiation-induced bystander effect. Most researchers agree that high LET radiation produces the most noticeable changes in reporter cell cultures. The highly variable results seen with low LET radiation have led some members of the community to question whether a bystander effect can be induced at all. The results of the majority of studies point to a significant difference between bystander outcomes produced by different radiation types. However, the etiology of this remains unclear.

1.13 The Chosen Factors: Dose Rate

Dose rate is one of the most variable factors within the bystander effect literature. The availability of different equipment causes significant variability in the dose rates used by different researchers. As a radioactive material decays, the dose rate can change as well. Furthermore, placement of donor cells is critical, since the distance from the source can

affect the dose rate imparted on the cells (Kim, 2018). The applied dose is always mentioned in the literature; however, there are many instances where the dose rate was not identified. It is important to understand how this factor can influence radiation-induced bystander effect outcomes.

A study done by Gow *et al.* in 2007 had the goal of discovering how varying dose rate affects the bystander effect induced in cells at the same doses. In this study, cells were exposed to dose rates of 1.1, 1.7, 3.0, and 4.7 Gy/min. A bystander clonogenic assay and medium transfer technique were used to acquire survival fractions of reporter cells. When using a cobalt-60 source, a decrease in survival fraction was seen at 0.5 and 5 Gy, but no significant differences were attributed to the varying dose rates used at these doses. A higher exposure at 10 Gy showed increased cell survival relative to controls, but again, no significant differences were observed between different dose rates. The authors then switched to a LINAC source producing 20 MeV electrons. The dose rates were 1.0, 3.0, 5.0, and 10.0 Gy/min. Similar to the experiment with cobalt-60, the reporter cells experienced a reduction in survival at 0.5 and 5 Gy but no differences were seen between the different dose rates. The 10 Gy irradiation caused an increase in cell survival, however, unlike in the previous experiment, there was a statistically significant difference observed with the 10.0 Gy/min dose rate relative to the lower dose rates used (Gow *et al*., 2007). Although a dose rate-dependent effect was not seen while using a cobalt-60 source, there was a significant difference observed with high dose rates at high doses in the 20 MeV electron irradiation group.

Realizing that this experimental parameter had long been neglected within the literature, Vo *et al*. (2019) conducted experiments that aimed to elucidate how dose rate influences the survival fraction and mitochondrial membrane potential (a measure of damage to the mitochondrial physiology) outcomes in different epithelial cells. Gamma rays with dose rates of 24.6, 109, 564, and 1168 mGy/min were used to irradiate HaCaT, HCT 116 p53+/+, and HCT116 p53 -/- cells. In HaCaT cells, clonogenic survival results and mitochondrial membrane potential at all doses were similar between the various dose rates tested. In HCT116 cells with p53 wild-type and null expression, no significant difference in clonogenic survival was detected between all of the tested dose rates (Vo *et al*., 2019). However, it was found that mitochondrial membrane potential was dependent on both dose and dose rate in HCT116 p53 wild-type cells. The results for mitochondrial membrane potential in HCT166 p53 null cells were more nuanced and the authors concluded that, “when p53 is absent, the loss of [mitochondrial membrane potential] is more serious at low dose/dose rate but alleviated at high dose” (Vo *et al*., 2019).

A very recent publication presented a novel phenomenon in the radiobiology literature: the emission of acoustic waves from irradiated cell cultures. In the experiment, MCF-7 and HL-60 cells were exposed to 1 Gy of x-ray photons and the sound generated by the cells was recorded before, during, and after irradiation. Both cell lines produced an acoustic signal that was induced by the radiation. The magnitude of the emission was shown to be dependent on the dose rate and can be observed above 0.2 Gy/min (Matarese *et al*., 2023). Although this is not clear evidence for the role of dose rate in the biological bystander effect process, it shows the complexity of the processes, how they

can be influenced by many different factors, and how dose rate may play a larger role than previously thought.

Throughout the modern bystander effect era, there have been a limited number of publications that systematically explored the effect of radiation dose rates on the radiation-induced bystander effect. Those that did appear to show that dose rate has little to no effect on bystander outcomes except for at high doses with very high dose rates. Almost all publications pertaining to this area agree on one thing: more research needs to be conducted to truly understand the effects and to ensure that future researchers do not unknowingly introduce a confounding variable into their experiments.

1.14 The Chosen Factors: Dose

It is well documented that, in conventional radiation experiments, as radiation dose increases, cellular damage increases, levels of markers of DNA damage increase and cell survival is reduced (Heeran *et al*., 2019). This phenomenon was also investigated in bystander cultures. In 1998, Prise *et al*. irradiated human fibroblasts with either x-rays or alpha particles to discern whether any bystander effects were induced. Both micronuclei formation and apoptotic cell numbers were investigated. The authors reported a “dose-dependent production of micronucleated and apoptotic cells three days after irradiation for both x-rays and a-particles” (Prise *et al*., 1998).

However, this was not an isolated publication. Many studies indicated that total dose influences the radiation-induced bystander effect. While investigating the contribution of bystander cell killing to the low-dose region of the radiation dose-response curve, Seymour and Mothersill (2000) found that, in human keratinocytes, clonogenic cell

death at the 0.01 – 0.5 Gy range occurs solely from bystander effects. There is a "saturation" of the effect around the 0.03 – 0.05 Gy range. At doses over 0.5 Gy, any cell killing is, "the result of a dose-dependent non-bystander effect and a dose-independent bystander effect" (Seymour & Mothersill, 2000). This publication showed that bystander signals induce the same magnitude of effect across the dose range studied and that the bystander effect is predominant in exposures under 0.5 Gy (Seymour & Mothersill, 2000).

Furthermore, increased mitochondrial mass within cells is a known marker of chromosomal damage as is increased expression of BCL2, a gene critical to the regulation of apoptosis in cells. Researchers explored clonogenic survival, mitochondrial mass, and BCL2 expression in human keratinocytes that were exposed to an irradiated cell-conditioned medium. As the dose increased, bystander colonies had reduced survival relative to controls. Moreover, at doses of 5 mGy and 0.5 Gy, a significant increase in mitochondrial mass was observed. There was not a significant effect seen at 5 Gy. Reporter cells also saw a decrease in BCL2 expression at 5 mGy and 0.5 Gy, whereas a significant increase in expression was observed at 5 Gy (Maguire *et al*., 2005). The data show how radiation dose can influence the biological changes and outcomes seen in bystander cultures.

The overall understanding of these effects *in vivo* is important for radiotherapy and risk calculations. The majority of experiments are conducted in an *in vitro* environment and, seeking to take it one step closer to an *in vivo* model, Vines *et al*. (2009) conducted a study to see how the bystander effect manifests in a multicellular primary tissue culture system *in vitro*. Medium from irradiated murine bladder samples were used to culture

reporter HPV-G cells. It was shown that c-myelocytomatosis (a protein linked to apoptosis) and uroplakin III (a protein linked to cellular differentiation) saw dose-dependent changes occur in cells exposed to 0.5 Gy or 5 Gy (Vines *et al*., 2009).

Tomita *et al*. (2012) noticed that normal diploid human lung fibroblasts experienced various levels of cell killing in microbeam irradiation experiments at various doses. There was a significant reduction in cell survival at doses of 0.47 Gy and it reached a maximum reduction at 1.2 Gy. However, no significant effect was seen between 2.3 and 7 Gy. The survival fraction stayed at around 90% consistently after 14 Gy. The authors concluded that the x-ray induced bystander effect must be dose dependent (Tomita *et al*., 2012).

Not all studies observe a dose-dependent bystander effect, however. Soleymanifard and Toossi Bahreyni (2012) conducted medium transfer experiments on QU-DB cancer cells and MRC5 normal lung cells. Micronuclei formation was used as an endpoint for cellular damage. The authors found that there was an increased number of micronucleated cells in QU-DB cultures as the dose increased, pointing towards dose-dependence. However, the same effect was not seen in the normal cell cultures. MRC5 cultures had a similar number of micronucleated cells across all doses, indicating that, “their response was not dose-dependent” (Soleymanifard & Toossi Bahreyni, 2012).

Contrasting this, normal human skin fibroblasts were shown to have a dose-dependent increase in micronucleus formation during an experiment that tested how different qualities of radiation affect the bystander effect. Autsavapromporn *et al*. (2013) exposed cells in a gap-junction communication experiment to various doses of x-rays, carbon ions, neon ions, and argon ions. Across all radiation types, as the dose increased,

so did the percentage of cells with micronuclei, confirming that there was a dose-dependent response (Autsavapromporn *et al*., 2013).

Within the radiation-induced bystander effect literature, most studies indicate a dose-dependent effect up to a certain threshold dose. After the threshold is reached, the dose-response is "saturated" and higher doses will not impart more bystander signalling or cellular response. Markers of DNA damage, such as BCL2 expression or micronuclei formation, indicate that dose does affect the magnitude of effect in reporter cells. However, some studies show that not all cell lines react in a dose-dependent manner. As is the case with many of the factors discussed in this section, more research is required to further understand this relationship.

Chapter 2: Methodology

2.1 Original Study Design

It is important to note that this thesis has gone through different phases, and to acknowledge the work done previously to bring it to the current state. Originally, it was intended to be an overall database consisting of all the data from relevant studies that were researching the radiation-induced bystander effect. This would allow the scientific community to find experimental outcomes quickly. Trends and outliers within the radiation-induced bystander effect literature would be easier to identify and discuss. Approximately 178 papers were manually reviewed and had their data extracted into the original bystander datasheet (Appendix 1). However, due to the variability in research endpoints, this was not feasible as originally intended. Endpoints that were recorded included cell

survival fraction, intracellular calcium levels, percentage of cMyc-positive cells, and levels of nuclear fragmentation, among many others. Unfortunately, including all of recorded endpoints would be outside the scope of this project.

2.2 Second Iteration of Study Design

It was determined that the best way to move forward from the original study design was to focus on one specific bystander endpoint. This would make the studies more directly comparable and would make any trends identified more compelling. As such, the second collection of data only included studies that looked at cell survival outcomes and provided the data in the form of survival fractions and percentages (Appendix 2). It was decided to focus on studies that shared a very similar experimental design. Inclusion/exclusion criteria (Section 2.5) were revised to allow for the most unambiguous analysis of the data. This led to the creation of the final dataset (Appendix 3) and thesis iteration that will be discussed at length hereinafter.

2.3 Final Study Design

After a thorough review of the radiation-induced bystander effect literature, it was decided that the most suitable method of conducting this investigation would be through a rapid review style study design. To begin, a research question was developed and read,

> *"How do different intrinsic and external factors influence cell survival outcomes during in vitro, radiation-induced, bystander effect experiments?"*

To answer this question, a rapid review design would be best. This type of design would allow for ample data collection and synthesis without the need for extra reviewers

or lengthy time frames as is required during full systematic reviews. It still allows for useful quantitative analysis but within a shorter period of time. Unfortunately, one must think critically about the conclusions discussed in a rapid review, as there are fewer studies included and could suffer from selection bias. A systematic review is usually recommended as a follow-up afterwards (Ganann *et al*., 2010).

Databases such as Ovid, PubMed, Web of Science, and Google Scholar were utilized to ensure that similar research did not exist within the literature at the time of the start of this review. Content experts and academic supervisors were referenced to ensure no duplication of a similar review. As such, this review contains completely original research keeping in line with the requirements laid out by the McMaster School of Graduate Studies.

2.4 Thesis Hypotheses

In order to give structure to the thesis and aid in answering the overall research question, 8 hypotheses were formulated based on the common variables found within the radiation-induced bystander effect literature. They are as follows:

1. The reporter cell line has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

2. The organ of origin of the reporter cell line has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

3. The sex of the reporter cell line has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

4. The p53 status of the reporter cell line has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

5. The radiation source has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

6. The radiation quality/type has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

7. The dose rate has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

8. The dose has a significant effect on cell survival outcomes in radiation-induced bystander effect experiments.

2.5 Inclusion and Exclusion Criteria

The list below outlined the inclusion criteria for the studies that comprised the review:

- Peer-reviewed, journal articles from 1980-2023.
- English language.
- The primary purpose of study was to explore cellular bystander effects/interactions.
- *In vitro* studies.
- The study used a medium-transfer technique.
- Experimental design used ionizing radiation.

- Investigated survival fraction endpoint (or a normalized survival endpoint).
- Articles that stated the cell-line that was used.
- Articles that stated the doses that were used.
- Experiments where irradiation did not exceed 10Gy.
- Article provided source data in the form of a chart or graph.

To allow for a more homogeneous, high-quality pool of data, the following exclusion criteria were implemented:

- Articles written in a non-English language.
- Experiments used *in vivo* techniques or explant cultures.
- Review papers or poster presentations.
- Studies that did not investigate the radiation-induced bystander effect.
- Articles whereby source data were not provided or displayed in a chart/graph.
- Articles that did not state the cell-lines that were used.
- Studies with doses above 10Gy.
- Studies in which priming doses were used.
- Studies that did not pursue a survival fraction endpoint.
- Non-ionizing radiation bystander effect experiments.

- Studies that used alternate methods of inducing a bystander response, like co-culture studies and microbeam irradiation studies.
- Experiments that explored inter-generational effects.
- Studies that measured the magnitude of effect at more than one time after treatment.
- Studies that introduced altering factors, such as chemicals or metals, into the bystander effect experiment design.

2.6 Study Selection and Literature Search

After consulting with experts in the radiation-induced bystander effect field, top research groups were identified and their papers were manually reviewed for any articles that would fit the inclusion criteria. The lab groups identified included authors C. Mothersill, C. Seymour, E. Wright, T. Hei, E. Azzam, O. Kovalchuk, M. Kadhim, F. Lyng, K. Price, and K. Held. Any applicable papers were recorded in the Microsoft Excel database.

Afterwards, a literature search strategy was developed (Appendix 4). The strategy was implemented within three major databases. Ovid MEDLINE, Embase, and Web of Science were searched for journal articles from 1974 (earliest database date) to the present day. Search results were then exported from each database into the reference management software, Endnote. Endnote automatically filtered through the overall reference list from the three databases and removed any duplicate articles.

2.7 PRISMA

PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) search guidelines were adhered to and the PRISMA flowchart (Figure 1) outlines how the database results were screened and how the final review articles were selected. After the elimination of any duplicate articles, 10,959 papers entered the “Abstract and Title Search” phase of screening. Each paper was assessed on its title and abstract to determine if it was relevant to the research question. Only 120 studies were eligible for the final, full-text phase of screening of which 21 were selected for addition into the final database.

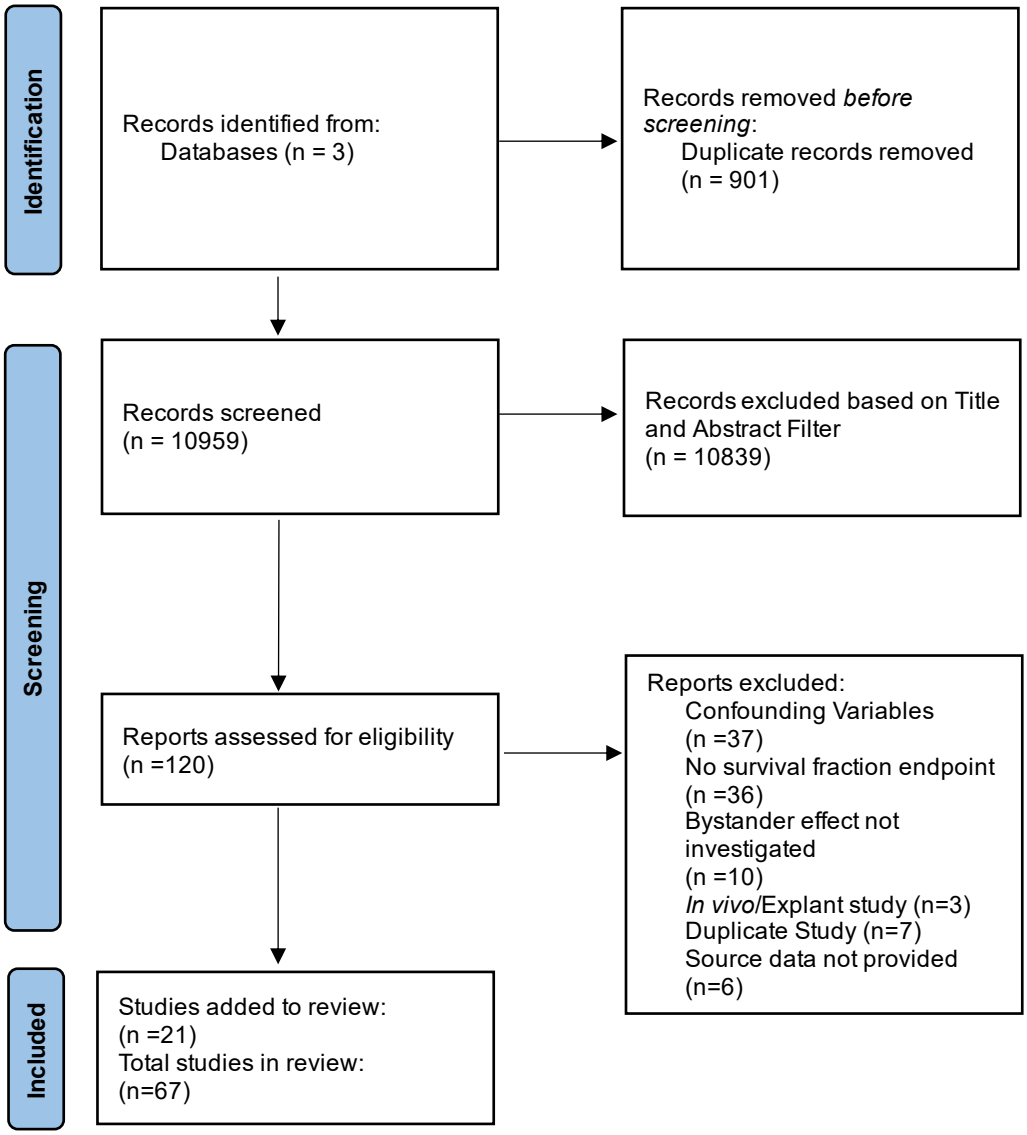

Figure 1: PRISMA Flowchart outlining study selection process.

2.8 Data Extraction

Each study was manually reviewed and had the following data extracted:

- Reference
- Donor Cell Line
- Reporter Cell Line
- Organ of Origin of the Donor Cell Line
- Organ of Origin of the Reporter Cell Line
- Sex of the Donor Cell Line
- Sex of the Reporter Cell Line
- Endpoint Examined
- p53 Status of the Reporter Cell Line
- Radiation Source
- Radiation Type/Quality
- Passage Number
- Dose Rate
- Dose
- Survival Percentage Change
- Overall Increase or Decrease

The final spreadsheet was edited and streamlined to eliminate redundant or useless data and provide more useful information for synthesis. The updated headings are as follows:

- Reference
- Donor Cell Line

- Reporter Cell Line
- Organ of Origin of the Donor Cell Line
- Organ of Origin of the Reporter Cell Line
- Sex of the Donor Cell Line
- Sex of the Reporter Cell Line
- p53 Status of the Reporter Cell Line
- Radiation Source
- Radiation Type
- Radiation Quality (LET)
- Dose Rate
- Dose Rate Range
- Dose
- Dose Range
- Survival Percentage Change

Dose Rate Ranges were defined as follows:

- Low Dose Rate: ≤0.02 Gy/min
- Moderate Dose Rate: 0.02 – 1 Gy/min
- High Dose Rate: ≥1 Gy/min

Dose Ranges were defined as follows:

- Low Dose: ≤0.5 Gy
- Moderate Dose: 0.5 – 2 Gy
- High Dose: ≥2 Gy

2.9 Quality Assessment of Included Studies

After an exhaustive search, it was determined that there is currently no standardized tool for the quality assessment of *in vitro* studies. It was noted, however, that a study by Khan *et al*. (2014) adapted an established QA tool for their research pertaining to the management of contaminated anterior cruciate ligament grafts. The original tool is known as the Methodological Index for Non-Randomized Studies (MINORS) and was developed by Slim *et al*. in 2003. As such, a further adaptation of the MINORS tool was created for the quality assessment of *in vitro*, radiation-induced bystander effect experiments included in this study and has the following criteria:

1. Clearly stated purpose: “The question addressed in the study is explicitly stated and testable by statistical means” (Khan *et al*., 2014).

0 - Purpose not explicitly stated.
1 - Purpose is explicitly stated but not testable by statistical means.
2 - Purpose is explicitly stated and testable by statistical means.

2. Endpoints Appropriate to Study Aim: “unambiguous explanation of the criteria used to evaluate the main outcome which should be in accordance with the question addressed by the study. Also, the endpoints should be assessed on an intention-to-treat basis” (Slim *et al*., 2003).

0 - Criteria used to evaluate the main outcome are not stated.
1 - Criteria are explained but are ambiguous or unrelated to the purpose of the study.
2 - Criteria are unambiguous and related to the purpose of the study.

3. Prospective Calculation of Study Size: “Data were collected according to a protocol established before the beginning of the study” (Slim *et al*., 2003).

0 - The data collection protocol is not stated or explained.
1 - Use of novel data collection protocol.
2 - The protocol was established before the beginning of the study.

4. Cell line type: Cell line information is explicitly stated and well described.

0 - No cell line information present.
1 - The cell line is stated but does not include the rationale for the usage of the cell line or origin.
2 - Cell line is mentioned and includes rationale and origin for the cell line.

5. *In vitro* culture techniques: The culture techniques are well-described.

0 - Not adequately described.
1 - Described but missing pertinent information (culture time, storage conditions, medium used, etc.)
2 - Culture techniques are well-described and reproducible.

6. Baseline equivalence of groups: The groups should be similar regarding the criteria other than the studied endpoints. Absence of confounding factors that could bias the interpretation of the results.

0 - Groups are not similar and there are many confounding factors.

1 - Groups are similar, but confounding factors are not accounted for.

2 - Groups are similar are there are minimal/no confounding factors.

7. Adequate control groups: Using a sufficient number of control groups to minimize error and increase the statistical significance of results.

0 - Control groups not adequately described.
1 - A Control group of 1 flask.
2 - A control group of 3 or more flasks.

8. Irradiation protocol: The protocol for the irradiation of cells is described.

0 - Not adequately described.
1 - Protocol is described but with limited information (i.e., Dose is mentioned but not dose rate).
2 - Protocol is described fully (i.e., Dose, dose rate, and timing are mentioned).

9. Bystander assay/technique: Description of how bystander effect was achieved and studied.

0 - Bystander assay not adequately described.
1 - Bystander assay described but insufficient (Seeding density, flask size, timing, medium transfer, or filtration not described).
2 - Bystander assay is well-described and includes all pertinent information.

10. Appropriate statistical analysis: Description and implementation of statistical tests appropriate to the dataset (Khan *et al*., 2014).

0 - No statistical analysis mentioned.
1 - Statistical analysis described but insufficient (missing P values).
2 - Statistical analysis well-described with reported P values.

The tool is a checklist of 10 items. Each item can be scored anywhere from 0-2. A score of “0” indicates that item was not reported. A score of “1” indicates that the item was reported but was inadequate in the outlined way. A score of “2” idicates that the item was reported in an adequate way. A total score above 18 is considered low risk for bias. Any total score between 15 and 18 is considered at medium risk for bias. Any total score below 15 is considered a high risk for bias. The ideal total score is 20 for *in vitro* bystander effect experiments. Each included study has a corresponding score which can be found in the last column of the Quality Assurance Scoring Sheet (Appendix 5).

2.10 Data Analysis – Graphing Techniques

Over 600 individual data points were entered into the Excel spreadsheet, each with multiple intrinsic and external factors attached to them. Due to the amount of data that was collected, scatter plots were used as a visual tool. Scatter plots allow for easy visualization of trends and are suitable for the non-linear relationship exhibited by this dataset. GraphPad Prism 9 was the graphing software used for the initial graphs. Dose values were plotted on the x-axis. Survival percentage change values were plotted on the y-axis. The overall scatter plot (Figure 3) shows the distribution of data without any grouping variables. All the subsequent scatter plots have grouping variables included and the legend for each graph shows the groups and their associated colour. This allows the

reader to easily discern any trends or areas of interest related to each intrinsic or external factor. Due to time constraints, only reporter cell line data were plotted and analyzed.

Due to the nature of radiation-induced bystander effect experiments, data points were highly concentrated at commonly used doses such as 0.5 Gy, 1 Gy, 2 Gy, and 5 Gy. It was extremely difficult to distinguish between different data points. As such, a different graphing software, Orange, was used to provide an alternate view of the data. Scatter plots were still implemented, however, a technique known as “jittering” was applied to each point. The technique applies a random noise variable to each data point, allowing for increased spatial resolution and readability.

Unfortunately, the data were still incomprehensible. Some variables, such as “Reporter Cell Line”, had as many as 50 separate groups within the dataset. It was decided that, for ease of comprehension, only the top 10 most populous groups within each variable would be plotted on graphs. This resulted in a simpler visual as fewer data were displayed.

Box plots were also generated in Orange to allow for easier visualization of the distribution of data within each variable. The comparison of quartiles, as well as the mean and median, allows for more robust data analysis.

2.11 Data Analysis – Statistical Techniques

The large amount of data, combined with the heterogeneity of studies, made statistical analysis a challenge. Using Orange, the data were shown to be normally distributed. As such, this allowed for the use of parametric statistical tests. However, there were multiple variables and multiple groups within each variable. Therefore, it was decided that the best

test to perform would be a Two-Way Analysis of Variance (ANOVA). A Two-Way ANOVA would be executed on six out of eight of the chosen factors (due to time constraints) and would show how that particular factor, along with dose, affects the survival percentage change of bystander cells.

The statistical analysis software, R, was used to perform these calculations. Any blank or "N/A" rows were removed and only the top 10 most populous groups within each variable would be included in the ANOVA results. ANOVA tables were generated and the F-ratio as well as the resulting p-value were recorded.

2.12 Outlier Detection and Normality Confirmation

To determine if there were any statistical outliers in the dataset, the generalized extreme studentized deviate (ESD) test was used. This test was developed in 1983 by Bernard Rosner and is useful to determine outliers when the exact number of outliers are unable to be specified exactly. The survival percentage change values were tested within R and, for each variable, it was determined that the value of 139 was a statistical outlier. As such, it was removed before any ANOVA tests were run.

A visual confirmation to assess the normality of data was done through the use of a histogram. The distribution of the survival percentage change values was plotted and a curve of best fit was overlayed (Figure 2). Based on the large sample size ($n \geq 300$), skewness and kurtosis were assessed in R to ensure the visual confirmation was accurate (Gupta *et al*., 2019). The dataset has a skew value of -0.21 (slightly negative) and a kurtosis value of 1.60. The distribution of data are considered to be normal if the

absolute values of skewness and kurtosis are ≤ 2 and ≤ 4, respectively (Gupta *et al*., 2019). As such, it was concluded that the data follows a normal distribution.

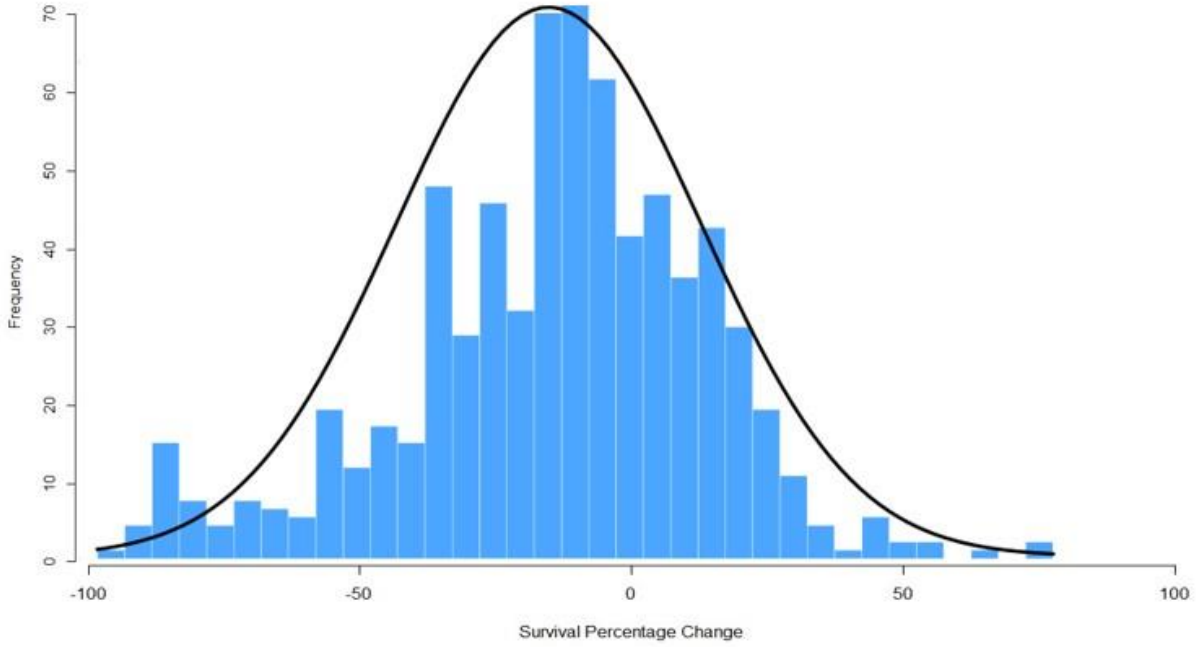

Figure 2: Histogram of Survival Percentage Change Values.

Chapter 3: Results

3.1 Brief Overview

There are 674 total data points from 67 studies that were compiled into an "overall" scatter plot (Figure 3). The scatter plot shows the wide range of survival fraction values that are recorded in the included literature. The x-axis displays dose values used to irradiate donor cells during each experiment. The y-axis demonstrates "Survival Percentage Change" which is the change in percentage cell survival of recipient cells after being grown using medium from irradiated donor cells, relative to control cultures. A "jittered" scatter plot

was added to aid in visualization (Figure 4). Each hypothesis has been addressed with the use of two scatter plots, a conventional plot, and a jittered plot. Box plots were also used to help show the dispersion of data within each grouping variable. Due to time constraints, only six of the hypotheses were tested with Two-Way ANOVA. The reporter cell line used, the p53 status of the reporter cell line, and the type of radiation showed a significant effect on the survival percentage change seen in bystander cells.

3.2 Overall Results

Graphs produced with the overall dataset indicate that the majority of experiments resulted in a reduction in survival percentage (approximately 71.6% of the data). The overall dataset had a mean survival percentage change of -17.4 and a standard deviation of 27.9. Figure 3 shows that data are highly concentrated at commonly studied doses such as 0.5 Gy, 1 Gy, 2 Gy, and 5 Gy, which make it difficult to visualize individual points. Due to the nature of the graph and the fact that most experiments use discrete dose values, points are arranged in vertical columns with many of the points overlapping. The jittered version of this graph (Figure 4) attempts to remedy this by spreading out the plotted values. Studies seldom investigated interval dose values (ie. 1.5 Gy, 2.5 Gy, 3.5 Gy, etc.) and there are noticeably fewer plotted values between 5 Gy and 10 Gy. These two graphs do not show an obvious trend when comparing survival percentage change with increasing dose. The overall dispersion of points at each dose value is skewed towards a reduction in survival percentage, but increased survivability is demonstrated as well, albeit, to a lesser degree. Among the included studies, the greatest increase in survival percentage was 74, while the largest reduction in survival percentage was -99.

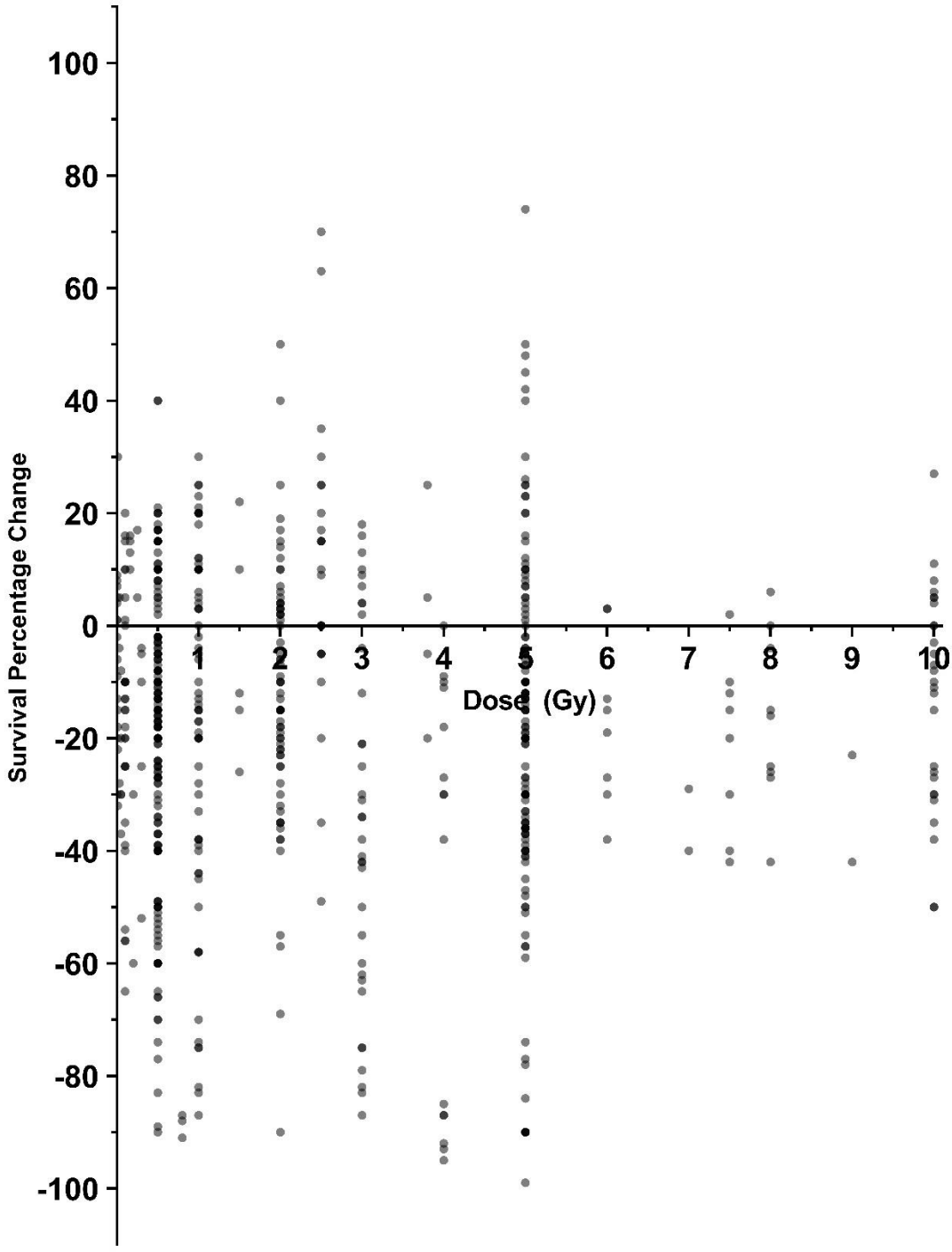

Figure 3: Overall Survival Percentage Change Data from Included Studies. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

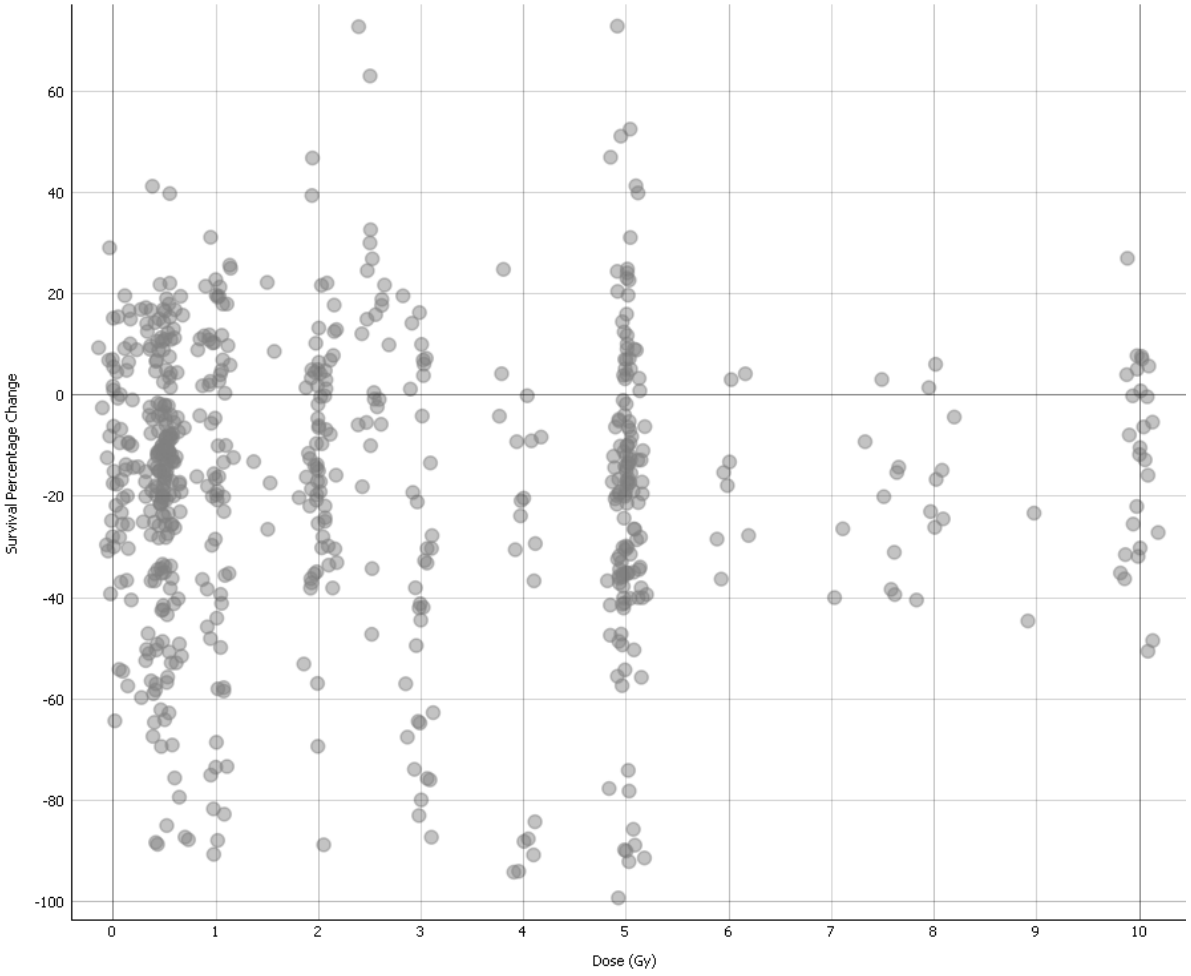

Figure 4: Alternate Graph of Overall Bystander Effect Data from Included Studies. Data points have been jittered and opacity reduced for better visualization. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

3.3 Reporter Cell Line Results

Over 40 unique reporter cell lines were recorded within the final included studies. For ease of visual interpretation, the top 10 most prevalent reporter cell lines were chosen to be displayed in the scatter plot. Figure 5 shows that HPV-G was the most common reporter cell line used and accounted for 26.8% of the data, while HeLa was the least common of the top 10 and accounted for only 1.8% of the data. Unfortunately, the high concentrations of plotted values at the commonly used doses made it difficult to identify trends, even with the addition of a coloured grouping variable. The jittered data displayed in Figure 6 made it easier to see where different cell lines are clustered and help to show how each reporter cell line's bystander survivability is affected by increasing dose. A Two-Way ANOVA was performed and results are summarized in Table 1. Simple main effects analysis showed that the reporter cell line did have a statistically significant effect on survival percentage change *(p = <2e-16)* while the dose did not have a statistically significant effect on survival percentage change *(p = 0.19).* The cell line, GM637H, did not show any reduction in survival within the included studies (M = 17.7, SD = 8.2). HeLa, on the other hand, exhibited a noticeably large decrease in survival within the included studies (M = -88.1, SD = 3.8). The other eight cell lines show less drastic changes in survival percentage with mean values ranging from -3.3 (CH0-K1) to -47 (SW48). Box plot data for each individual cell line are displayed in Figure 7. Table 2 shows the number of studies within each subgroup.

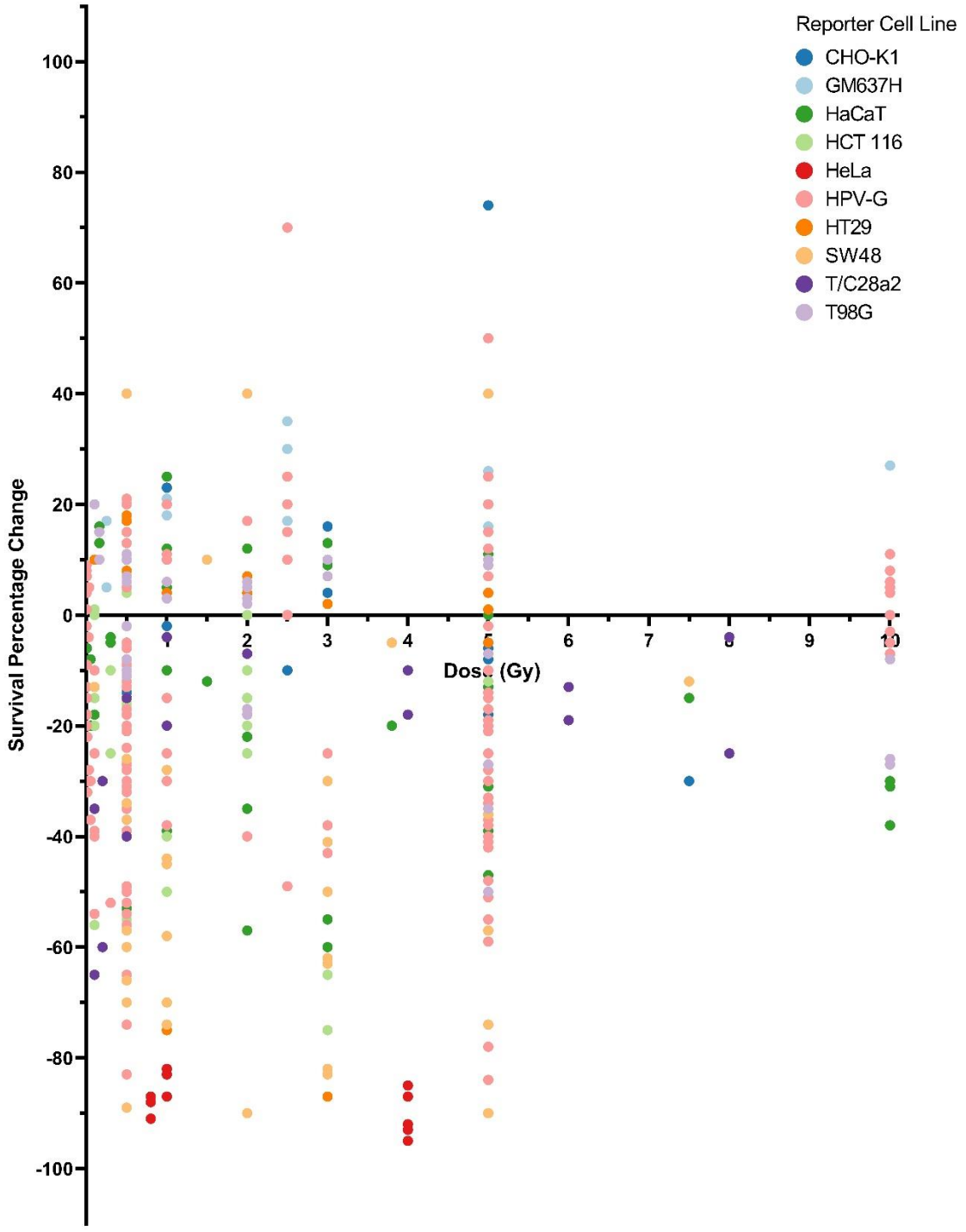

Figure 5: Survival Percentage Change Data for the 10 most prevalent Reporter Cell Lines in Included Studies. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

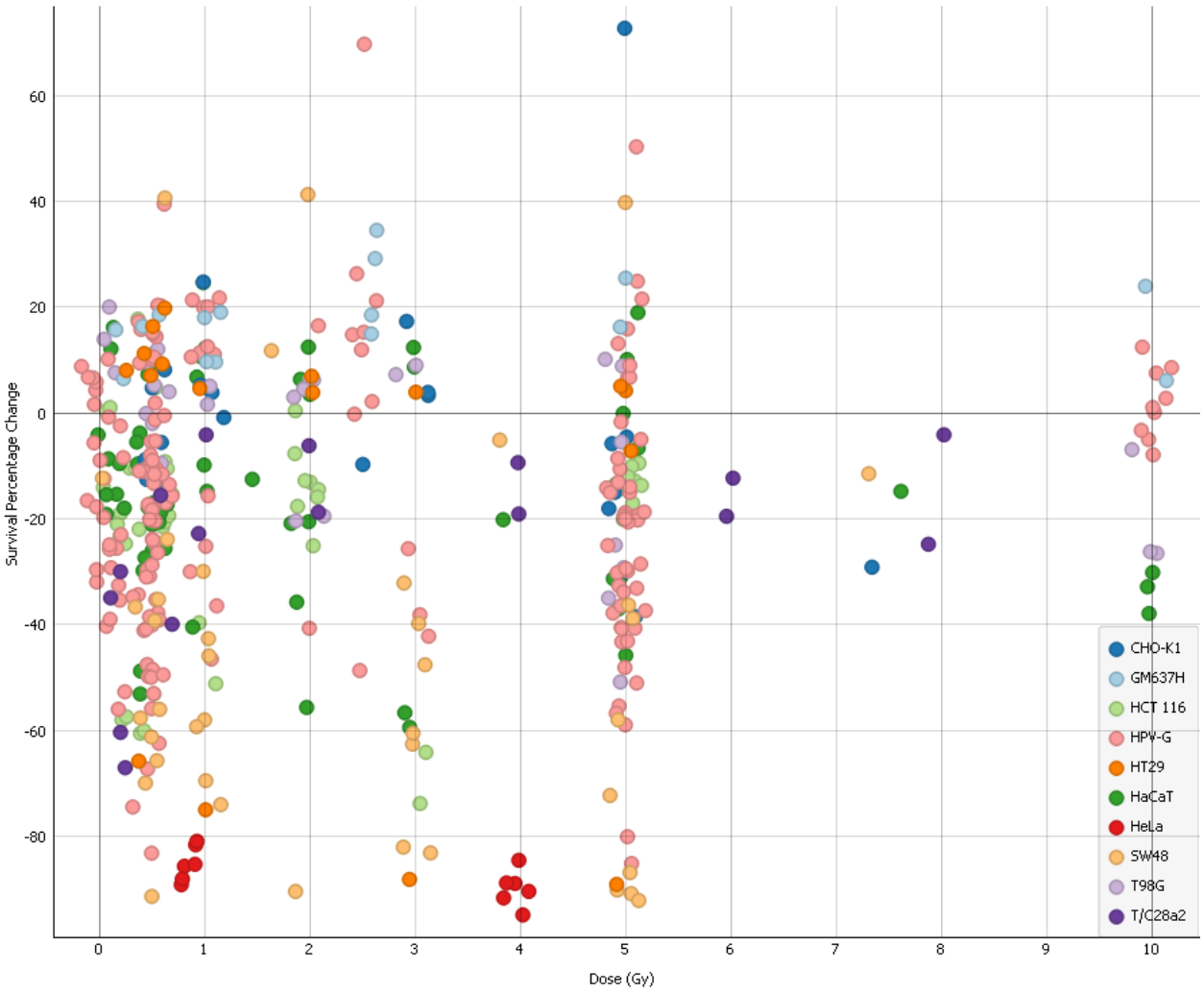

Figure 6: Alternate Graph of Survival Percentage Change Data for the 10 most prevalent Reporter Cell Lines in Included Studies. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

Source of Variation	***Df***	***SS***	***MS***	***F***	***p-value***
Cell Line	9	126149	14017	24.255	<2e-16 ***
Dose	2	1913	956	1.655	0.19
Residuals	420	242708	578		

*Table 1: Two-Way ANOVA Table Results for Reporter Cell Line variable. *** indicates significance where α = 0.05.*

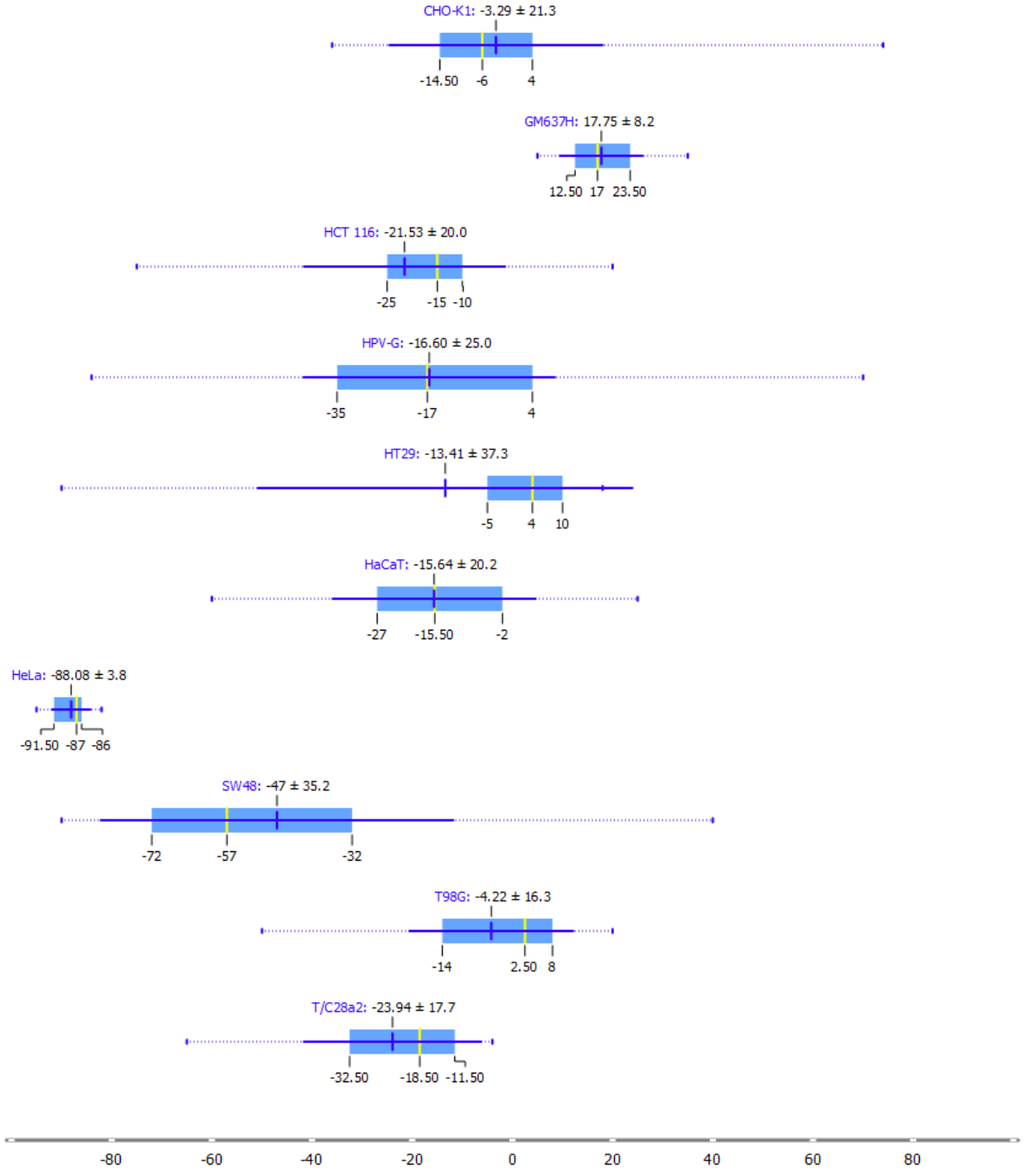

Figure 7: Box Plot of Survival Percentage Change Data for the 10 most prevalent Reporter Cell Lines in Included Studies.

Cell Line	Number of Plotted Data
CHO-K1	24
GM637H	16
HCT 116	45
HPV-G	181
HT29	17
HaCaT	66
HeLa	12
SW48	40
T98G	31
T/C28a2	16

Table 2: Table displaying the number of plotted data within each Cell Line subgroup.

3.4 Organ of Origin of Reporter Cell Line Results

It was difficult to plot the organ of origin results due to the fact that there were over 20 unique organs of origin, an issue identical to the one faced while attempting to plot the reporter cell line results. Therefore, an identical solution was used. The top 10 most prevalent organs of origin were subsequently plotted. All organs were of human origin with the exception of “H. Ovary” which represents “Hamster Ovary”. Figure 8 shows that skin was the most common organ of origin for reporter cell lines and accounted for 43.9% of the data. The least common organ of origin within the top 10 was the spleen which only accounted for 1.3% of the data. The alternate, jittered scatter plot (Figure 9) shows that skin, colon, hamster ovary, and brain outnumber other organs by a substantial margin. Together, they account for 78.4% of all data within the included studies. A Two-Way ANOVA was not performed on this variable due to time constraints. The organ of origin box plot (Figure 10) shows that the spleen was the only organ with a positive mean survival percentage change of 2.7. The only cell line originating from the uterus is the HeLa cell line which shares the same notable decrease in survival that was discussed in the previous section (M = -88.1, SD = 3.8). All other organs of origin results are concentrated closer to the overall mean of -17.4 with colon and skin having the largest standard deviations of 33.2 and 26.3, respectively. Table 3 shows the number of studies within each subgroup.

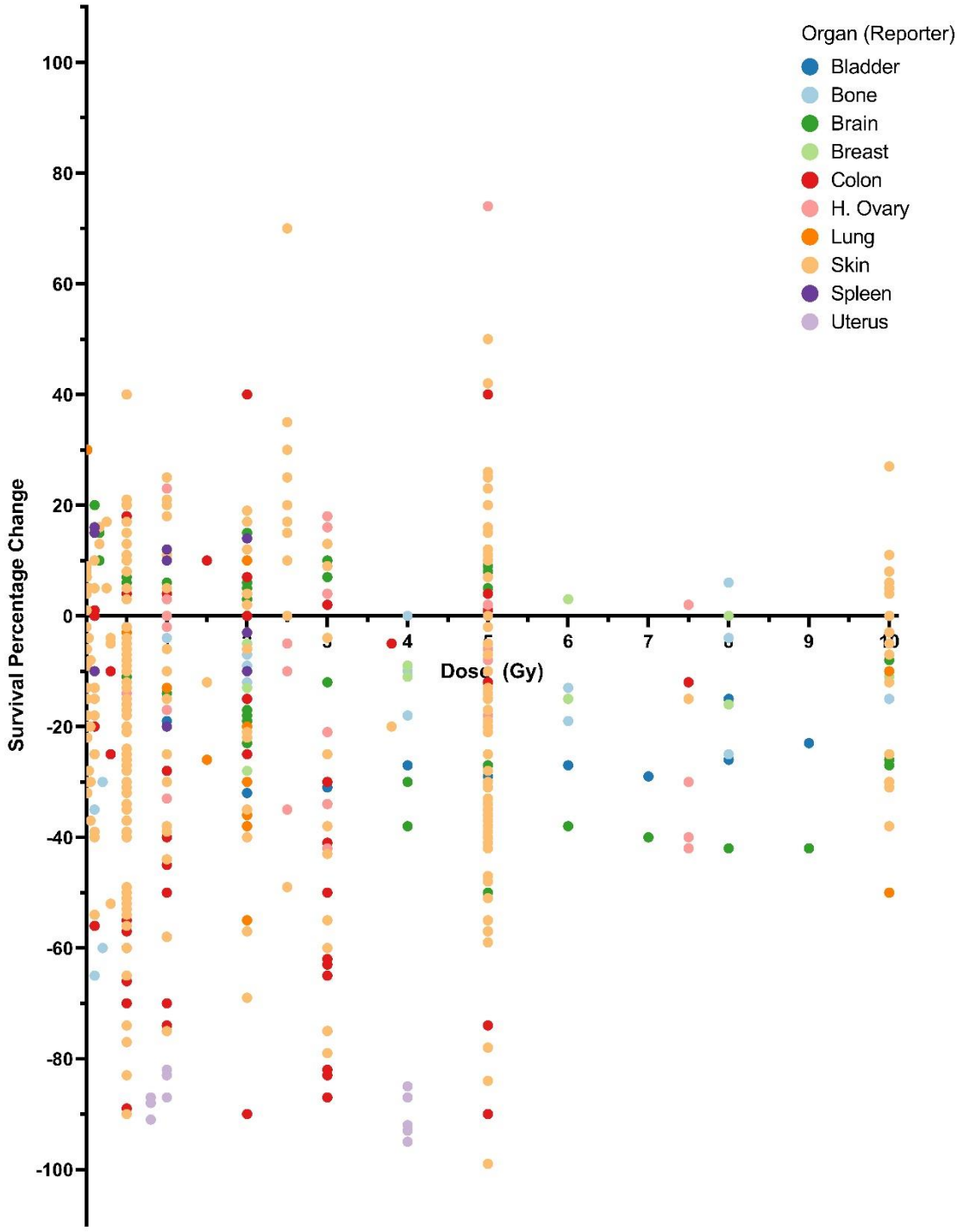

Figure 8: Survival Percentage Change Data for the 10 most prevalent Organs of Origin from Reporter Cell Lines in Included Studies. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

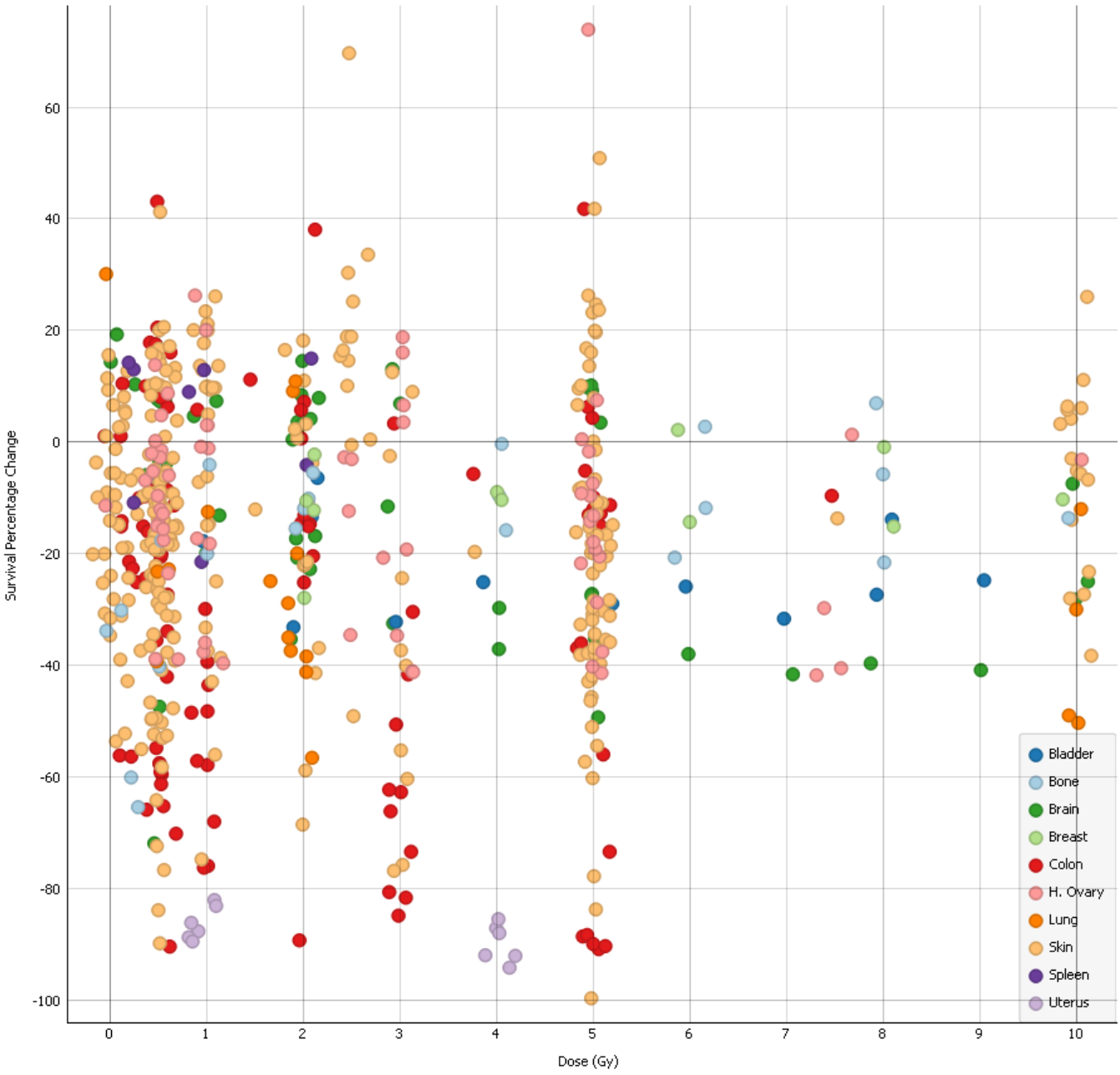

Figure 9: Alternate Graph of Survival Percentage Change Data for the 10 most prevalent Organs of Origin from Reporter Cell Lines in Included Studies. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

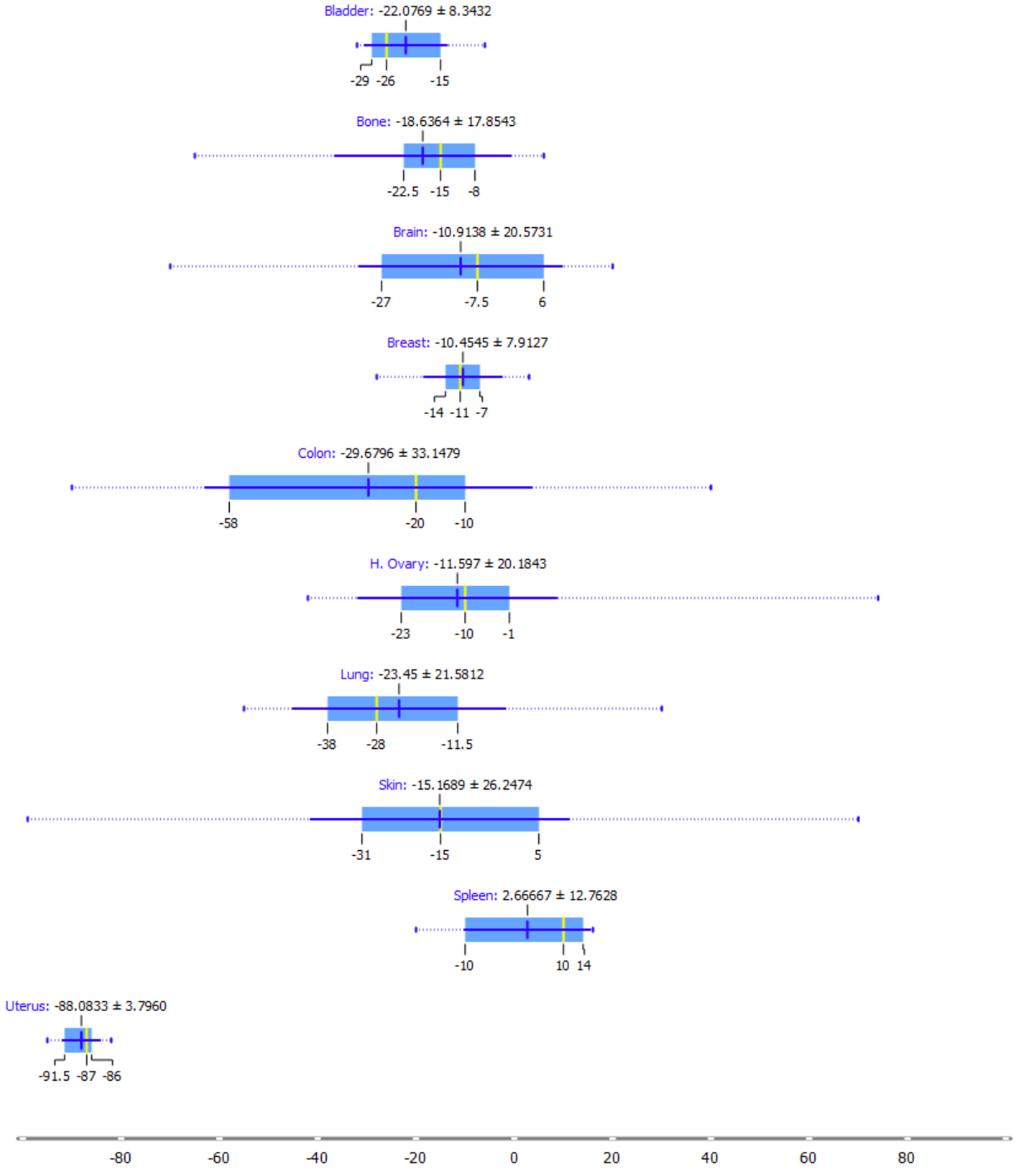

Figure 10: Box Plot of Survival Percentage Change Data for the 10 most prevalent Organs of Origin from Reporter Cell Lines in Included Studies.

Organ	Number of Plotted Data
Bladder	13
Bone	22
Brain	58
Breast	11
Colon	103
H.Ovary	67
Lung	20
Skin	296
Spleen	9
Uterus	12

Table 3: Table displaying the number of plotted data within each Organ subgroup.

3.5 Sex of Reporter Cell Line Results

Only four groups were needed when sorting the data by sex of the reporter cell line. Male was the most common and accounted for 63.6% of the data. Studies that used female cell lines accounted for 31.7% of the data. The third group is "M/F" which denotes a mixed karyotype whereby the reporter cell lines were isolated from a mixed population. This is attributable to experiments that used fish cell lines, such as RTG-2. The M/F group accounted for 3.4% of the data. The final group was for unspecified cell lines where sex could not be definitively determined and was labelled as "N/A". Studies that listed "normal human lung fibroblasts", for example, were included in this group and accounted for only 1.2% of the data. Both the M/F and N/A subgroups were excluded from the statistical analysis. Survival percentage change results are plotted in Figure 11. The alternate, jittered scatter plot (Figure 12) shows that there is not an obvious trend, even when grouping by sex of the reporter cell line. A Two-Way ANOVA was performed and a simple main effects analysis (Table 4) showed that the sex of the reporter cell line and dose did not have a significant effect on survival percentage change *($p = 0.07$* and *$p = 0.78$,* respectively*).* None of the groups varied drastically when comparing results in the box plot (Figure 13). The mixed karyotype group, M/F, was the only group with a positive mean survival percentage change value of 4.1. Both male and female cell lines displayed similar results. Male cell lines had a mean of -16.7 and a standard deviation of 24.6 while female cell lines had a mean of -20.8 and a standard deviation of 33.3. Table 5 shows the number of studies within each subgroup.

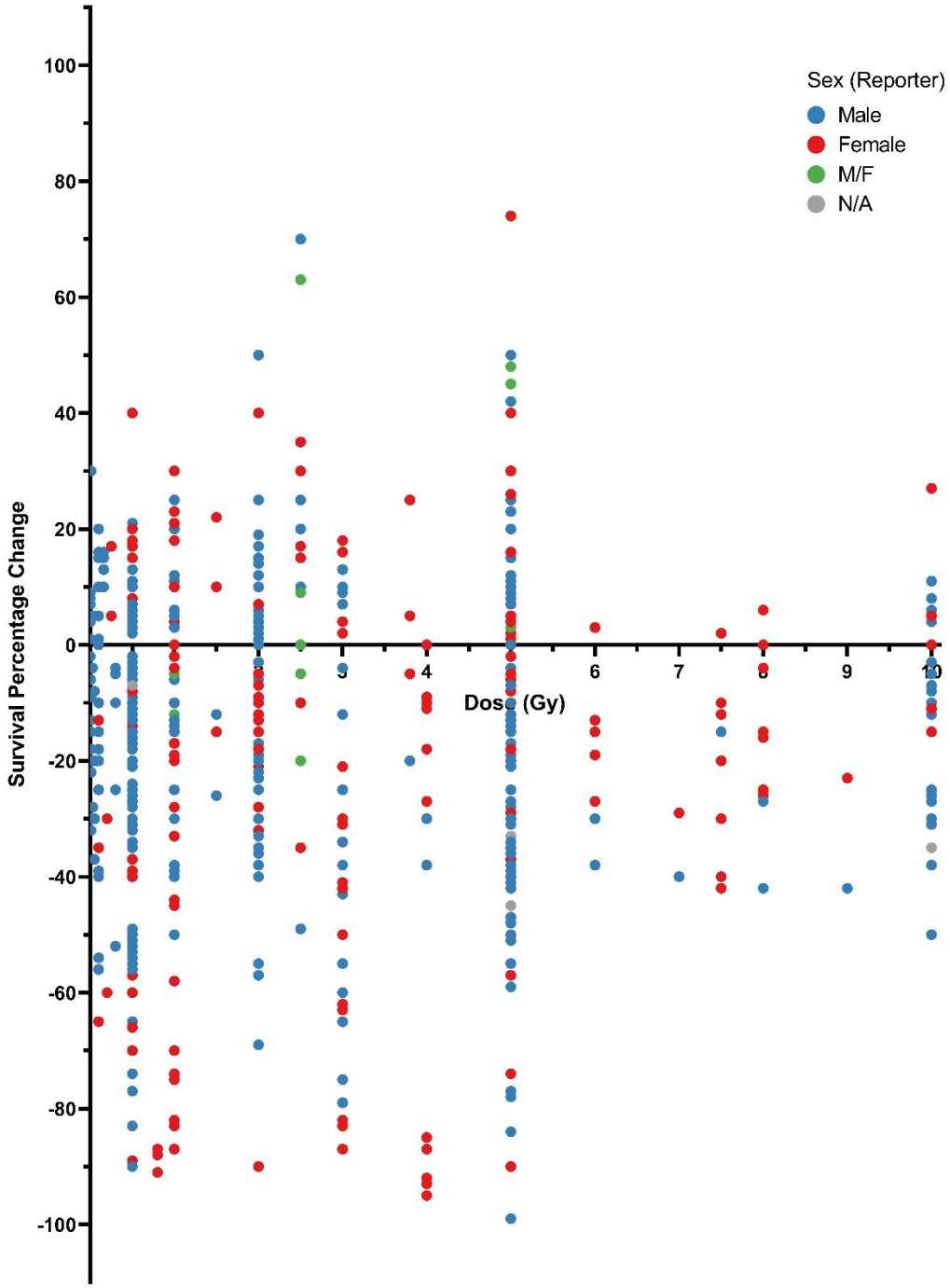

Figure 11: Survival Percentage Change Data for Included Studies grouped by Sex of the Reporter Cell Line. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "M/F" denotes animal cell lines where sex was potentially mixed. "N/A" denotes studies where sex information was unable to be obtained.

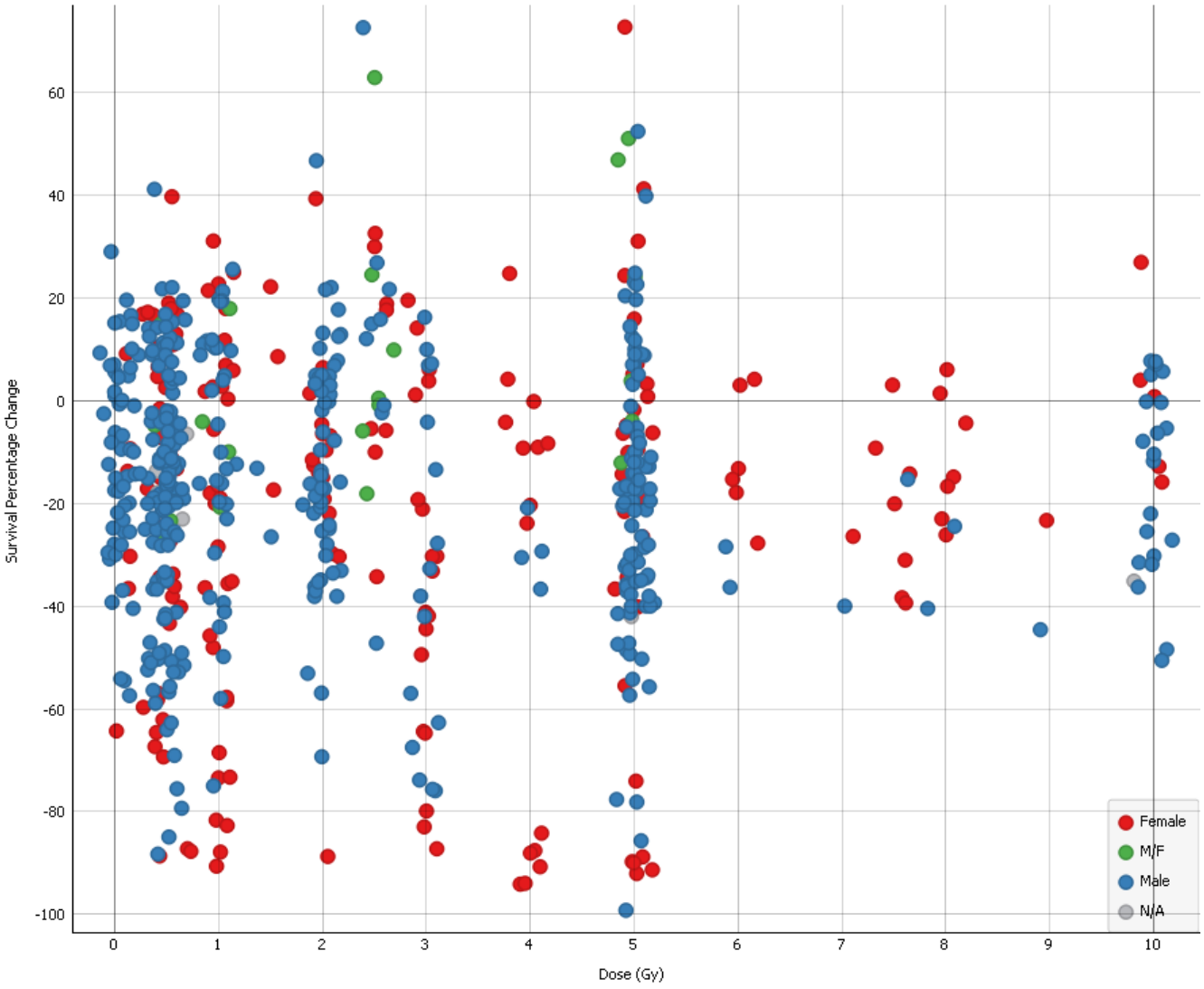

Figure 12: Alternate Graph of Survival Percentage Change Data for Included Studies grouped by Sex of the Reporter Cell Line. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "M/F" denotes animal cell lines where sex was potentially mixed. "N/A" denotes studies where sex information was unable to be obtained.

Source of Variation	***Df***	***SS***	***MS***	***F***	***p-value***
Sex	1	2505	2504.5	3.242	0.07
Dose	2	381	190.4	0.246	0.78
Residuals	636	491315	772.5		

*Table 4: Two-Way ANOVA Table Results for Sex of Reporter Cell Line variable. *** indicates significance where α = 0.05.*

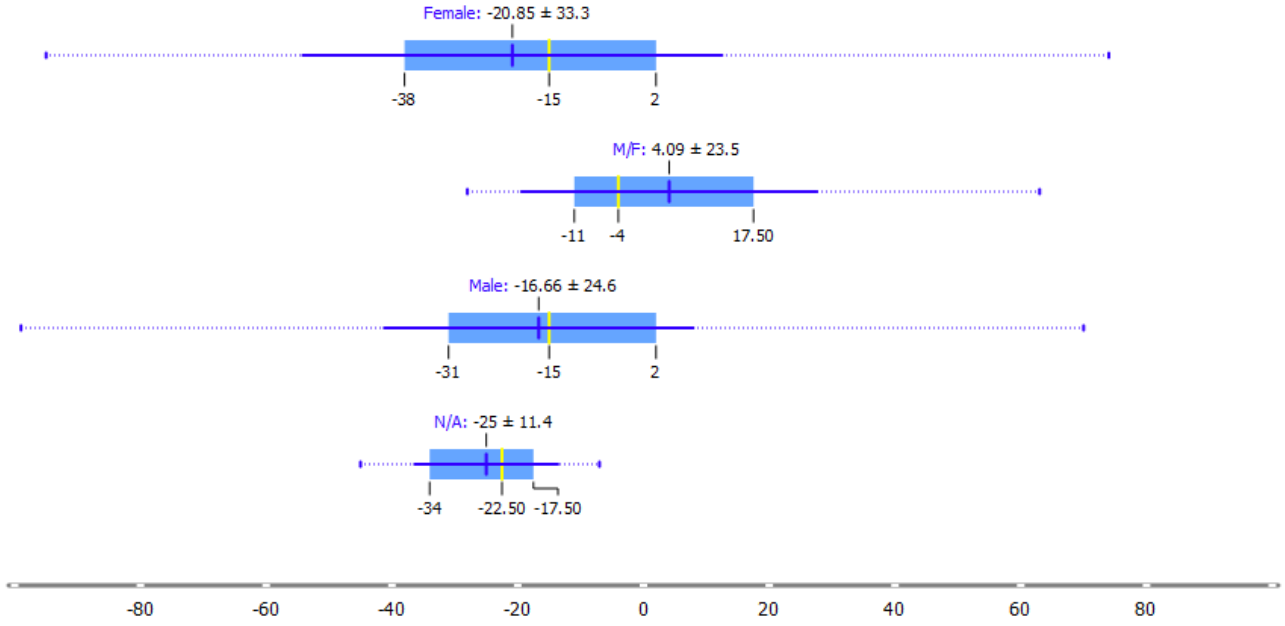

Figure 13: Box Plot of Survival Percentage Change Data for Included Studies grouped by Sex of the Reporter Cell Line.

Sex	Number of Plotted Data
Female	213
M/F	23
Male	429
N/A	8

Table 5: Table displaying the number of plotted data within each Sex subgroup.

3.6 p53 Status of Reporter Cell Line Results

The p53 status of reporter cells was grouped into four categories. “Mutant”, where p53 proteins exist but in an altered state from normal. The wild-type group, labelled as “wt”, is where the tumour suppressor gene p53 is functioning as expected in nature. The third category, “null”, is for cell lines that have no functioning p53 proteins. Lastly, “N/A” is used to denote cell lines where the p53 status could not be confirmed from the information given in the study or by searching online. Most studies used cell lines with mutant p53 status as can be seen in Figure 14. It accounts for 38.3% of data. The second-most prevalent group was “null” with 34.7% of the data. Wild-type was less pronounced in the included studies and made up 23.3% of included data. Only 3.7% of the cell lines were unable to have their p53 status confirmed and were added to the “N/A” group. Due to the large amount of included data, it is important to reference Figure 15, which provides an alternate view and allows one to discern otherwise overlapping points. A Two-Way ANOVA was performed and results are summarized in Table 6. Simple main effects analysis showed that the p53 status of the reporter cell line did have a statistically significant effect on survival percentage change *($p = 1.08e\text{-}05$)* while the dose did not have a statistically significant effect on survival percentage change *($p = 0.78$).* Both mutant (M = -16.9, SD = 27.4) and null (M = -13.9, SD = 25.2) had similar means and standard deviations which can be seen in Figure 16. Wild-type p53 status did produce, on average, slightly more negative survival fraction outcomes (M = -26.98, SD = 29.6). Table 7 shows the number of studies within each subgroup.

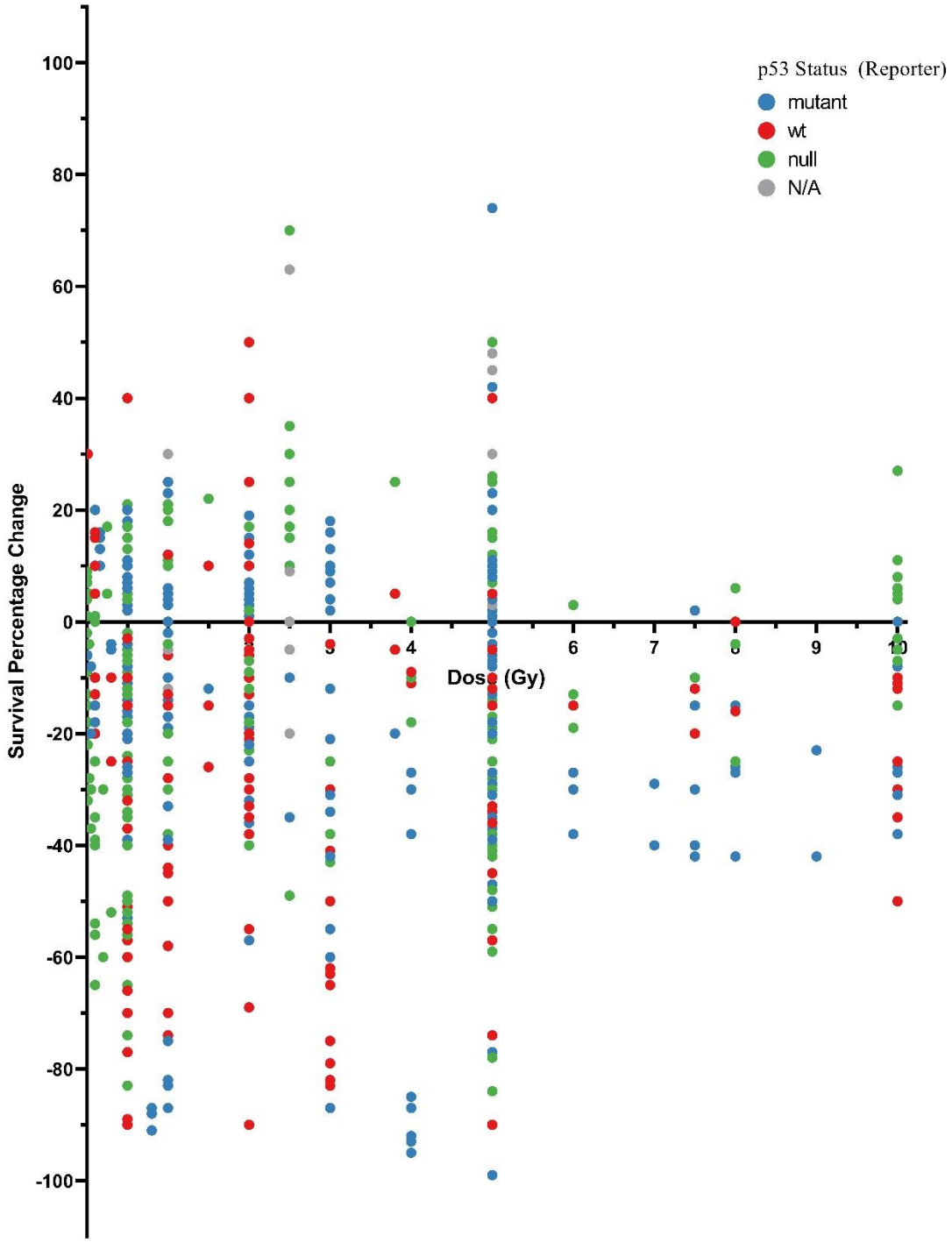

Figure 14: Survival Percentage Change Data for Included Studies grouped by p53 Status of the Reporter Cell Line. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "N/A" denotes studies where p53 status was unable to be confirmed.

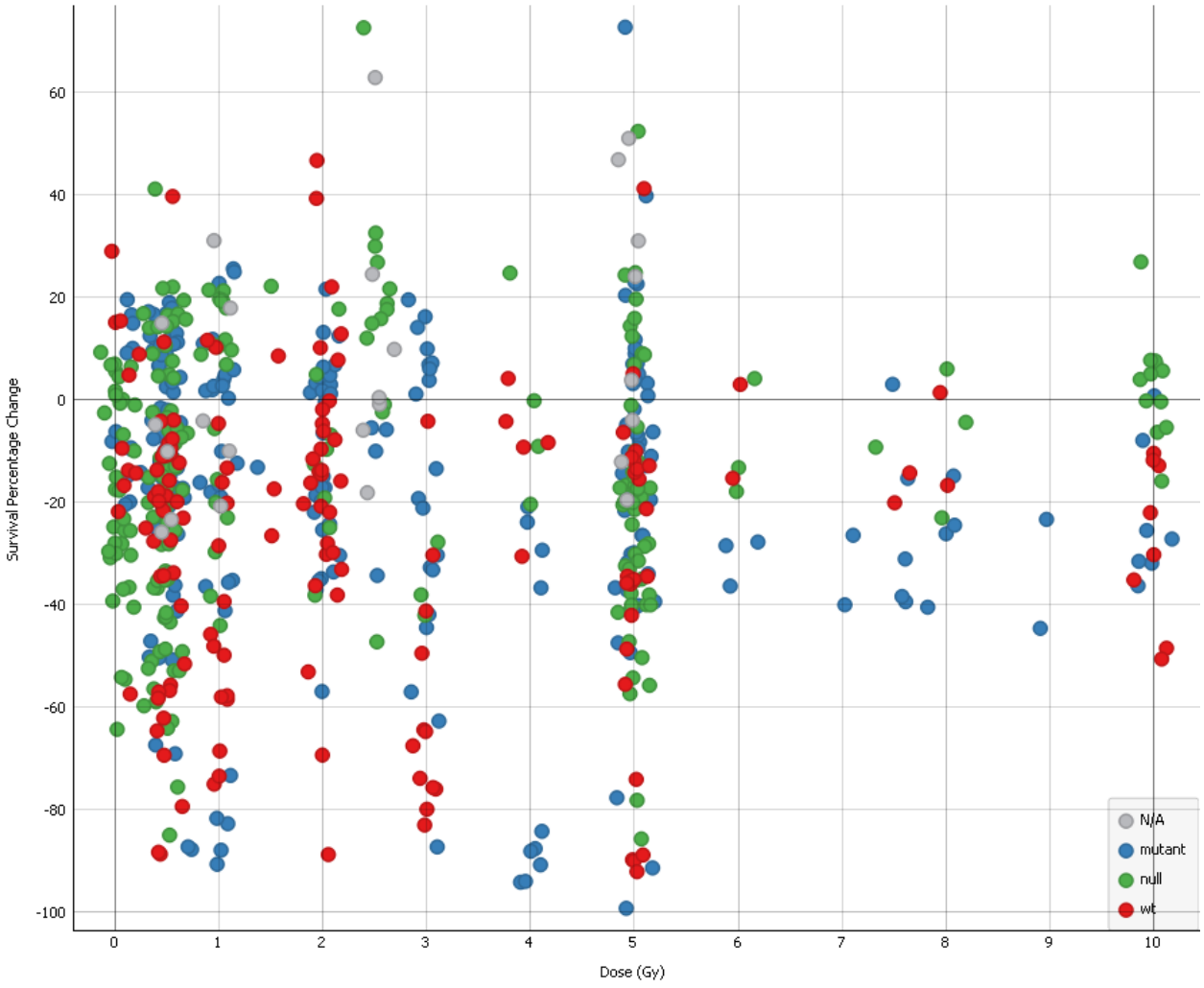

Figure 15: Alternate Graph of Survival Percentage Change Data for Included Studies grouped by p53 Status of the Reporter Cell Line. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "N/A" denotes studies where sex information was unable to be obtained.

Source of Variation	***Df***	***SS***	***MS***	***F***	***p-value***
p53 Status	2	16791	8396	11.641	1.08e-05***
Dose	2	356	178	0.247	0.78
Residuals	639	460877	721		

*Table 6: Two-Way ANOVA Table Results for p53 Status of Reporter Cell Line variable. *** indicates significance where α = 0.05.*

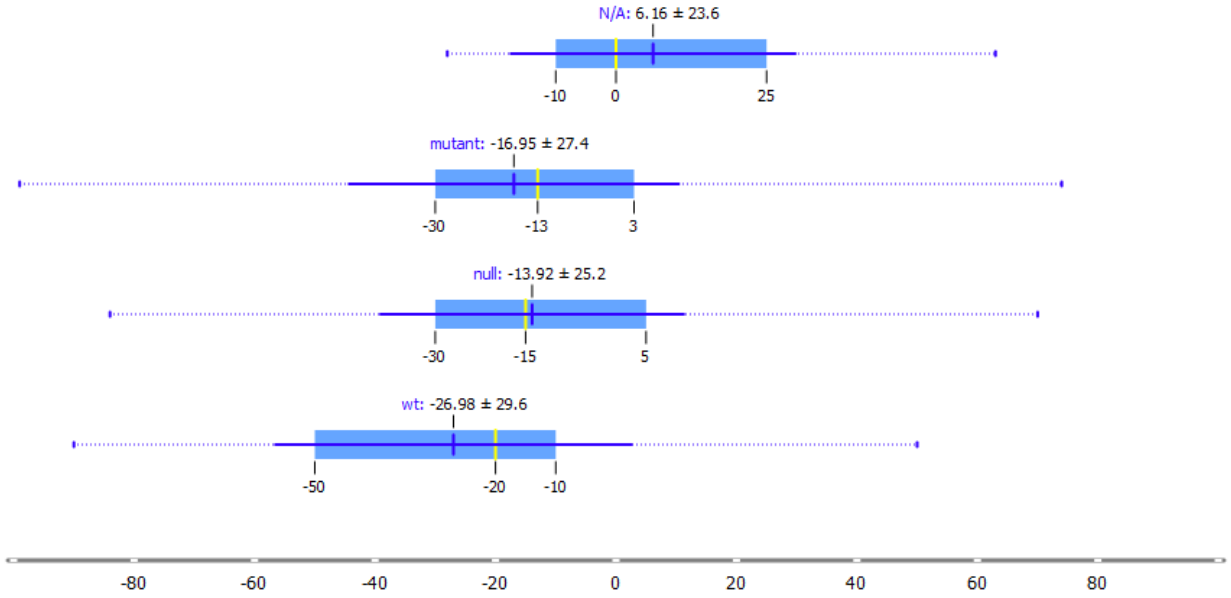

Figure 16: Box Plot of Survival Percentage Change Data for Included Studies grouped by p53 Status of the Reporter Cell Line.

p53 Status	Number of Plotted Data
N/A	25
mutant	257
null	234
wt	157

Table 7: Table displaying the number of plotted data within each p53 Status subgroup.

3.7 Radiation Source Results

There were 7 different radiation sources that were listed within the included studies and groups can be seen in Figure 17. Cobalt-60, a radioactive isotope of cobalt that produces gamma rays, was the most common radiation source and accounted for 54.2% of the data. The least common source found within the included studies was Plutonium-238, a radioactive isotope of plutonium that emits alpha particles and only accounted for 0.2% of the data. Only 1 study included plutonium which can be easily seen near the y-axis of Figure 18. Unfortunately, mean and standard deviation were not able to be calculated for plutonium and a box plot was not produced for that source (Figure 19). “X-Ray Irradiation Unit” is a broad term used for any devices or sources that produced x-rays as a means of experimental irradiation. LINACs were grouped into “Particle Accelerators” for ease of data plotting and statistical analysis. Figure 19 shows that experiments that used Americium-241 (M = -72.7, SD = 27.6) produced markedly reduced survival percentages compared with the other radiation sources. Table 8 shows the number of studies within each subgroup.

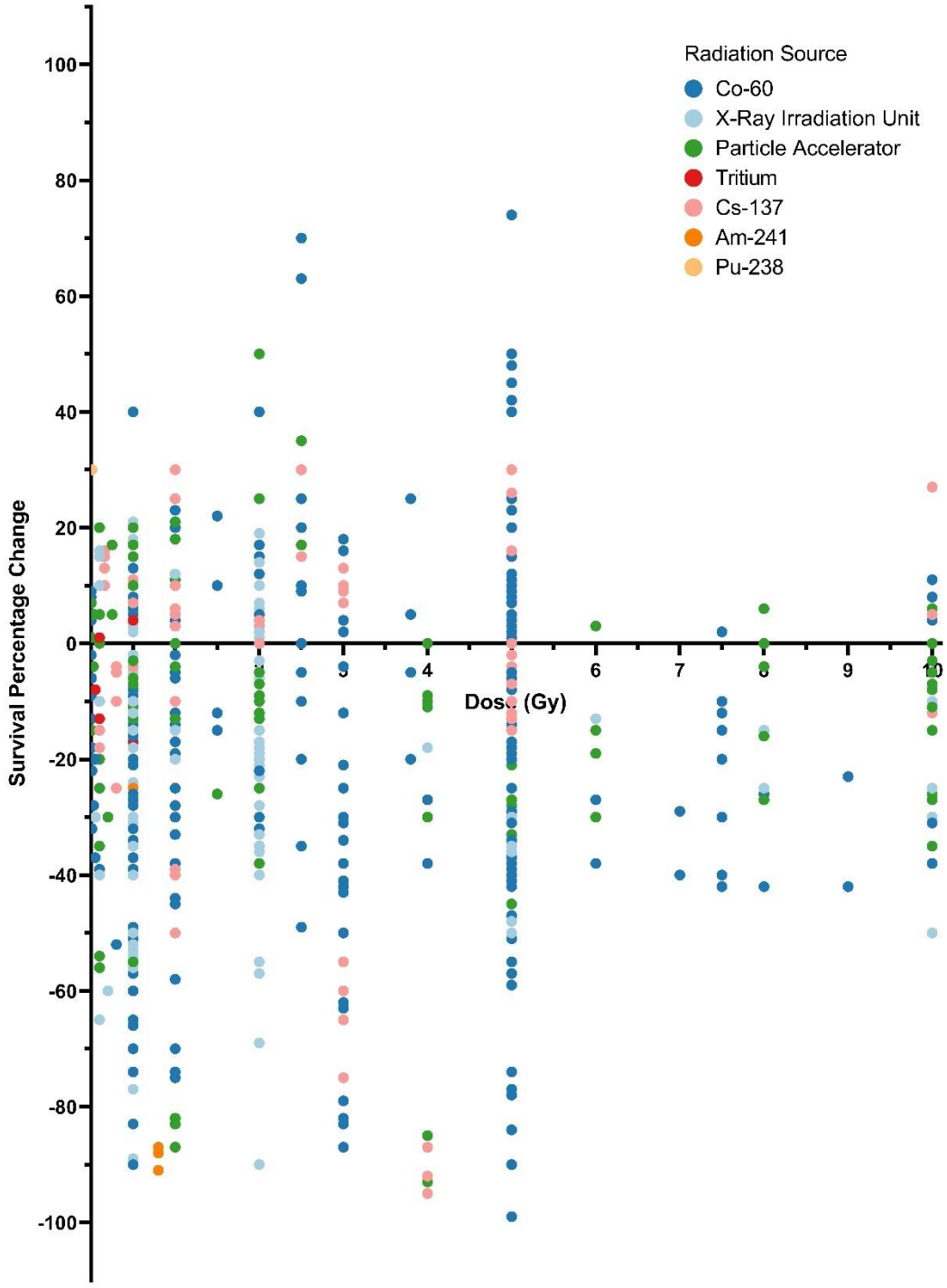

Figure 17: Survival Percentage Change Data for Included Studies grouped by Radiation Source used. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

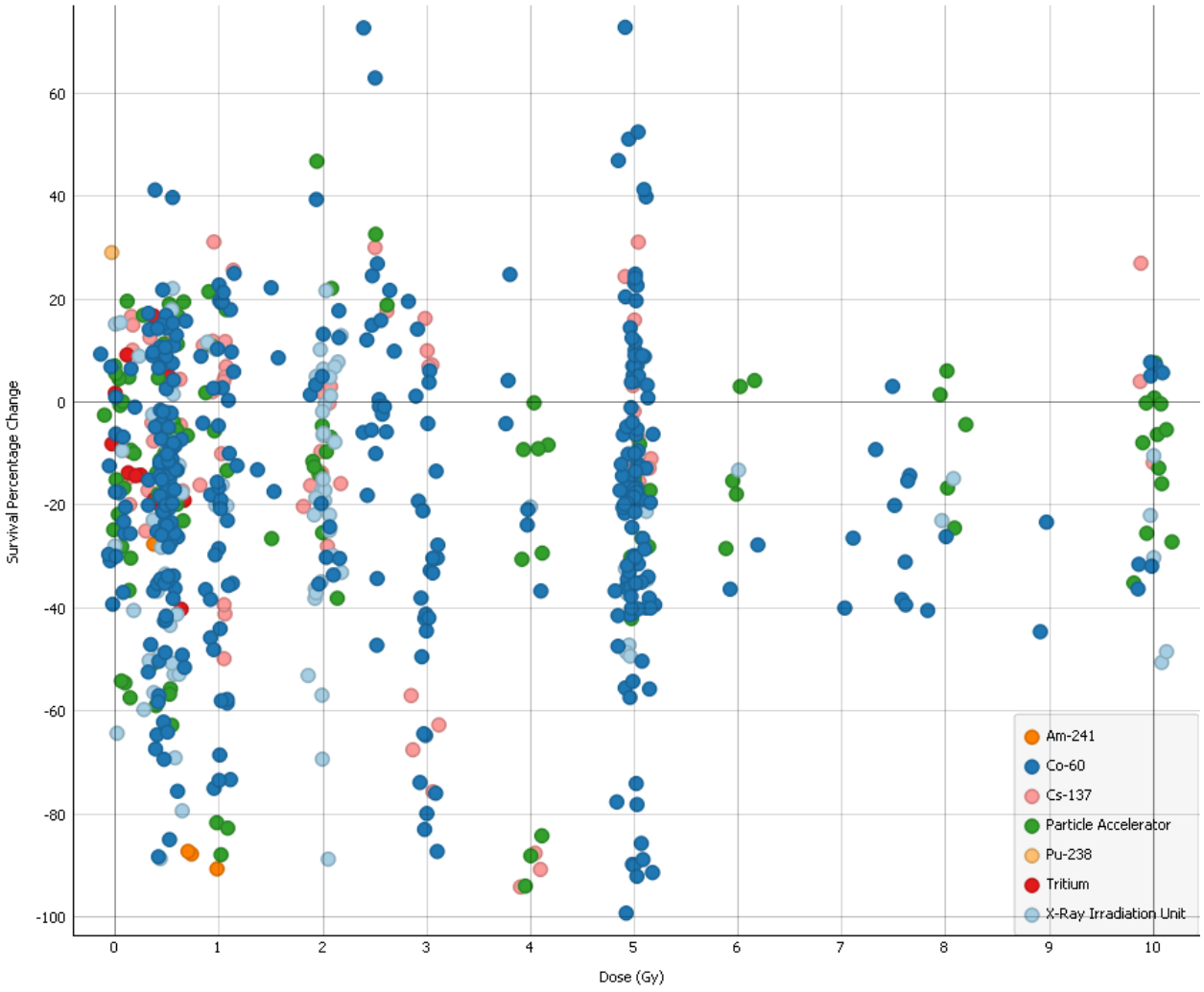

Figure 18: Alternate Graph of Survival Percentage Change Data for Included Studies grouped by Radiation Source used. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

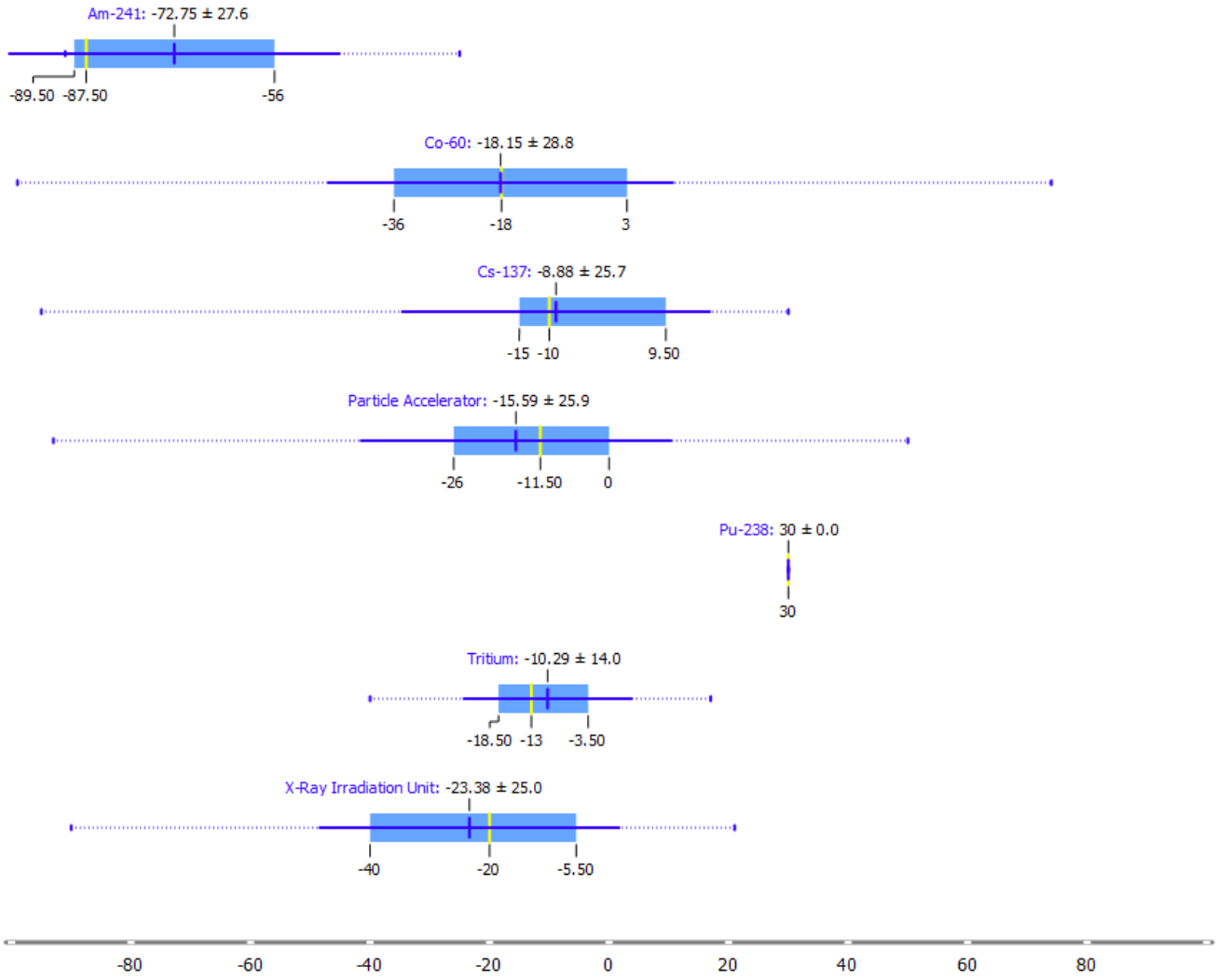

Figure 19: Box Plot of Survival Percentage Change Data for Included Studies grouped by Radiation Source used. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. Unable to generate box plot for Pu-238 due to small sample size (n=1).

Radiation Source	Number of Plotted Data
Am-241	4
Co-60	365
Cs-137	83
Particle Accelerator	114
Pu-238	1
Tritium	14
X-Ray Irradiation Unit	92

Table 8: Table displaying the number of plotted data within each Radiation Source subgroup.

3.8 Radiation Type Results

There were nine different radiation types within the included studies. Gamma radiation was the most common with 66.6% of the total data and can be seen throughout the survival percentage change scatter plot (Figure 20). Proton radiation was the least common accounting for just 0.5% of the data. “Electron” represents electron beam radiation and is grouped separately from beta radiation. A significant portion of beta, alpha, Fe ion, proton, and Carbon ion radiation data are confined to doses under 1 Gy as shown in Figure 21. A Two-Way ANOVA was performed and results are summarized in Table 9. Simple main effects analysis showed that radiation type did have a statistically significant effect on survival percentage change *(p = 3.67e-05)* while dose did not have a statistically significant effect on survival percentage change *(p = 0.92).* Figure 22 shows that the included proton radiation data were particularly dissonant from the other radiation type data (M = -88.3, SD = 3.4). Neutron radiation was the only type that produced a positive mean survival percentage change (M = 1.4, SD = 8.0). Table 10 shows the number of studies within each subgroup.

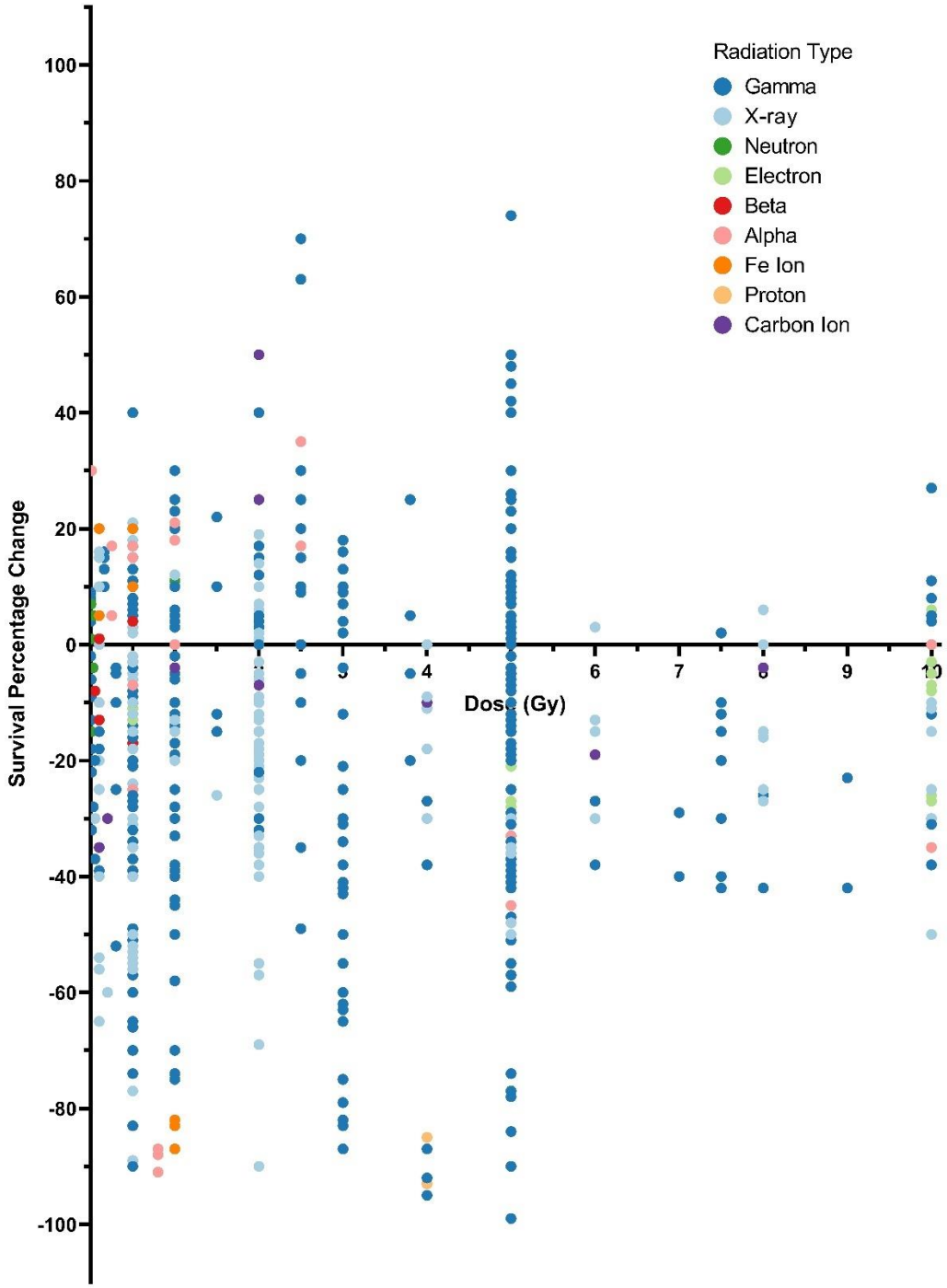

Figure 20: Survival Percentage Change Data for Included Studies grouped by Radiation Type. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

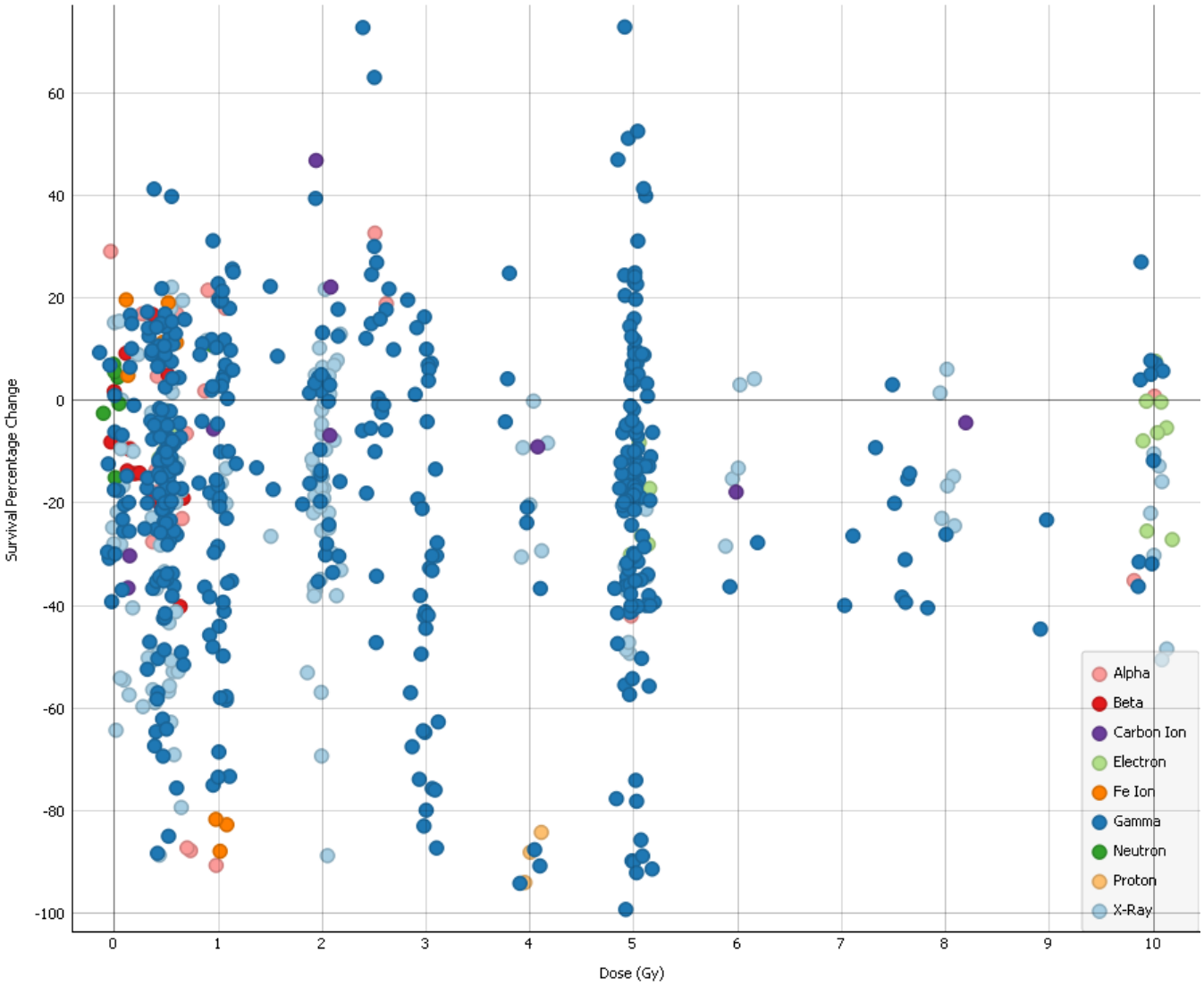

Figure 21: Alternate Graph of Survival Percentage Change Data for Included Studies grouped by Radiation Type. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

Source of Variation	***Df***	***SS***	***MS***	***F***	***p-value***
Rad Type	8	25149	3143.6	4.374	3.67e-05 ***
Dose	2	124	62.0	0.086	0.92
Residuals	653	469300	718.7		

*Table 9: Two-Way ANOVA Table Results for Radiation Type variable. *** indicates significance where α = 0.05.*

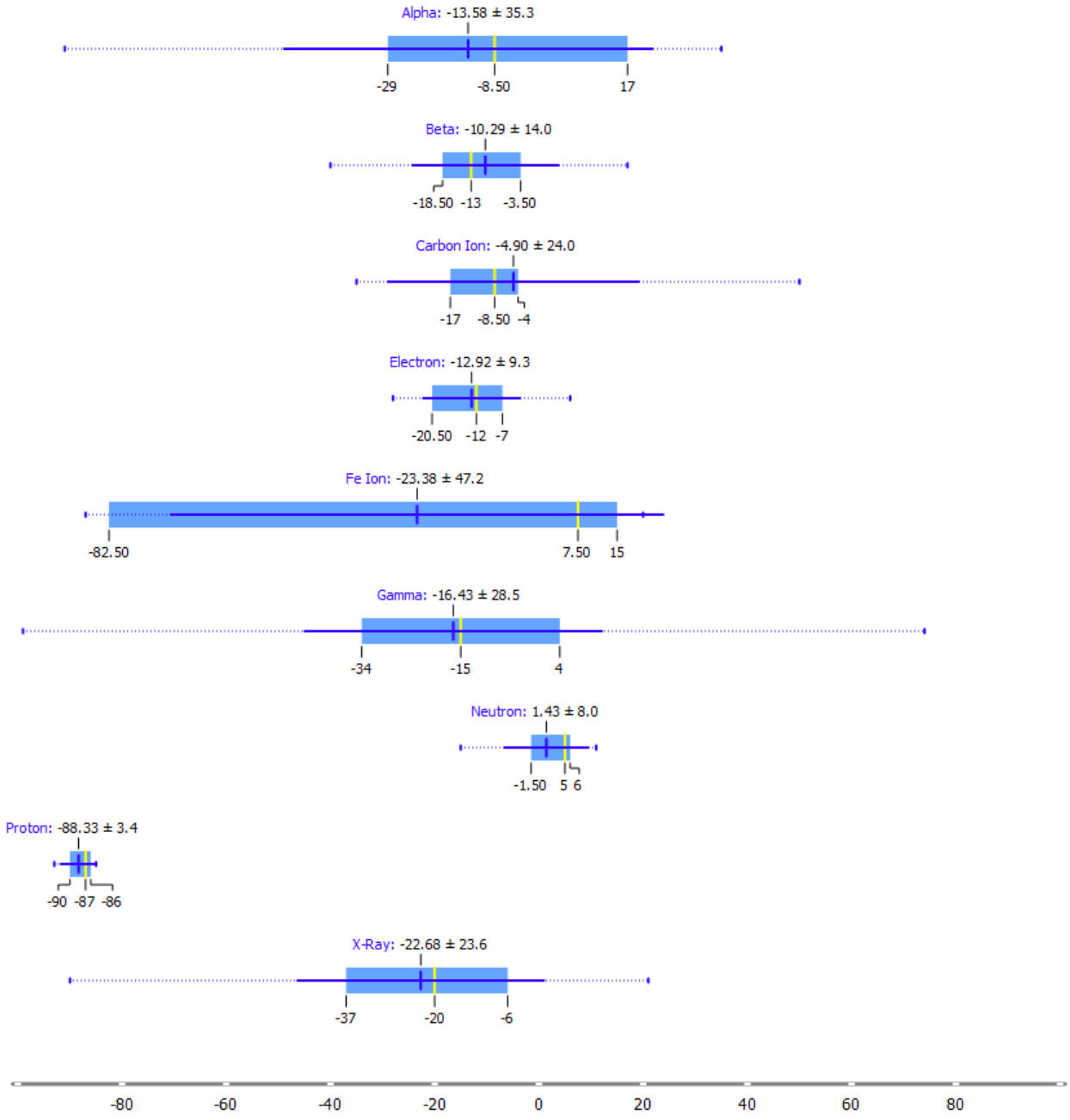

Figure 22: Box Plot of Survival Percentage Change Data for Included Studies grouped by Radiation Type. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis.

Radiation Type	Number of Plotted Data
Gamma	448
X-ray	134
Neutron	7
Electron	24
Beta	14
Alpha	24
Fe Ion	8
Proton	3
Carbon Ion	10

Table 10: Table displaying the number of plotted data within each radiation type subgroup.

3.9 Dose Rate Results

Dose rates were recorded within the final bystander results spreadsheet (Appendix 3). However, many studies used different dose rates and some only had negligible differences (i.e., 1.2 Gy/min vs 1.99 Gy/min). To reduce the number of groups and allow for less complex statistical analysis, it was decided to group dose rates into ranges. As such, “Low” is for dose rates less than or equal to 0.02 Gy/min, “Moderate” is for dose rates between 0.02 and 1 Gy/min, and “High” is for dose rates greater than or equal to 1 Gy/min. “N/A” was reserved for studies that did not disclose the dose rate used. The overall dispersion of the dose rate data can be seen in Figure 23. Most studies used high or moderate dose rates which accounted for 45.2% and 36.3% of the data. Only 1.5% of the included studies used a source that delivered a dose rate of less than or equal to 0.02 Gy/min. Logically, low dose rates were used for low doses while moderate and high dose rates were commonly used for doses higher than 1 Gy (Figure 24). 16.9% of included studies did not record or disclose the dose rate of the source that was used. A Two-Way ANOVA was performed and results are summarized in Table 11. Simple main effects analysis showed that dose rate and dose did not have a statistically significant effect on survival percentage change *($p = 0.57$ and $p = 0.42$, respectively).* High and moderate dose rates resulted in similar survival outcomes and their means only differed by a value of 0.3 (Figure 25). Table 12 shows the number of studies within each subgroup.

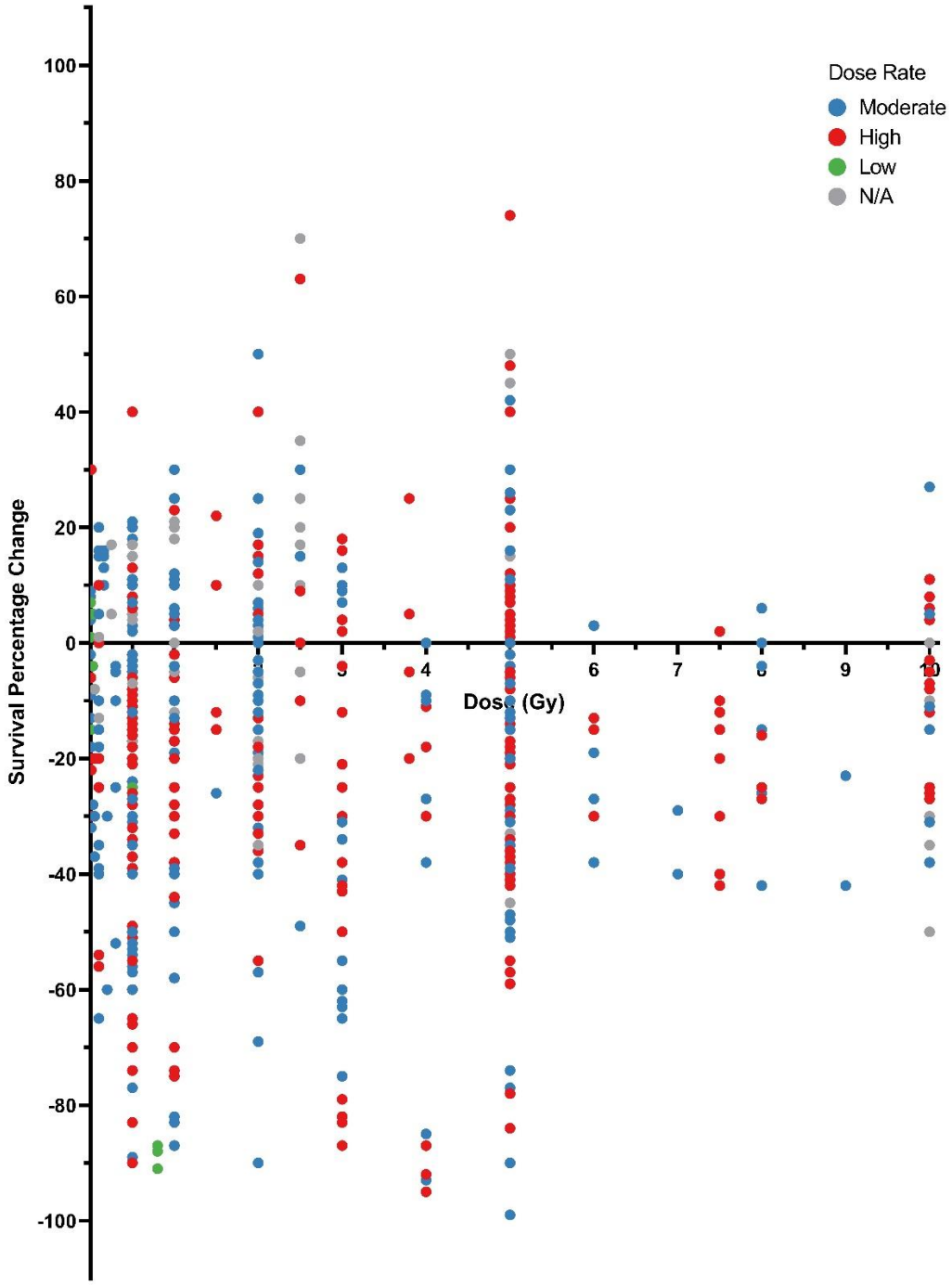

Figure 23: Survival Percentage Change Data for Included Studies grouped by Dose Rate Range used. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "N/A" denotes studies where Dose Rate information was unable to be obtained.

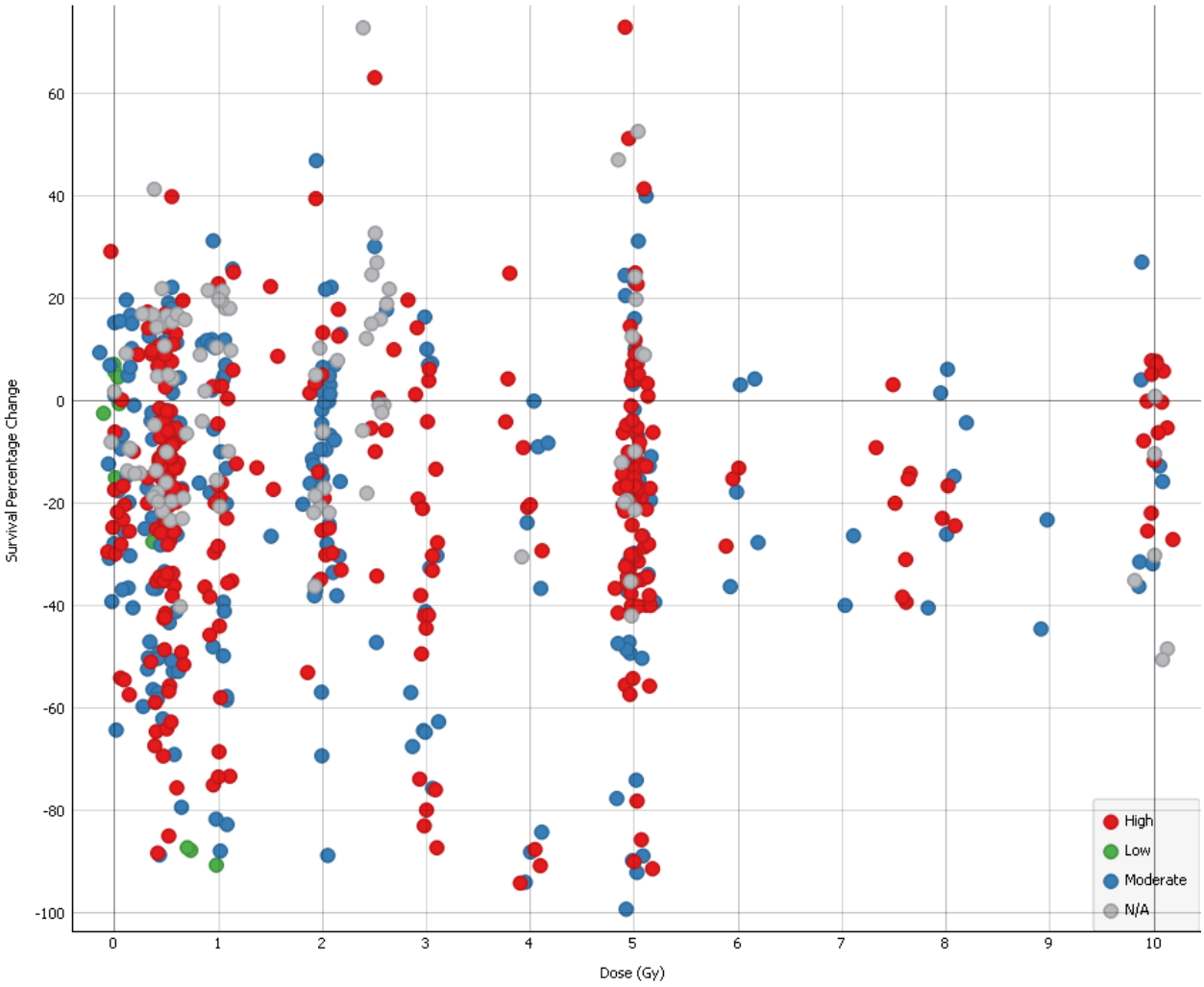

Figure 24: Alternate Graph of Survival Percentage Change Data for Included Studies grouped by Dose Rate Range used. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "N/A" denotes studies where Dose Rate information was unable to be obtained.

Source of Variation	***Df***	***SS***	***MS***	***F***	***p-value***
Rate	2	852	426	0.564	0.57
Dose	2	1318	659	0.872	0.42
Residuals	569	430019	756		

*Table 11: Two-Way ANOVA Table Results for Dose Rate variable. *** indicates significance where α = 0.05.*

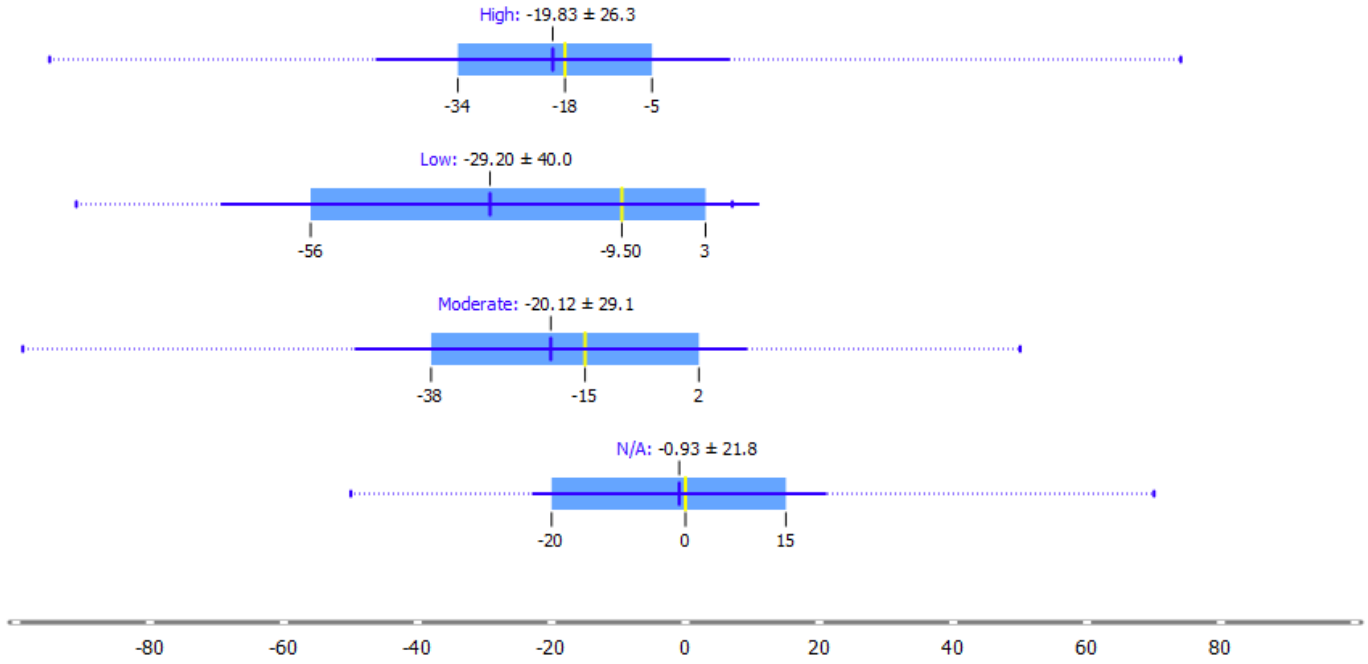

Figure 25: Box Plot of Survival Percentage Change Data for Included Studies grouped by Dose Rate Range used. Dose values are on the x-axis. Survival Percentage Change values are on the y-axis. "N/A" denotes studies where Dose Rate information was unable to be obtained.

Dose Rate	Number of Plotted Data
High	322
Low	10
Moderate	245
N/A	96

Table 12: Table displaying the number of plotted data within each dose rate subgroup.

Chapter 4: Discussion

4.1 Overall Discussion

There are numerous experimental parameters, present in the current bystander effect literature, that are often different or variable between experiments. Although they were investigating the same endpoint, it was not uncommon to find lab groups that used different cell lines which had their own intrinsic differences such as organ of origin, sex of the original donor, and p53 status. Available tools and resources also differed between lab groups. A common variable factor identified was the radiation source that was used. These produced different types of incident radiation that often had variable dose rates. Certain experimental designs have been standardized due to their usefulness and accuracy in reproducing significant results. As such, these rarely differed in any meaningful way between lab groups. Furthermore, experiments regularly explored cellular responses at discrete dose levels, such as 0.5, 1, 2, and 5 Gy. The aim of this thesis was to document and bring to light the heterogeneity of the bystander effect literature as well as investigate how the chosen factors influence cell survival outcomes during *in vitro* radiation-induced bystander effect experiments.

Although the individual parameters were explored separately, the overall results, shown in Figure 3 and Figure 4, provided some useful insights. There was a wide range of survival outcomes present within the included studies. The greatest increase in survival percentage was 74 relative to control groups, while the largest decrease in survival percentage was 99 relative to control groups. The mean survival percentage change was -17.4 and 71.6% of the included experiments saw an overall reduction in survival across

all dose values. Although dose can be described as a continuous variable, most experiments investigated discrete dose values, as mentioned in the previous paragraph. The relatively high density of data points at 0.5, 1, 2, and 5 Gy show how common these dose levels were within the literature. Only a few research groups investigated the effects of doses between 5 and 10 Gy. Overall trends were difficult to discern due to the sheer amount of overlapping data that was visualized, but one can see that, although there were many plot points in the “negative” region (indicating a reduction in survival), there were also a fair number of recorded instances where exposure to bystander signals caused an increase in survival in reporter cultures.

The results demonstrated the marked variation in survival outcomes are in the bystander effect literature. This could be due to differences in any of the chosen factors that will be discussed in subsequent sections. The data do not contradict the current understanding of the radiation-induced bystander effect. Similarly volatile results were reported by Mothersill *et al*. (2002) after exposing 13 different cell lines to bystander signals. Many showed a reduction in cloning efficiency, but others showed cloning efficiencies that were higher than controls. Some fish cell lines were included in the present review and it is understood that fish cell lines can exhibit a “protective” bystander response as demonstrated by Ryan *et al*. (2008). Moreover, some studies used neutrons to irradiate donor cultures, which have been shown to induce a radio-adaptive response (Suzuki *et al*., 2020). Both of these factors could have increased survival in bystander cultures, resulting in the enhanced variability between the included studies. Ultimately, the data displayed is expected based on previous and current literature.

4.2 Discussion of the Effect of Reporter Cell Line

There were many different reporter cell lines present within the included literature. As such, only the top 10 most common cell lines were graphed and analyzed. HPV-G was by far the most common reporter cell line used and exhibited an overall mean reduction in survival after exposure to bystander signals, but also showed increases in survival in some cases. This variability is expected due to the large number of studies that use HPV-G cells as reporter cultures. Some of the experiments used fish cell lines as donor cultures which resulted in an increased survival of HPV-G recipient cells relative to controls (O'Neill-Mehlenbacher *et al*., 2007). Other publications, like the one by Seymour and Mothersill (2000), showed a reduction in the survival of HPV-G reporter cells. Due to their relative radiosensitivity, HPV-G cells exhibit a wide range of responses depending on other experimental factors. HeLa cells showed extremely low bystander survival relative to controls. This may have been due to the small number of included studies that used HeLa cells as the reporter cell line. However, there are a number of publications that demonstrated a pronounced bystander effect in HeLa reporter cell cultures (Autsavapromporn *et al*., 2012; Giovanna Gomes Lara *et al*., 2018; Ilyas *et al*., 2021). HeLa cells have been used in radiation biology experiments for many years and exhibit impaired survival when exposed to direct radiation (Puck & Marcus, 1956). It would not be unexpected to see a similar trend in HeLa cells exposed to bystander signals. GM637H, on the other hand, only exhibited an increased survival percentage. Experiments with priming doses showed that GM637H fibroblast cells produced a protective bystander response while colorectal cancer cells did not (Schwarz *et al*., 2008). Furthermore, it was demonstrated that GM637H cells exposed to irradiated cell-

conditioned medium from different donor cell lines consistently exhibited an increase in survival (Rajamanickam Baskar *et al*., 2007). As such, the results seen here were in line with the current scientific understanding. Another cell line, SW48, differed notably from the rest of the group with a mean survival percentage change of -47. These colon cancer cells are known to be highly radiosensitive, even compared with other colon cancer cell lines, so their marked reduction in survival was expected (Dunne *et al*., 2003). The remaining cell lines that were investigated all saw slight reductions in survival and had mean values similar to the overall mean.

The Two-Way ANOVA results supported the theory that the reporter cell line had a significant effect on survival percentage change *($p = <2e-16$)*. The statistical significance is in line with the first hypothesis outlined in Section 2.4. These results should be considered during future radiation-induced bystander effect experiments. Using cell lines that are known to consistently produce higher or lower survival outcomes may skew a researcher's results one way or another. To make comparisons more direct and produce more powerful results, it is suggested that a standardized reporter cell line be established for bystander effect experiments. Unless the purpose of the study is to compare differences between cell lines, the literature would benefit from a consistent reporter system. From the results presented here, the ideal candidate cell lines would be HCT 116, HPV-G, or HaCaT cell lines. All responded notably to bystander signals but did not exhibit extreme radiosensitivity. More research is required to determine what reporter cell line would be the best suited for a standardized reporter system.

4.3 Discussion of the Effect of Organ of Origin

As mentioned previously, there are few publications that investigated *in vitro* bystander effects at a tissue-specific level. Tissue-specificity is more easily identifiable in an *in vivo* model. Therefore, most studies failed to mention the organ of origin of the cell lines that were used during the experiment. This made it difficult to determine the current scientific understanding at a strictly cellular level. The included literature contained over 20 different organs of origin so, to make the data more manageable and presentable, only the top 10 most prevalent organs were graphed. There are a few organ groups that had visually distinct differences and should be mentioned. Both colon and skin exhibited a wide range of survival outcomes within the included literature. This may be due to the fact that skin cell lines and colon cell lines were the two most prevalent reporter cells used, thus exposing them to a wide variety of experimental conditions. Schanz *et al*. (2012) mentioned that cells from quickly renewing tissues such as the skin or colon, have a stronger response to bystander signals, which is in line with the results seen here. The data from the brain cell lines further support this theory since, although bystander effects were still evident, a more muted response was noted compared with the skin and colon cell lines.

Spleen cell lines had a positive mean survival percentage change which evidently contradicts a large portion of the literature that reported a significant reduction in survival of reporter cells as well as increased presence of markers of DNA damage in spleen cell lines (Grifalconi *et al*., 2007; He *et al*., 2014; Zhang *et al*., 2008). However, this is likely due to experimental differences instead of a factor that is intrinsic to spleen cells. Uterine

cell lines also produced notable results; However, HeLa cells were the only reporter cell line included in this group. The large reduction in survival fits within the current understanding of responses to bystander signals as mentioned in the previous section. This could also be due to the small within-group sample size of the uterus grouping variable which may have skewed results to suggest that there was a significant difference which was not material (Forstmeier *et al*., 2016). Other organs that were graphed did not show an obvious trend or differ from the mean in an obvious way. It should also be noted that immortalized cells used for *in vitro* experimentation may not yield results that are directly comparable to cells from the organ of origin. Tumour cells are known to behave differently than original host cells and the organ of origin may not have any significant meaning during these *in vitro* experiments.

Due to time constraints, a Two-Way ANOVA was not performed on this factor. As such, interpretations were made solely on visual data provided in Figure 8, Figure 9, and Figure 10. While previous research has focused on *in vivo* models to compare organ responses to bystander effects, these results attempted to demonstrate differences seen within *in vitro* models. Only data from the uterus was visually distinct compared with the rest of the organs that were investigated and this was likely due to a small sample size within the group. It is possible that tissue-specificity is only relevant *in vivo*, where cells of the organ are in their natural state, however, further research is needed to confirm this theory.

4.4 Discussion of the Effect of Sex of the Reporter Cell Line

Grouping the data by sex of the reporter cell line was relatively simple. Most cell lines were able to be traced back to their original host and the host's sex was recorded. Only three of the included studies described cell lines in very generic terms, such as "normal human lung fibroblast" or "human skin fibroblasts". As mentioned previously, the sex of these cell lines was simply recorded as "N/A" and was not included in any statistical analysis. Female cell lines appeared to, on average, produce a larger reduction in cell survival according to Figure 11 and Figure 12. This is supported by the box plot in Figure 13 as female cell lines had a mean survival percentage change of -21 with a standard deviation of approximately 33. In comparison, male cell lines had a mean survival percentage change of -17 and a standard deviation of 25. The difference between the sexes didn't reach statistical significance ($p = 0.07$), however, this still contradicted previous *in vivo* results which suggested that males may be more susceptible to the radiation-induced bystander effect (Koturbash *et al*., 2008; Noshchenko *et al*., 2001; Yahyapour *et al*., 2018). It is likely that the effects seen *in vitro* are not directly comparable to those seen *in vivo*. Cell lines that used a mixed karyotype were labelled "M/F" and the group consisted solely of fish cell lines. Taking this into consideration, a positive mean survival percentage change relative to controls made sense as it has been documented previously that fish cell lines produced a protective bystander response (Ryan *et al*., 2008). The sex of the reporter cell line is a parameter that is often overlooked in the current literature, although based on the data presented here, that shouldn't be the case. A larger systematic review with wider-reaching inclusion criteria would help elucidate whether the sex variable should be explored further on an *in vitro* level.

4.5 Discussion of the Effect of p53 Status

Due to the high concentrations of data present at the commonly investigated dose values, it was difficult to discern precise trends displayed in Figure 14. The alternate, jittered view shown in Figure 15 made it slightly easier to identify patterns within the data. Cells with p53 wt status rarely exhibited an increase in survival in bystander cultures. A large majority were within the negative values, indicating an overall reduction in survival fraction compared with controls. Those with mutant p53 status or null p53 status had more data points concentrated in the positive change area compared with wt cells. However, this increase in survival commonly stayed below 20% and rarely surpassed 40%. Bystander survival percentage change for all p53 statuses was near or below 0 after 5 Gy, although this is likely due to the relatively small amount of data for doses between 5 and 10 Gy. The largest reductions in survival were seen at 5 Gy or less for all cell lines. After this threshold, the limited amount of data that was present didn't drop below a 50% reduction relative to controls. Fish cell lines were unable to have their p53 status confirmed and their status was recorded as "N/A". These were not included in any statistical analysis.

Results from the Two-Way ANOVA indicated that the p53 status of the reporter cell lines did have a statistically significant effect on survival percentage change ($p = 1.08e\text{-}05$). This was predicted and is in accordance with the current understanding of how p53 plays a role in the radiation-induced bystander effect. Wt cells had the lowest mean bystander survival percentage, with an average change of -27, while mutant and null cells did not exhibit such a drastic reduction. These groups had mean survival percentage change values of approximately -17 and -14, respectively. Wt cells have functioning p53

proteins which ensure cells with DNA damage do not proliferate uncontrollably and aid in the initiation of apoptosis. Considering this, it made sense that wt cells would have the greatest reduction in survival out of the three p53 statuses. Cells with a mutant p53 status have mutations that affect the TP53 gene meaning that the protein will work abnormally but will not necessarily cause a complete lack of function. Different point mutations likely result in the cell having an inhibited function of p53 or even an overactivity of the protein, producing extremely high or extremely low bystander outcomes (Le *et al*., 2017). In line with this, mutant cells had the greatest increase and decrease in bystander survival. On the other hand, p53 null cell lines have no functioning p53 protein at all and the G1 arrest does not occur. As such, one would expect these cells to have the highest mean bystander survival relative to mutant or wt cultures. Figure 16 shows this hypothesis to be true within the included literature.

Although this is not a novel discovery, it shows how important the p53 status of the reporter cell line is within bystander effect experiments and clearly indicates that it is a factor that should not be ignored. Many publications failed to state the p53 status of the various cell lines that were present during their experiment. Statuses had to be confirmed either through database searches or using previous publications for reference. In an ideal scenario, the p53 status of the donor and reporter cell lines would be confirmed by each lab before conducting their experiment. Since this factor has the ability to influence various outcomes, it is recommended that future research includes the p53 status of each cell line in the methods section of their respective manuscript.

4.6 Discussion of the Effect of Radiation Source

As mentioned in Section 1.11, the main source of inspiration for investigating this factor was the discrepancy seen in the strength of bystander effect outcomes in experiments that used cobalt-60 as a source and those that used cesium-137 as a source. Both emit gamma rays, albeit with different energies. Cobalt-60 was the most common radiation source used and dominated much of Figure 17 and Figure 18. Results show both increases and decreases in cell survival relative to controls. Cesium-137 produced a noticeable bystander response, however, plot points rarely reached the extreme values that cobalt-60 did, especially with regard to a protective bystander effect. The box plot in Figure 19 confirms this as cobalt-60 had a mean survival percentage change of -18 compared to -9 for cesium-137. It is difficult to hypothesize the reasoning for this discrepancy, however, it should be investigated in subsequent studies.

Americium-241 had a noticeably low mean survival percentage change (*-72.75*). This source is an alpha emitter and these strong bystander effects are in line with the current understanding of how high LET radiation, such as alpha particles, can induce the radiation-induced bystander effect (Kadhim *et al*., 2006; Shao *et al*., 2001; Sowa *et al*., 2011). Particle accelerators showed similar results to cobalt-60, but this group consists of various heavy particles and doesn't provide much insight into the specific "source". Tritium had a mean survival percentage change of -10 with the smallest standard deviation (+/- 14). This agrees with previous publications that demonstrate that beta particles can induce bystander effects in reporter cultures, although to a lesser degree when compared with alpha particles and heavy ions (Persaud *et al*., 2007). Furthermore, the included

studies only used tritium for very low-dose exposures, which likely contributed to this overall “muted” response. Experiments that used an x-ray irradiation unit usually saw reductions in bystander survival. These results do not contradict the current literature as x-rays are known to produce bystander effects in reporter systems.

Plutoniumm-238 was a source that only had one recorded use within the included studies. As such, a box plot was unable to be generated in Figure 19. Due to time constraints, a Two-Way ANOVA was not performed on this factor. Therefore, all comparisons were made solely on the visual evidence provided by Figures 17, 18, and 19. The primary objective of including this factor was to elucidate the differences seen between cobalt-60 and cesium-137. Future publications, that focus on the differences seen between sources that produce the same radiation type, would be beneficial to the bystander effect field. Ideally, these publications would seek to understand how their different decay structures may influence the number of “hits” that occur within the cell, and thus, how much damage is communicated. Although grouping the data by the source that was used provided an interesting insight, it is arguably more important to understand the differences between the types of radiation being used in various experiments, which will be discussed further in the next section.

4.7 Discussion of the Effect of Radiation Type

The majority of the included bystander experiments used sources that produced gamma radiation. Figures 20 and 21 are dominated by gamma and x-ray radiation types as these accounted for 84% of the data collectively. Both of these radiation types appear to produce less drastic results after 5 Gy, but this is likely due to the lack of data points

present between 5 and 10 Gy. The few studies which used neutron radiation saw relatively modest bystander outcomes which were investigated at very low doses. It is possible for neutron irradiations to be contaminated with gamma rays; however, the included studies used a lead shield to attenuate any gamma radiation, thus eliminating this confounding variable. Experiments that used electrons to irradiate cultures reported slight reductions in cell survival at low doses and the reduction peaked at 5 Gy. At the highest investigated dose of 10 Gy, electron irradiation still induced a reduction in cell survival, albeit to a smaller degree. Beta irradiation was concentrated in the low-dose region of the graph and showed a maximum reduction of bystander survival of 40%. Irradiation with alpha particles resulted in a wide range of bystander survival outcomes with increased survivability around 2.5 Gy and a notable decrease in survival at 4 Gy. The results from alpha particles plateaued around 5 Gy. Iron ions were only used for experiments that investigated doses of 1 Gy or less. These heavy particles produced an increased survival at very low doses and a very noticeable reduction of survival at 1 Gy. Protons were seldom used within the included literature but consistently produced a large reduction in bystander survival at 4 Gy. Lastly, carbon ions saw the greatest survival reduction at very low doses, with a protective bystander response around 2 Gy. At doses above 2 Gy, there was a slight reduction in survival but never more than 20% relative to controls.

All of the included radiation types had mean percentage change values that were similar to the overall mean with a couple of exceptions. Protons had a noticeably large mean reduction which is probably due to the limited number of studies present in the included literature, resulting in a mean reduction that is unlikely to be representative of true survival outcomes seen with this radiation type. Neutron radiation produced a mostly

positive survival percentage change which is in agreement with previous studies that demonstrated that neutrons produced a protective bystander effect in recipient cultures (Suzuki *et al*., 2020). Another phenomenon to highlight is that x-rays (low LET) had a lower mean reduction compared with alpha particles (high LET), but alpha particles had a much larger standard deviation. This is in line with most of the previous literature that stated that, although low LET radiation is able to induce a bystander effect, more pronounced results were seen with high LET radiation (Anzenberg *et al*., 2008; Buonanno *et al*., 2011; Kadhim *et al*., 2006; Shao *et al*., 2001).

A Two-Way ANOVA confirmed that radiation type did have a statistically significant effect on survival percentage change ($p = 3.67e\text{-}05$). Although this agrees with the majority of the current literature, it is recommended that future research focus on individual radiation types that often produce varying results, such as the protective bystander effect seen with neutron radiation or the considerable reduction in bystander survival seen with proton radiation. However, a standardized experimental design that minimizes confounding variables should be implemented for more conclusive results.

4.8 Discussion of the Effect of Dose Rate

As explained in Section 3.9, dose rates from the included studies had to be grouped into ranges to allow for simplified plotting and statistical analysis. The majority of the included literature used a high dose rate, most likely because the dose rate inherent to their source was unable to be altered, or to allow for the accumulation of the required dose in a shorter time frame. Both high and moderate dose rates were the most prevalent in the included literature and can be seen dominating Figure 23. No obvious trends were identified for

high or moderate dose rates within Figure 24. Low dose rates accounted for the smallest fraction of the data; under 2%. At very low doses, low dose rates were unable to induce a strong bystander response. However, as the dose increased to 1 Gy, there was a more obvious bystander effect with a survival reduction maximum of 91%. This pointed to a dose-dependent response in the low dose rate group, although more research that focuses on low dose rate effects is needed to confirm this phenomenon. Figure 25 showed that high and moderate dose rates resulted in similar bystander outcomes, while low dose rates induced a larger reduction in survival, on average. However, low dose rates also had a much larger standard deviation, presumably due to the limited number of included studies that actually used low dose rates in this group. Approximately, 17% of studies did not mention the dose rate that was used during experimentation. These were labelled as “N/A” and were not included in statistical analysis.

A Two-Way ANOVA indicated that the dose rate did not have a statistically significant effect on survival percentage change ($p = 0.569$). This is in line with previous literature, which only reports a dose rate dependence at high doses and high dose rates or does not report a significant dose-rate dependence at all (Gow *et al*., 2007; Vo *et al*., 2019). It is recommended that future research focuses on elucidating the dose-response observed with very low dose rates, as this could have applications in radiation protection.

4.9 Discussion of the Effect of Dose

Radiation dose is an integral part of any radiobiology experiment. The included literature contained a wide range of dose values. A large majority of these studies researched effects seen at 5 Gy or less, however, studies with doses of up to 10 Gy were included in

this dataset. Each of the chosen factors was plotted with dose as the independent variable, similar to the bystander survival fraction data that was recorded from the included literature. As mentioned previously, common dose values were 0.5, 1, 2, and 5 Gy. Although dose is considered a continuous variable, the incremental dose values that were commonly investigated essentially produced graphs with a discrete independent variable. Only certain factors were analyzed with a Two-Way ANOVA and of those that did, none found that dose had a statistically significant effect on the survival percentage change. The dose was closest to significance while being analyzed alongside the reporter cell line variable ($p = 0.192$). However, this is most likely due to the fact that the complete dataset had to be modified to include only the top 10 most prevalent cell lines, which reduced the number of data being analyzed by the Two-Way ANOVA relative to other factors.

These results are in line with the current understanding of how dose affects the survival outcomes during bystander effect experiments. It is understood that bystander effect magnitude saturates at a very low dose (approximately 0.5 Gy or below) and that there is not a dose-dependent increase in magnitude as the dose to the donor cells increases (Seymour & Mothersill, 2000). This threshold dose is best observed within the "Reporter Cell Line" factor displayed in Figure 5. HPV-G, for example, showed the lowest bystander survival fractions at around 0.5 Gy. Similar survival percentages were seen in the same cell line at 5 Gy. The cell line, T/C28a2 is another good example of this phenomenon. This cell line showed the greatest reduction in bystander survival at 0.1 Gy. As the dose increased, the overall reduction was less pronounced and plateaued at a modest survival reduction of around 20%. As expected, an increased dose did not

necessarily result in a greater reduction in survival outcomes. In agreement with previous publications, the combined data displayed here indicated that bystander response is not dose-dependent and a threshold dose likely occurs around 0.5 Gy. It is difficult to come to any strong conclusions with regard to this factor due to the high level of variance between experiments. More research is needed to confirm if this phenomenon is seen in all cell lines and how different factors can influence this apparent threshold dose. This information would be beneficial for applications in radiation protection and environmental impact analysis.

4.10 Limitations

In general, the research question being investigated in this thesis would be better answered with a systematic review of the bystander literature. Multiple researchers with more allotted time would be able to have wider inclusion criteria, thus giving a better overall representation of the literature. Since there was only one researcher for this project, inclusion criteria had to be quite specific and narrow, which allowed for a manageable number of studies that could be synthesized and analyzed within the required time frame. This may impact the generalizability of the results found in this review. Furthermore, each factor had to be divided into representative groups. Unfortunately, some groups did not have a representative amount of data within them (such as the HeLa cell line group), and results may not be indicative of how these specific groups truly react to bystander signals. This limits the generalizability of these particular groups.

The extensive amount of data within the included studies was difficult to analyze. Smaller groups were created to allow for clearer graphing and easier statistical analysis. For example, within the “Radiation Source” factor, the group “Particle Accelerators” includes many different particle accelerators as well as LINACs. More specific differences seen within the groups themselves may be missed. Moreover, due to time constraints, not all of the chosen factors were analyzed by a Two-Way ANOVA. As such, any conclusions drawn from these factors are based solely on visual data presented within the figures of the thesis and do not have the backing of statistical tests, significantly impacting their reliability.

Lastly, it would be ideal for each of these factors to be isolated and tested individually. Due to the nature of this review, confounding variables were unable to be removed when comparing how each factor could influence bystander survival outcomes. A Two-Way ANOVA was chosen to test the main variables in question; the chosen factor and the dose used. However, it would be more representative to conduct an Eight-Way ANOVA that accounts for all of the chosen variables. This would, unfortunately, be outside of the scope of the current review.

Chapter 5: Conclusion

This review aimed to identify which chosen factors influence cell survival outcomes during *in vitro*, radiation-induced bystander effect experiments. Based on a quantitative analysis using a Two-Way ANOVA parametric test, it was shown that the reporter cell line, p53 status of the reporter cell line, and incident radiation type are important factors that impact the results of bystander effect experiments. Many factors had to be divided into manageable groups of unequal sizes which, overall, affects the generalizability of the results. Nonetheless, this review gives new insight into how these chosen factors influence bystander cell survival outcomes and highlights the heterogeneity of the literature and the difficulty of comparing one study to another within the bystander effect field. Based on the presented findings, it is essential for future researchers to include all of the chosen factors within the methods section of their manuscript. Additionally, it is recommended that a full systematic review of the literature be conducted to strengthen or contrast the claims made in this rapid review. Future research would benefit from using a multi-factorial ANOVA to analyze all of the confounding variables discussed here. The radiation-induced bystander effect is a complex biological phenomenon that can be influenced by many factors and the scientific community must work together to fully elucidate its intricacies. This review emphasizes the importance of creating a standardized bystander effect model and suggests ways to improve future research, paving the way for more compelling discoveries in the years to come.

Chapter 6: Appendices

Appendix 1: Original Excel Spreadsheet

Original Excel Spreadsheet.xlsx

Appendix 2: Second Version of Excel Spreadsheet

Second Version of Excel Spreadsheet.xl

Appendix 3: Final Excel Spreadsheet

Final Excel Spreadsheet.xlsx

Appendix 4: Literature Search Strategy

Search Strategy.docx

Appendix 5: QA Scoring Sheet

QA Scoring Sheet.xlsx

Chapter 7: References

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