Characterizing and modulating the effects of ionizing radiation to the juvenile hippocampus

Malin Blomstrand

Department of Oncology
Institute of Clinical Sciences
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Picture of the brain, modified by Malin Blomstrand using Nils Blomstrands I-pod5 and Powerpoint.

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malin.blomstrand@neuro.gu.se

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ABSTRACT

Survival rates after childhood cancer treatment have improved, leading to a growing population of survivors. Radiotherapy is an important tool for curing cancer in the brain. Unfortunately, radiotherapy is associated with late side effects e.g. of cognitive impairment. The mechanisms underlying radiation-induced cognitive dysfunctions are not fully understood but involve changes in the neurogenic niche of the hippocampus. The aim of this thesis was to characterize and modulate the effects of ionizing radiation to the hippocampus of the juvenile brain, with the future goal of ameliorating cognitive deficit of childhood cancer survivors following radiation for intracranial disease.

We investigated the effects of low-dose radiation of the brain in infancy. A total of 3,860 boys were treated with radiation for cutaneous hemangiomas before the age of 18 months. Of these, 3,030 were analyzed for military test scores at the age of 18 years and 2,559 for the highest obtained educational level. We also characterized and compared the radiation-induced reactions in the hippocampus of the juvenile and adult rat brain, as well as evaluated the modulating effect of amifostine, WR-1065 and N-acetylcysteine during cranial irradiation of the juvenile rat brain. This was done in a rat model. Further, we tried to modulate the dose to the hippocampus in medulloblastoma patients by the use of modern radiotherapy techniques. Different radiation prescription scenarios, by means of computer-based treatment, were used to evaluate the possibilities of sparing the hippocampus from radiation and to assess their potential benefits regarding cognitive outcome.

We did not find any effect on the highest obtained education when we investigated the risk of cognitive dysfunctions after exposure to low doses of cranial radiation in infancy. There was no decrease in logical, technical or spatial test scores after radiation doses up to the highest dose category (median 680 mGy). Verbal test scores displayed a very small but statistically significant trend for decreasing scores with increasing doses to the hippocampus. We concluded that the juvenile brain, from a clinical perspective, was not sensitive to doses overlapping the range used for diagnostic purposes, contrasting with earlier findings. For therapeutic doses of radiation in rodents, we found that the radiation reaction in the hippocampus differed in the juvenile brain compared to the adult brain in terms of density of resident microglia, number of activated microglia, levels of apoptosis, specific cytokines/chemokines and growth factors. In rodents, we did not find any protection by amifostine, WR-1065 or N-acetylcystein using tolerable doses during cranial radiation. However, in children we could conclude that sparing the hippocampus from radiation during cranial radiotherapy is feasible by the use of modern treatment techniques. We found that the greatest potential for hippocampal sparing was offered by intensity-modulated proton therapy. Interestingly, we also found that the use of different techniques influenced the dose to the hippocampus to a higher extent, than the use of smaller treatment volumes for the tumor boost. Further, we estimated that a hippocampal sparing strategy could ameliorate the cognitive impairment seen after cranial radiotherapy.

Keywords: low-dose radiation, CNS, hippocampus, apoptosis, cytokines, growth factors, microglia, medulloblastoma, hippocampal sparing, tumor bed boost, cognitive risk estimation

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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Barn överlever cancer i allt större utsträckning tack vare bättre behandlingsmetoder. Strålbehandling är en viktig del av behandlingen för att bota cancer i hjärnan hos barn. Dessvärre ger strålbehandling av hjärnan ofta biverkningar i form av besvär med minne och inlärning. Risken för dessa biverkningar är större hos flickor än hos pojkar och ökar ju yngre barnet är vid behandlingstillfället. Besvären är också progressiva, d.v.s ökar med tid efter behandlingen. Det är därför viktigt att finna strategier som minskar risken för skada av den normala hjärnvävnaden, med samtidigt bibehållen behandlingseffekt.

I denna avhandling har vi dels utforskat några av de mekanismer som leder till försämrat minne efter strålbehandling av hjärnan och dels provat olika strategier för att minska risken för biverkningar.

Vi kunde visa att pojkar som fått mycket låga doser av strålning mot hjärnan i unga år inte hade lägre utbildningsnivå i vuxen ålder än pojkar som inte fått strålning. Vi uppmätte inte heller någon försämring av deras resultat i logiska, tekniska eller spatiala tester, men en minimal försämring i verbala tester. Vi kunde således inte finna några bevis för att låga stråldoser, i nivå med upprepade skiktröntgenundersökningar av hjärnan, i unga år, har någon praktisk betydelse i det dagliga livet.

Nybildning av nervceller sker bland annat i en del av hjärnan som heter hippocampus, sjöhästen. Denna del av hjärnan är betydelsefull för minne och inlärning. I en råttmodell kunde vi visa att strålning mot hippocampus med högre doser gav olika resultat i den unga- jämfört med den vuxna råtthjärnan. Om detta visar sig vara sant även i den mänskliga hjärnan talar det för att det delvis är olika mekanismer som ligger till grund för minnes- och inlärningssvårigheter hos barn och vuxna efter strålbehandling. Detta talar för att det kan behövas olika strategier för att minska risken för biverkningar hos barn och vuxna i samband med strålbehandling av hjärnan.

Vi testade också om substanserna amifostin, WR-1065 och N-acetylcystein kunde skydda stam/progenitorcellerna under strålbehandling, för att på så vis minska risken för biverkningar. Vi kunde emellertid inte se någon effekt av dessa substanser.

För att se om man med moderna strålbehandlingstekniker kunde undvika att bestråla hippocampus under strålbehandling av hjärnan genomförde vi två simulerade dosplaneringsstudier. Vi kunde visa att det går att minska stråldosen betydligt i hippocampus, framför allt om vi använde protonstrålning. Vi kunde också visa att valet av behandlingsteknik påverkade dosen till hippocampus mer än storleken av säkerhetsmarginalerna runt området där tumören tidigare suttit. Genom att använda oss av resultat från en tidigare studie som utvärderat bl.a. minne efter cancerbehandling kunde vi arbeta fram en matematisk modell för att uppskatta den potentiella vinsten med att sänka stråldosen till hippocampus. Vi kunde visa att minnesförmågan skulle komma att påverkas mindre vid användande av behandlingstekniker som sänker dosen till hippocampus. Det återstår dock att i framtida kliniska studier verkligen bevisa att våra beräkningar stämmer.

Sammanfattningsvis bidrar denna avhandling till ökad kunskap om strålbehandling av den unga hjärnan och hur vi kan minska risken för sena biverkningar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Blomstrand M, Holmberg E, Åberg M A I, Lundell M, Björk-Eriksson T, Karlsson P and Blomgren K. **No clinically relevant effect on cognitive outcomes after low-dose radiation to the infant brain: a population-based cohort study in Sweden.** *Acta Oncologica (2014) Early Online:1-8.*

II. Blomstrand M, Kalm M, Grandér R, Björk-Eriksson T and Blomgren K. **Different reactions to irradiation in the juvenile and adult hippocampus.** *Submitted manuscript*

III. Blomstrand M*, Brodin P N*, Munck af Rosenschöld P, Vogelius I R, Sánchez Merino G, Kiil-Berthlesen A, Blomgren K, Lannering B, Bentzen S M and Björk-Eriksson T. **Estimated clinical benefit of protecting neurogenesis in the developing brain during radiation therapy for pediatric medulloblastoma.** *Neuro-Oncology (2012) 14(7):882-889*

IV. Brodin P N, Munck af Rosenschöld P, Blomstrand M, Kiil-Berthlesen A, Hollensen C, Vogelius I R, Lannering B, Bentzen S M and Björk-Eriksson T. **Hippocampal sparing radiotherapy for pediatric medulloblastoma: impact of treatment margins and treatment technique.** *Neuro-Oncology (2014) 16(4):594-602*

*Authors contributed equally to this work

CONTENT

ABBREVIATIONS

BER	base excision repair
BBB	blood-brain-barrier
PBS	phosphate-buffered saline
BrdU	bromodeoxyuridine
CCL2	chemokine (C-C motif) ligand 2
CNS	central nervous system
CRT	cranial radiotherapy
CT	computed tomography
CTV	clinical target volume
DG	dentate gyrus
DNA	deoxyribonucleic acid
DSB	double stand break
GCL	granule cell layer
GTV	gross tumor volume
Gy	Gray
HR	homologous recombination
Iba-1	ionized calcium-binding adaptor molecule 1
IGF-1	insulin like growth factor 1
IHC	immunohistochemistry
IL-6	interleukin 6
IMAT	intensity modulated arc therapy
IMRT	intensity modulated radio therapy
IMPT	intensity modulated proton therapy
i.p.	intraperitoneal injection
LET	linear energy transfer
LQ-model	linear quadratic model
M-CSF	macrophage colony stimulating factor
MB	medulloblastoma
NAC	n-acetylcysteine
NHEJ	non-homologous end-joining
NTCP	normal tissue complication probability
OAR	organ at risk
OER	oxygen enhancement ratio
P9	postnatal day 9
PPAR	peroxisomal proliferator-activated receptor
PTV	planning target volume
RAS	renin angiotensin system
RBE	relative biological effectiveness
SGZ	subgranular zone

SSB	single strand break
SSBR	single-strand break repair
SVZ	subventricular zone
TCP	tumor control probability
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

1 INTRODUCTION

The survival after childhood cancer improves continuously. Along with this gratifying progress comes a growing population of long-term survivors, some of which will suffer from treatment-related side effects. Today, in Sweden, it is estimated that 1/700 adult people (25 to 35 years of age) is a long-term survivor after treatment of childhood cancer (Hjorth et al., 2010). The incidence of central nervous system (CNS) tumors in children is 4.59/100000, constituting about 30% of all childhood cancer in Sweden (Gustafsson G, 2013). Medulloblastoma (MB) is a primitive neuro-ectodermal tumor located in the posterior fossa of the brain and accounts for about 20 % of the CNS tumors in children, with a peak incidence at 5 years of age (Fossati, Ricardi, & Orecchia, 2009; Gustafsson G, 2013). MB is characterized by a high frequency of seeding within the cerebrospinal pathways (Harisiadis & Chang, 1977) and therefore the whole CNS is treated with an adjuvant dose of radiotherapy, apart from the boost of a higher dose to the primary tumor site. The 5-year survival rates of MB are now approximately 75-85% for standard risk patients (Lannering et al., 2012). In general, cranial radiotherapy (CRT) is an important tool for curing cancer in the brain. Many of the children surviving CNS tumors suffer from late side effects (emerging more than 6 months after radiation) including perturbed growth, cognitive impairments and secondary malignances (Armstrong et al., 2010; Lannering, Rosberg, Marky, Moell, & Albertsson-Wikland, 1995; Neglia et al., 2006; Packer et al., 2003). Research has indicated that CRT is the most important treatment modality for induction of neurocognitive deficits (Grill et al., 1999; Kieffer-Renaux et al., 2000). Cognitive impairments are irreversible and progressive over time (S. L. Palmer et al., 2013) and comprises impaired attention, working memory, processing and other executive functions, as well as a measurable decrease in IQ scores (Rodgers, Trevino, Zawaski, Gaber, & Leasure, 2013). Further, an increased risk of hearing loss and impaired vision after CRT might contribute to the disability (Brodin et al., 2011). The impact of cognitive dysfunction of the brain tumor survivors are also reflected in the increased risk of poor quality of life and poor economic status later in life (Armstrong et al., 2009). The mechanisms responsible for the cognitive impairments after CRT are not fully clarified, but involve decreased hippocampal neurogenesis, altered neuronal function, neuroinflammation and changed vascular and glial clonogenesis (Greene-Schloesser, Moore, & Robbins, 2013). The studies of this thesis focus on characterization of radiation-induced changes in the hippocampus and on ways of diminishing theses effects.

1.1 Ionizing radiation in medicine

External beam radiation therapy can be delivered using high-energy photons, electrons, neutrons, light ions, e.g. hydrogen ions (=protons), helium, boron, carbon ions or heavy ions. The type of radiation and its kinetic energy will govern the mode of interaction with matter. Ionizing radiation can eject bound electrons from the target atoms, hence the name *ionizing radiation.*

Linear energy transfer (LET) is a term describing the density of ionization in a particle track. It is defined as Δ energy/Δ length. Photons and protons are examples of low-LET radiation and heavier light ions are examples of high-LET radiation.

The energy absorbed in tissue is measured in the unit Gray (Gy). 1 Gy is equivalent to 1 Joule of energy per 1 kilogram of mass.

1.1.1 Radiobiology

The ions formed by radiation react with cellular elements causing damage to the tissue directly through breakage of chemical bonds and indirectly by production of damaged molecules, so called free radicals, which are highly reactive. This leads to damage to all molecules in the cell, but deoxyribonucleic acid (DNA) is the most critical target for radiation induced cell killing (Warters & Hofer, 1977). Damage to DNA consists principally of double strand-breaks (DSB), single strand-breaks (SSB) and altered bases. The DSBs are the most important and lead either to apoptosis, cell cycle checkpoint-activation and attempts to DNA repair or cell cycle arrest. The two ways of repairing DSBs are non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ repairs about 80% of the DSB and can be carried out in all parts of the cell cycle but is more prone to errors than HR. HR, in contrast, repairs without errors but can only operate in the S and G2 phase. SSB and base damage are much more frequent than DSB, but generally easier to repair since the unaffected DNA strand can be used as a template during single-strand break repair (SSBR) and base excision repair (BER).

Cells can die from irradiation in many different ways. *Apoptosis* is a highly controlled, "programmed", way of dying. It involves elevated levels of p53, release of cytochrome-c and eventually activation of caspases that will lead to apoptosis. This is a common way of cell-death post irradiation in neural progenitor cells (A. Fukuda, Fukuda, Jonsson, et al., 2005). *Senescence* is a permanent arrest in G0 caused by overload of DNA damage. The cells are still metabolically active but have lost their ability to proliferate. This is a

common response to irradiation in fibroblasts and endothelial cells. *Autophagy* represents an unspecific protein degradation process that involves lysosomes. However, the role in radiation-induced killing is unclear. *Necrosis* is a way of dying where disrupted transport of ions across the cell membrane leads to swelling and cell rupture, inducing an inflammatory response to the cellular components released. This seems not to be very common post irradiation. Mitotic catastrophe is when the cell does not manage to repair the DNA damage before entering mitosis. By time the cell will accumulate DNA aberrations and this will eventually lead to death. Mitotic catastrophe is a common path leading to cell death post irradiation in tumor cells due to dysfunctional repair systems, lost growth regulation and lost capacity of undergoing apoptosis (Gudkov & Komarova, 2003).

Different biological factors that modulate the effect of fractionated radiotherapy were concluded by Withers et al in the 1970s in the **4 R´s of radiotherapy**; *Repair, Reoxygenation, Redistribution* and *Repopulation* (Withers, 1975). *Repair* stands for the ability to repair DNA damage after irradiation, which is varying in different cell types. *Reoxygenation* involves the pattern of oxygenated tissue at the time of radiation. The oxygenation in a tumor differs with time. In low-LET the oxygenation of the tissue is very important for the effect. By giving fractionated doses, the probability to hit different parts of the tumor that are well oxygenated increases. *Redistribution* involves the cell cycling. Cells are most radiosensitive in late G2 and M. Directly after one fraction all cells will synchronize in less sensitive cell cycle phases and are thereby less sensitive to irradiation. Tumor cells usually cycle much faster than normal cells (at least cells in late reacting tissue). Because of that, the tumor cells will quickly redistribute again in all cell cycle phases and more tumor cells compared to normal cells, will be in a sensitive cell cycle phase at the next fraction. Hence a larger proportion of tumor cells than normal cells will be killed by each fraction. *Repopulation* means that the tumor cells will start to grow faster over time due to growth factors secreted when cells are dying. If the time span between two fractions becomes too long the tumor cells will have time to repopulate. In contrast, if the time span is too short there will not be enough time for repair of normal tissue. A fifth R –*Radiosensitivity* has also been proposed (Steel, McMillan, & Peacock, 1989). The fifth R is of special importance in the setting of this thesis, since childhood cancers often are more sensitive to radiation than cancer in adulthood (Bjork-Eriksson, West, Karlsson, & Mercke, 1998; Deacon, Peckham, & Steel, 1984).

The total dose and the dose per fraction are both highly associated to radiation induced cell death and to the biological effect of an organ or

organism. In radiotherapy usually the dose 2 Gy per fraction is used. To be able to compare the biological effectiveness of different treatment schedules, in which smaller or larger fractionated doses are delivered, a mathematical model has been developed. This model is referred to as the linear quadratic (LQ) model (Fowler, 1989). As a rule of thumb, this model might be adequate to use when comparing treatments of 1-6 Gy per fraction. For higher or lower doses, other mathematical models are recommended. In the LQ model the term α/β is used. This ratio describes the shape of the fractionation response. For late reacting normal tissue this value is usually low (0.5-6 Gy) and for early reacting tissue and tumor tissue this value is generally higher (7-20 Gy) (Kogel, 2009). In clinical practice typically α/β of 3 Gy is used for late reacting tissue and α/β of 10 Gy is used for tumor tissue.

High-LET radiation and low-LET radiation exert their detrimental effect to the cells slightly different. It is estimated that low-LET damage the tissue by direct effect to the DNA in 25% and indirect effect via formation of free radicals to 75%. In high-LET the ratio is the reversed. Since oxygen is important for fixation of the free radicals, low-LET is more dependent on the oxygen level in the tissue for effectiveness than high-LET. This relationship is described as oxygen enhancement ratio (OER) (Kogel, 2009).

To be able to estimate the biological effects of different radiation qualities (e.g. photons and light ions, including protons and carbon ions) the concept Relative Biological Effectiveness (RBE) is used. RBE is the ratio between *a dose of reference radiation* (for instance low-LET 250 kV X-rays) and *a dose of test radiation*, required to achieve the same biological effect. The biological effect can be measured by e.g. the dose for which 37% of cells remain clonogenic. Apart from radiation quality, RBE is also dependent on the dose per fraction, the dose rate (Gy per time) and the target tissue or endpoint. In current clinical practice, the RBE for high-energy photons is usually defined to be 1 and for protons usually considered to be 1.1, and for high-LET radiation in the range of 2-10 depending e.g. on dose per fraction.

1.1.2 Diagnostic and therapeutic radiation

Ionizing radiation is used for diagnostic purposes e.g. X-rays and computed tomography (CT) as well as for therapeutic radiation to treat cancer. The energy used for diagnostics (kV) is much lower than for therapeutic radiation (MV), in general. For comparison, the dose from a CT brain scan in a child is in the range of 28 – 44 mGy (Pearce et al., 2012) and the dose to a brain tumor can be up to 60 Gy.

In therapeutic radiation different delivery techniques can be used. In conventional therapy usually 1-3 fields of photon beams are used. In intensity modulated radiotherapy (IMRT) and intensity modulated arc therapy (IMAT) multiple fields of photon beams are used. In intensity modulated proton therapy (IMPT), multiple proton beams are typically used. The different techniques are illustrated in ***fig 1***. Protons deposit their energy in the tissue in a different way than photons. Whereas photons deliver a quite homogeneous dose throughout the penetrated tissue, the protons enter with a low dose and deliver almost all their energy at a certain depth, the so called Bragg peak. The depth of the Bragg peak is depending on the proton energy, and behind the Bragg peak almost no dose is deposited. Using proton therapy, one can typically better avoid irradiating radiation-sensitive organs.

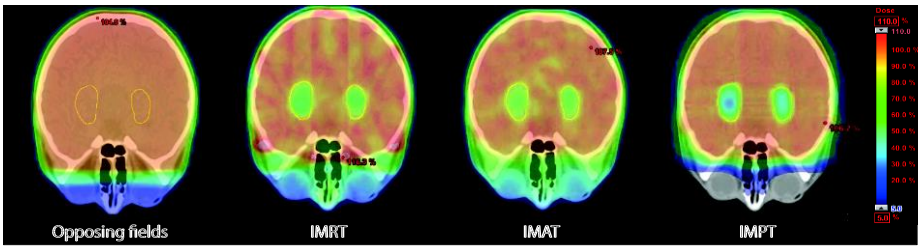

Figure 1. Color-wash showing absorbed radiation dose for (from left to right) opposing fields, IMRT, IMAT and IMPT, with the hippocampi outlined within the brain.

To define the parts that should be irradiated, several volumes are delineated. Gross tumor volume (GTV) is the known tumor or the operation cavity, defined by radiological techniques. Clinical target volume (CTV) is the volume of subclinical tumor and planning target volume (PTV) also comprises a safety volume for variations of set up, movements of the target and character of the beam.

1.1.3 TCP versus NTCP

In radiotherapy the goal is to eradicate (curative treatment) or reduce (palliative treatment) the number of cancer cells. However, for the individual to benefit from the treatment, the normal tissue has to be spared and uncompromised by radiation. Fortunately tumor cells are generally more sensitive to radiation than cells of the normal tissue (in regard to late side-effects) primarily due to quicker cell cycling and dysfunctional DNA repair systems. This difference is the prerequisite for a successful radiation treatment and usually referred to as the therapeutic ratio or window. The probability of curing the cancer is referred to as tumor control probability (TCP) and the risk of harming normal tissue is referred to as normal tissue complication probability (NTCP) (Bentzen et al., 2010; Burman, Kutcher, Emami, & Goitein, 1991; Emami et al., 1991; Holthausen, 1936). The relation between TCP and NTCP is illustrated in ***fig 2.*** To widen the therapeutic ratio, the NTCP curve must be moved

to the right (e.g. with radioprotecting drugs) and/or the TCP curve to the left (e.g. with radiosensitizing drugs). Another way of separating the curves is to use particle radiation with e.g. proton therapy.

The toxicity of the normal tissue can be divided in to early (acute) and late (chronic) effects. The early radiation effects hit rapidly proliferating cells like the mucosa. It appears days to weeks from start of the irradiation and usually heals completely. However, it may introduce patient discomfort and even lack of treatment compliance. Late sequelae appear months to years after irradiation and are typically irreversible and progressive over time (chronic), making them the most dose-limiting factor.

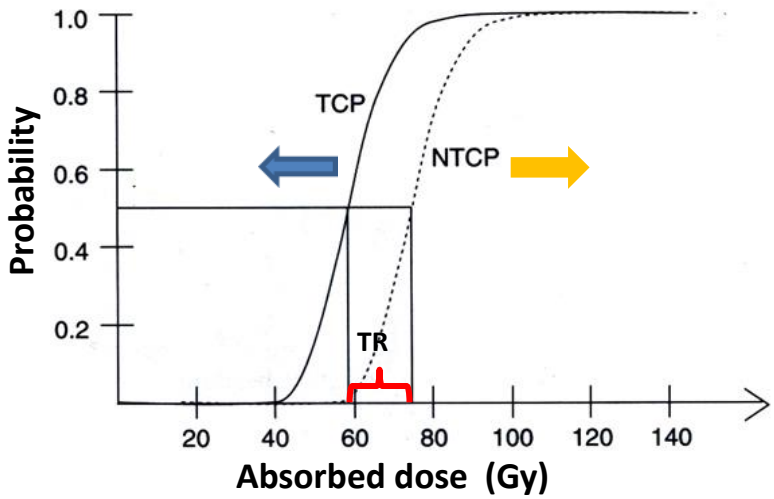

Figure 2. Picture showing the relationship between the tumor control probability (TCP) and the normal tissue complication probability (NTCP). The therapeutic ratio (TR) is the difference in absorbed dose between the curves. To widen the therapeutic ratio the TCP curve can be moved to the left or the NTCP curve moved to the right.

1.2 The neurogenic niches and irradiation

Neurogenesis in the adult mammalian brain was proposed by Altman already in the early 1960s, though met with skepticism by the scientific community (Altman, 1962). However, it was definitely demonstrated in rodents in the 1990s (T. D. Palmer, Takahashi, & Gage, 1997; Reynolds & Weiss, 1992). Peter Eriksson and coworkers in Gothenburg, Sweden, managed to prove that this was true also in humans (Eriksson et al., 1998). There are two major sites

of neurogenesis in the adult mammalian brain, the subventricular zone (SVZ) in the lateral wall of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Eriksson et al., 1998; T. D. Palmer et al., 1997). In rodents the SVZ neurons migrate along the rostral migratory stream (RMS) to the olfactory bulb (Lois & Alvarez-Buylla, 1994). This has also been demonstrated in humans (Curtis et al., 2007). Other studies have shown that there is no neurogenesis in the human olfactory bulb (Bergmann et al., 2012) and that the migratory path from the SVZ to the olfactory bulb in humans is only present for the first 6 months of life (Sanai et al., 2011). It has recently been suggested that neural progenitors migrate from the SVZ to the striatum throughout life in humans (Ernst et al., 2014).The functional role of neurons formed in the SVZ is still elusive though. In this thesis I have focused on the neurogenic niche of the hippocampus.

1.2.1 The neurogenic niche of the hippocampus

In the neurogenic niche of the hippocampus, there is an intimate interplay between many different cell types. They all have their specific role and the microenvironment created in the interplay of these cells seems to be critical for the function of the niche.

Adult stem cells are defined as cells capable of self-renewal in combination with the ability to give rise to at least two different cell types. The immediate progeny of stem cells are called progenitor cells. Neural progenitor cells are capable of self-renewal but to a more limited extent than stem cells and they can give rise to both neurons and macroglia (astrocytes and oligodendrocytes). Compared to stem cells, progenitor cells are more proliferative and hence they are termed transiently amplifying progenitor cells. The two cell types can be distinguished in vitro (Seaberg & van der Kooy, 2002) but in vivo the difference between true stem cells and progenitor cells is vague and the term *neural precursors* has therefore been proposed to cover both (Gerd Kempermann, 2011).

New neurons formed in the neurogenic niche migrate into the granule cell layer (GCL), where they mature and integrate into the neuronal network (G. Kempermann, Wiskott, & Gage, 2004). However, many of the newborn cells never manage to integrate and die (C. Zhao, Deng, & Gage, 2008). It was recently shown that 1400 new neurons (700 per hippocampus) are generated daily in humans (Spalding et al., 2013).

Neural precursors are in close contact with the vasculature in the neurogenic niche, implying that important factors may be secreted by the cells comprising the vessels, like pericytes and endothelial cells (T. D. Palmer, Willhoite, & Gage, 2000). For example vascular endothelial growth factor (VEGF) has been shown to promote proliferation in the SGZ, which can be congested by receptor blockage (Cao et al., 2004). The inside of the blood vessels are lined with endothelial cells, which are part of the blood-brain-barrier (BBB), restricting and regulating the passage of molecules and fluid in and out of the brain. Pericytes are perivascular mesenchymal cells stabilizing and supporting the endothelial cells (Lindahl, Johansson, Leveen, & Betsholtz, 1997). Pericyte loss is associated with increased permeability of the BBB, indicating the importance of pericytes (Armulik et al., 2010). Pericytes and endothelial cells also play an important role in modulating the passage of immune cells into the brain (Hurtado-Alvarado, Cabanas-Morales, & Gomez-Gonzalez, 2014).

Astrocytes are the star shaped macroglia of the brain. They used to be regarded only as supporting cells for neurons. We now know that they interplay with neurons in a critical way by modifying the micro environment as well as regulating neuronal synaptic function and plasticity (Bernardinelli, Muller, & Nikonenko, 2014). Later research has also illuminated their potential role as neural precursors in the form of radial glia-like progenitor cells (Alvarez-Buylla, Garcia-Verdugo, & Tramontin, 2001; Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997). Oligodendrocytes are the other type of macroglia and functions as structural support for neurons. Both astrocytes and oligodendrocytes can be derived from neural progenitor cells in the SGZ.

Microglia are the resident macrophages and antigen-presenting cells of the CNS. They originate from primitive macrophages in the yolk sac, from where they invade the CNS very early in life (Cuadros, Martin, Coltey, Almendros, & Navascues, 1993; Ginhoux et al., 2010). Under normal conditions the roles of microglia are homeostatic functions and immune surveillance (Cronk & Kipnis, 2013; Greter & Merad, 2013). During hippocampal neurogenesis many of the newborn neuroblasts undergo apoptosis and it is critical to have functional microglia to phagocytose these cells to maintain a healthy microenvironment (Sierra et al., 2010). The question of how adult microglia homeostasis is maintained, via recruitment of circulating monocytes and/or via microglia proliferation, is debated. This question was addressed in a recent review where the conclusion was that under physiological conditions it is predominantly through self-renewal (Greter & Merad, 2013). Microglia density is generally higher in gray matter compared to white and particularly dense in the hippocampus, olfactory telencephalon, basal ganglia and

substantia nigra (Lawson, Perry, Dri, & Gordon, 1990). Under physiological conditions microglia appear with a small cell body and long fine branches and are identified by the cell marker ionized calcium-binding adaptor molecule 1 (Iba-1) (Ito et al., 1998). Activated microglia express CD68 (ED-1), which is considered a general marker of activation of these cells (Damoiseaux et al., 1994) ***fig 3***.

In humans, macroglia represent more than 80% and microglia around 10% of the cells of the brain (Greter & Merad, 2013).

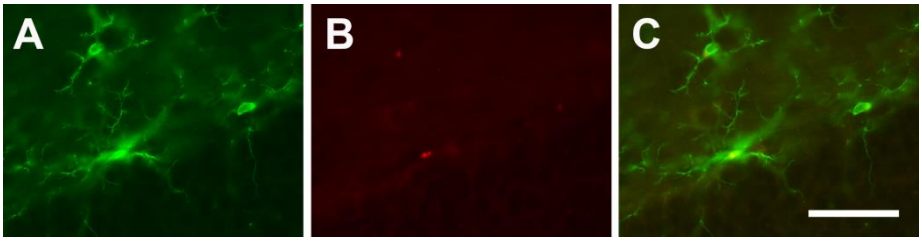

Figure 3: Microglia in the DG. (A) Iba-1, (B) CD-68, (C) A merged picture of Iba-1 and CD-68. Scalebar=50µm.

1.2.2 Effects of irradiation to the hippocampus

Irradiation of the hippocampus in rodents results in a dose and age dependent apoptosis of the cells in the SGZ, peaking around 12 hours, corresponding to a decrease in proliferating cells and new neurons (A. Fukuda, Fukuda, Swanpalmer, et al., 2005; H. Fukuda et al., 2004; Mizumatsu et al., 2003). In juvenile rodents, neural precursors have been demonstrated to recover from a moderate dose of radiation in the SVZ, but not in the SGZ, implying dissimilarities of the two major sites of neurogenesis (Hellstrom, Bjork-Eriksson, Blomgren, & Kuhn, 2009).

Damage to the microvasculature used to be considered as the main reason for cognitive dysfunction after CRT (O'Connor & Mayberg, 2000). However, the theory was challenged by a study showing that depletion of neural precursor cells after local brain irradiation was due to the radiation dose to the parenchyma (Otsuka et al., 2006). Still, capillary loss has been shown to precede cognitive impairment induced by radiotherapy (W. R. Brown et al., 2007). Also a disrupted neurovascular relationship has been proposed as a potential reason for hampered neurogenesis (Monje, Mizumatsu, Fike, & Palmer, 2002; T. D. Palmer et al., 2000), though questioned in the juvenile brain (Bostrom, Kalm, Karlsson, Hellstrom Erkenstam, & Blomgren, 2013). A reduction of endothelial cell density in the spinal cord after irradiation

doses of 19.5 to 50 Gy has been observed and this was associated with acute and transient increase of BBB permeability in young adult rats. However, there was no significant effect endothelial cell density in the spinal cord from doses up to 8 Gy (Li, Chen, Jain, Reilly, & Wong, 2004). Nevertheless, an intact BBB is important for maintaining a healthy microenvironment in the neurogenic niche.

A changed microenvironment after cranial irradiation of adult rodents has been reported to alter the fate of neural precursor cells from neural towards glial linage, partly due to chronic inflammation (Mizumatsu et al., 2003; Monje et al., 2002). By the use of the anti-inflammatory drug indomethacin, adult neurogenesis was restored (Monje, Toda, & Palmer, 2003). Support for chronic irradiation-induced inflammation in adult rodents has also been presented by others (Moravan, Olschowka, Williams, & O'Banion, 2011). In line with these preclinical data, radiation-induced inflammation, along with impaired neurogenesis was also demonstrated post mortem in humans treated for cancer (Monje et al., 2007). Cranial irradiation induces an up-regulation of pro-inflammatory mediators like tumor necrosis factor alpha (TNF-α), interleukin beta (IL-β) interleukin 6 (IL-6) and chemokine ligand 2 (CCL2 also known as MCP-1) in adult rodents (W. H. Lee, Sonntag, Mitschelen, Yan, & Lee, 2010; Monje et al., 2003).

However, in the juvenile rodent brain, there have been indications of a different inflammatory response to cranial radiation (Hellstrom et al., 2009; Kalm, Fukuda, et al., 2009) and this will be further discussed in this thesis.

1.3 The hippocampus, cognition and the effects of radiation

The importance of hippocampus in memory formation became evident in the 1950s when the patient Henry Molaison (HM) underwent an operation for epilepsy, where both hippocampi were removed (Scoville & Milner, 1957). Immediately after surgery, HM was unable to imprint any new memories but had an intact memory of the past. Neither his working memory nor his motor skills were affected. Since then the knowledge about memory and memory formation has expanded, though still not fully understood. It is now common knowledge that the memory impaired in HM was the *declarative memory*. This is the conscious part of the memory and can be divided into the *episodic* (memories of events) and the *semantic* (learning of facts, not connected to time or space) memory. The declarative memory is dependent on the hippocampus and its related structures. The hippocampus is also important

for spatial memory in humans (Maguire et al., 2000) and rats (Eichenbaum, Stewart, & Morris, 1990). In contrast, the *procedural* or *implicit memory*, which is unconscious and related to motor skills like walking, swimming or playing music, is not primarily related to hippocampal functions.

In rodents, adult neurogenesis has been shown to be important for hippocampal-dependent memory formation (Shors et al., 2001). Further, radiation-induced impaired neurogenesis is correlated to a decline in hippocampal dependent cognitive tests in rodents (Barlind, Karlsson, Bjork-Eriksson, Isgaard, & Blomgren, 2010; Kalm, Karlsson, Nilsson, & Blomgren, 2013; Karlsson et al., 2011; Madsen, Kristjansen, Bolwig, & Wortwein, 2003; Raber et al., 2004; Rola et al., 2004), which can be ameliorated by reestablishing neurogenesis, for example through physical exercise (Naylor et al., 2008), lithium treatment (Huo et al., 2012) and transplantation of new stem cells (Acharya et al., 2009).

In humans, several studies have demonstrated a correlation between absorbed dose to the brain and cognitive outcome (Grill et al., 1999; Kieffer-Renaux et al., 2000; Kieffer-Renaux et al., 2005; Mulhern et al., 1998; Silber et al., 1992) and more specifically an association between dose to the temporal lobe, including the hippocampus, and neurocognitive sequelae (Armstrong et al., 2010; Gondi, Hermann, Mehta, & Tome, 2012; Jalali et al., 2010; Redmond, Mahone, & Horska, 2013). In children, cognitive decline after irradiation is known to be more severe after treatment at younger age (Fouladi et al., 2005; Mulhern et al., 2005) and more severe in girls than boys (Lahteenmaki et al., 2007; Waber et al., 1990). It has also been shown to be progressive over time (S. L. Palmer et al., 2013). However, in adults the relation is partly opposite, with older age as a risk factor for cognitive decline after CRT (Brandes, Rigon, & Monfardini, 2000; Swennen et al., 2004).

Finally, CRT is associated with multiple cognitive dysfunctions including reduced attention, working memory, processing and executive functions as well as measurable decrease in IQ scores (Rodgers et al., 2013). As implied above, not all these dysfunctions can be explained by pathological conditions in the hippocampus after irradiation, illustrating the complexity of cognitive impairment after CRT. However, in this thesis I have chosen to focus on the role of the hippocampus.

2 AIM

The aim of this thesis was to characterize and modulate the effects of ionizing radiation to the neurogenic niche in the hippocampus of the juvenile brain, with the future goal of ameliorating cognitive deficit after cranial radiation in children cancer survivors.

2.1 Specific aims

I. To investigate if very low doses of ionizing radiation to the juvenile brain can cause cognitive deficit.

II. To evaluate if the dose to the hippocampus would be a better predictor of cognitive dysfunction than the dose to the anterior or the posterior part of the brain.

III. To explore and compare the reactions to irradiation in the juvenile and the adult rat brain.

IV. To investigate the possibility of lowering the hippocampal dose using modern techniques as compared to CRT and to estimate the benefit from this on cognitive performance.

V. To investigate the possibility of protecting the neurogenic niche of the hippocampus by modulating the effects of ionizing radiation with radioprotective substances.

3 MATERIAL AND METHODOLOGICAL CONSIDERATIONS

All individual papers contain detailed descriptions of materials and methods. In this section I have chosen to highlight some of them, to enable a discussion of pros and cons and also to elucidate the prerequisites and considerations leading us to perform the studies the way we did.

3.1 Paper I

A total of 3 860 men who had received radiation treatment for cutaneous hemangiomas in their childhood between 1950 and 1960 were collected. Cutaneous hemangioma is a benign malformation of the vessels in the skin and it usually disappears spontaneously with time (Callahan & Yoon, 2012). However, after radiation treatment, the hemangiomas disappeared promptly and hence some cases in the past were treated primarily for cosmetic reasons. The children were up to 18 months old when they received their first treatment and they were treated on average 1.3 times each. By correlating the absorbed radiation dose in different parts of the brain to test scores from the *Swedish Military Service Conscription Register* at the age of 18 years and the highest obtained educational level, we were able to assess the impact of low doses of radiation to the juvenile brain without having to contact and examine the whole study population. Considering our hypothesis being that even low-doses of irradiation could be harmful to the brain, it would have been an ethical dilemma to contact the participants, especially since many of them are not even aware that they have been treated. By accessing this data set of conscription tests, we were also able to analyze a very large set of cognitive testing data. Collecting such a data set only for the specific purpose of correlating radiation dose with cognition would not be feasible, given the sheer numbers of tests to perform. Obviously, the use of cognitive tests collected for another purpose might influence quality of the data. For example a fraction of the men performing the cognitive test might have been poorly motivated to perform well for various reasons, where avoiding conscription could be one. However, we do not see how that could systematically affect the data nor – therefore – compromise the conclusions reached by our study. All data was handled in accordance with the ethical approval obtained from the Regional Ethical Committee in Gothenburg, Sweden (Dnr 215-05, T 019-10). Since the hemangiomas could be located

anywhere on the body and some of them were so distant from the head that the treatment of them did not contribute to any dose at all to the brain, they could be used as internal controls, thereby minimizing the risk of selection bias influencing the data. Further, it was a large study-population, with long time to follow-up and limited loss of follow-up. The use of two independent parameters for assessment strengthened the conclusion. A limitation to the generalizability of the study was that it only included men and it its known that the cognitive function in girls is affected more by radiation than boys, at least at higher doses (Lahteenmaki et al., 2007; Waber et al., 1990). Further, no specific cognitive testing could be performed and psychological factors influencing cognitive outcome were not assessable.

3.2 Paper II and unpublished data

3.2.1 Animal model

Our model for radiation of rats was set up to mimic the clinical situation for children receiving CRT. We used a clinical treatment machine (Varian Clinac 600 C; Radiation Oncology Systems LLC, San Diego, CA) with 6 MV nominal photon energy and a dose rate of 2.1 Gy/min or 4 MV nominal photon energy and a dose rate of 2.3 Gy/min.

The animals were anesthetized with an intraperitoneal injection (i.p.) of tribromoethanol (50 mg/kg) and placed in a prone position on a polystyrene bed. To obtain a homogenous dose throughout the brain, the head was covered with a 1 cm tissue equivalent bolus material. The whole brain was irradiated using a field of 2 x 2 cm for postnatal day 9 (P9) rats and a 3.5 x 4 cm field for 6 months old rats, with a source-to-skin distance of 99.5 cm, to yield a single absorbed dose of 6 Gy to the whole brain. However, in the first pilot study of WR-1065 300mg/kg, only one hemisphere was irradiated (unpublished data). Control animals were anesthetized but not subjected to irradiation. After irradiation the pups were returned to their dams and sacrificed 6 hours, 7 days, 2 or 4 weeks post irradiation.

By using the LQ model, a single dose of 6 Gy corresponds approximately to 11 Gy given as 2 Gy fractions, assuming an α/β of 3. For comparison, children conditioned with total body irradiation before stem cell transplantation receive approximately 12 Gy. Many children get higher doses to the brain, e. g. medulloblastoma patients receive 23.4-36 Gy to the whole brain and further a boost of the tumor bed up to 54-55.8 Gy. Hence, the dose in our model is in the lower spectrum of the clinical setting.

The question of radiosensitivity of the rat brain compared to the human brain is still elusive. When our model was set up, different single doses that would cover the dose range commonly used for children in the clinical setting were applied; 4 Gy (EQD2~6) to 12Gy (EQD2~43Gy). Single doses of 4, 8 and 12 Gy all caused measurable detrimental effects to the tissue in rodents (H. Fukuda et al., 2004).

Our model was also set up to mimic the susceptibility of the brain irradiation in childhood. A direct comparison between the rodent and the human brain is very difficult since the development is not congruent in the two species. We used P9 rats which arguably corresponds to very young children (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). This age was chosen because the rat brain at that age is in a liner growth phase and hippocampal neurogenesis is prominent. In **paper II** the model was further modified to enable irradiation of adult rodents.

3.2.2 Labeling proliferating cells

Bromodeoxyuridine (BrdU) is a thymidine analog that can be incorporated into the DNA in dividing cells. The BrdU residues can be stained with specific antibodies and therefore this method is often used as a marker of proliferating cells. BrdU can be administered intraperitoneally, usually at several different time points to allow for more extensive labeling. However, BrdU has been shown to be toxic to neural precursors at higher doses, hence an adequate dose is important for assessment of for example neurogenesis (Caldwell, He, & Svendsen, 2005). BrdU is also a radiosensitizer, which means that the substance makes the cells more sensitive to irradiation (Sano, Sato, Hoshino, & Nagai, 1965). Hence, it is crucial not to administer it before radiation, if neurogenesis and normal tissue complications introduced by radiation alone are to be evaluated. We used a dose of 50 mg/kg, as an intraperitoneal injection once daily for four consecutive days (day 2-5 after irradiation).

3.2.3 Tissue preparation, immunohistochemistry and caspase activity assay

Animals were deeply anesthetized with sodium pentobarbital before being transcardially perfused with either 4% paraformaldehyde (unpublished data) or phosphate-buffered saline (PBS) (**paper II**). The rinsing of blood vessels is important for minimizing the amount of red blood cells left in the tissue, since they can disturb further measurements by autofluorescence. The brains were then fixed in paraformaldehyde or put in sucrose or flash frozen.

For immunohistochemistry (IHC) the brains were either paraffin embedded and cut coronally on a microtome into 5 µm thick sections, or infiltrated with 30% sucrose solution, frozen with dry ice and sagitally cut into 30 µm thick sections in a series of 12 on a sliding microtome (free floating). The paraffin sections were mounted on glass slides and stored at room temperature and the free floating sections were stored in a cryoprotection solution containing 25% ethylene glycol and 25% glycerol, at 4˚C.

The advantage of paraffin sections is that some antibodies have problems to penetrate the thick free-floating sections. If there is a big lesion in the brain due to trauma, the paraffin also stabilizes the brain during cutting.

The advantages of free-floating sections are that more cells can be assessed in the same section and that the full morphology of a cell can be seen, e. g. all the branches of a microglia would not be visible in a 5 µm thick section. It is also a much quicker procedure than paraffin preparation.

Staining procedure of paraffin sections (WR-1065 and N-acetylcysteine (unpublished data)): Deparaffinization with xylene/histofix and graded alcohol. Ten minutes boiling in 0.01 M citrate buffer and after cooling and rinsing in phosphate-buffered saline (PBS) 3x5 minutes, incubation with 0.1M Tris-HCl buffer (pH 7.5) containing 3% BSA for 30 minutes at room temperature. After rinsing in PBS 3x5 minutes, incubation with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture (Roche Diagnostics GmbH, Roche Applied Science, Nonnenwald 2, 82672 Penzberg, Germany) for 60 minutes in a humidified chamber at 37 ˚C. Rinsing with PBS 3 x 5 minutes and blocking endogenous peroxidase (POD) activity with 0.3 % H_2O_2 in methanol at room temperature for 10 minutes and then rinsing with PBS 3 x 5 minutes again. Incubation with POD 1:5 in buffer (0.1 M Tris-HCl, pH 7.5, containing 3% BSA) in a moist chamber at room temperature for 30 minutes. Rinsing with PBS 3x5 minutes and washing with 0.1 M sodium acetate (NaAc) solution, pH 6.0, for 5 minutes. Incubation with diaminobenzidine (DAB) for 10 minutes and then again washing with 0.1 M NaAc solution, pH 6.0, for 5 minutes. Finally, dehydration with graded alcohol and xylene/histofix.

Staining procedures and antibodies used in **paper II** are described in detail in the paper.

The caspase activity assay is described in detail in **paper II**. The advantage of this method compared to for example IHC with TUNEL is that it is a much quicker method to detect and quantify cell death. The disadvantage is the loss

of information on what cell types that are apoptotic and their location in the tissue.

3.2.4 Luminex

Luminex is a magnetic bead-based multiplex immunoassay. The beads are fluorescently labelled, each with a distinct color code to permit discrimination between different biomarkers. The capture antibodies of a desired biomarker are covalently coupled to the magnetic beads. When the antibodies/beads are mixed with the samples, the antibodies react with the biomarkers, the antigens of choice. After washing, a secondary biotinylated antibody for detection is added, to create a double sandwich. By adding streptavidin-phycoerythrin (SA-PE) conjugate, a final detection complex is formed. When the beads pass through the reader one-by-one (like in flow cytometry), the code for each biomarker is detected by a red laser (635 nm). At the same time a green laser (532 nm) excites the PE, which is detected by a photomultiplier tube and presented as the median fluorescence intensity. The concentration of the analyte bound to each bead is proportional to the median fluorescence intensity. The assay is very fast and allows for concomitant measurement of multiple cytokines in a small sample.

We used a prefabricated panel for quantification of 24 cytokines, chemokines and growth factors in hippocampal lysates (EPO, G-CSF, GM-CSF, GRO-KC, IFN-γ, IL-1α, IL-1β, MCP-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17α, IL-18, M-CSF, MIP-1α, MIP-3α, RANTES, TNF-α and VEGF). 5-7 animals per group were analyzed. Due to the high cost of the analysis unicate samples were assessed. The procedure is described in detail in **paper II**.

3.2.5 Stereology

Examining tissue by microscopy gives important information on morphology as well as the relationship between different cell types. However, quantification of cells in the microscope is very slow and time-consuming. To obtain unbiased quantitative data, stereology can be used to sample a representative fraction of cells of interest. We used StereoInvestigator (MicroBrightFields, Colchester, VT, USA), which is a computer-aided system coupled to a microscope with a motorized stage. On the computer, an area of interest and a grid with a counting frame can be defined. The computer will then randomly place the grid with the counting frame over the area of interest. By counting all the cells in the counting frame a representative sample will be obtained, that can then be used to estimate the total number of cells in the structure. Also the volume can be calculated

according to Cavalieri principle, V=SA x P x T, where V is the total volume, SA is the sum of area measurements, P is the inverse of the sampling fraction and T is the thickness of the sections.

This method was used for all cell counting and volume assessments in **paper II** and **unpublished data** in the amifostine project.

For **unpublished data** of WR-1065, cells were counted in one midsection of the hippocampus of each animal. For N-acetylcysteine counting of every fiftieth section was done throughout the hippocampus (paraffin sections 5 µm).

3.3 Papers III and IV

In **papers III and IV** we used historical data of MB patients in which we had access to both postoperative/preradiotherapy computed tomography (CT) and magnetic resonance (MR) scans. Adequate delineation of organs at risk (OARs) was performed by an experienced neuroradiologist. The patients were collected from Sweden (Gothenburg) and Denmark (Copenhagen) during a reasonably long time period (2002-2009), warranting not to include patients treated with different protocols, which could affect the outcome. The median age in **paper III** was 7.5 years and in **paper IV** 6 years, corresponding to the peak incidence of MB (5 years of age) (Fossati et al., 2009).

Papers III and IV are examples of so called *in silico planning comparative studies*. This kind of computer simulation is a first step to test the hypothesis before designing and moving further into clinical studies. It can also be used to form new hypothesis. *In silico planning studies* are frequently used for comparison between different techniques and it is a very important tool for development of new treatment techniques in radiotherapy.

3.4 Statistical analysis

The studies use very different statistics, because of different designs and different numbers of subjects in each group (=n). The statistical analyses for each study are described in detail under **papers I-V**. For results of unpublished data (WR-1065, amifostine and N-Acetylcystein) a one way-ANOVA with a Bonferroni post-hoc test was used.

4 RESULTS AND DISCUSSION

4.1 Characterizing the effects of ionizing radiation to the hippocampus

4.1.1 The effects of dose and time

The risk for neurocognitive dysfunction in children after therapeutic radiation of the brain has been known for decades. At what dose level the detrimental effects of irradiation to the brain starts is not clear, but support for a dose-response relationship is abundant. For example, one early study showed that patients treated with a dose of 36 Gy to the whole brain were estimated to score 8.2 points less on IQ testing than those treated with 24 Gy and 12.3 points less than those who received 18 Gy (Silber et al., 1992). Confirming this dose-dependence, children treated for MB with reduced brain doses of 23.4-25 Gy showed a better cognitive outcome than those treated with standard doses of 35-39.6 Gy (Grill et al., 1999; Mulhern et al., 1998; Mulhern et al., 2005). Further, Armstrong et al assessed the neuropsychological outcomes in adult survivors of childhood CNS malignances and found a dose-response relationship between cognitive dysfunction and increasing dose to the temporal lobe region (Armstrong et al., 2010). Survivors treated with CRT for acute lymphoblastic leukemia with 24 Gy, but not 18 Gy, did show reduced cognitive status and memory. However, the patients receiving 18 Gy were 1 year older when they received CRT and the evaluation was on average also performed almost 10 years earlier (25.6 years compared to 34.5 years), factors that could bias the results (Armstrong et al., 2013). In adults, a study of cognitive performance after postoperative pituitary radiotherapy with hippocampal doses of 2-14.2 Gy (to 70% of the volume) showed no difference in cognitive outcome compared to a non-irradiated patient group. (Brummelman et al., 2012). In children, not much is published on obvious cognitive decline after radiotherapy doses below 18 Gy and further studies in this spectrum are warranted.

Likewise, very low doses, within the range of diagnostic radiological examinations to the brain, are little explored. In rodent models, a single fraction of 1 Gy and less has been shown to impair neurogenesis (Mizumatsu et al., 2003; Tan, Rosenzweig, Jaffray, & Wojtowicz, 2011). A study on children receiving a mean brain dose of 1.3 Gy for treatment of tinea capitis at the mean age of 7.1 years, demonstrated a negative influence on cognitive performance (Ron, Modan, Floro, Harkedar, & Gurewitz, 1982). Further, a

Swedish population-based cohort study reported a reduced cognitive function as well as decreased proportion of high school attendance in boys receiving doses of less than 1 Gy at an early age for treatment of hemangiomas, as compared to controls (Hall et al., 2004). This study suggested a risk of cranial radiation within the range of diagnostic radiation like a few series of computed tomography (CT) of the brain. The results of this study prompted a debate on safety aspects of radiation for diagnostic purposes in children. We had the opportunity to analyze a similar, but larger, cohort of boys treated for hemangiomas. There was no overlap with the previous cohort. We deliberately designed our study in an analogous way to Hall et al., to enable comparisons between the two studies. With the knowledge of the hippocampus being of importance for cognitive outcome as discussed earlier, we also included the dose to the hippocampus in addition to the doses to the anterior and posterior brain, which were used in the study by Hall et al. Further we hypothesized that the hippocampal dose would better predict the cognitive outcome than the dose to the anterior or posterior brain. In our study, **paper I**, we could conclude that logical, technical and spatial test scores were not affected by radiation doses estimated for the study population. Verbal test scores showed a statistically significant trend for decreasing scores with increasing doses to the hippocampus ($p=0.005$) and to a lesser extent also to the anterior brain ($p=0.02$). The absolute mean difference between the zero dose and the highest dose category (median 680 mGy) was very small (0.64 stanine points) and it should be emphasized that it was the highest dose category, containing few subjects, that contributed the most to the dose-response relationship and the results hence should be interpreted with caution. The very small reduction in verbal test scores was not accompanied by any effect on the highest obtained educational level. Either the small measurable difference in verbal test scores could be compensated for somehow or the endpoint highest obtained education was to blunt to reveal any differences. Since the minimal differences in test scores were not reflected in scholastic performance we did not consider it to be clinically relevant. These results thus indicate that exposure of the brain to doses overlapping the range of a few diagnostic CT scans in infancy, might not be as detrimental as previously concluded by Hall et al. Still, a restricted use of CT scans is recommended, considering the risk of inducing secondary cancers (Pearce et al., 2012).

The phenomenon known as hyper radiation sensitivity, illustrating a higher sensitivity of many cell lines in the dose spectrum around 10 cGy is interesting in the setting of our study (Lambin, Marples, Fertil, Malaise, & Joiner, 1993). Cell cycle arrest is only triggered by doses exceeding about 10 cGy. This is thought to be the cause of hyper radiation sensitivity, since the

lower doses will not result in cell cycle arrest and hence not time for DNA repair. However, we could not observe this phenomenon in **paper I**.

Paper I expands the knowledge of effects of low-dose ionizing radiation to the juvenile brain and will hopefully contribute to future risk assessments in radiology.

The time-point of evaluation of cognitive decline is critical, since the cognitive decline is known to be progressive over time (S. L. Palmer et al., 2013; S. L. Palmer et al., 2003). In **paper I**, the children had their first radiation treatment before the age of 18 months and were examined for cognitive outcome at the age of 18 years. We believe that this was sufficient time for development of cognitive decline, which was in fact proven by the detectable small but significant decline in verbal stanine test scores.

4.1.2 The effects of age and gender

Younger age at treatment is a well-known risk factor for cognitive impairments after CRT in children (Danoff, Cowchock, Marquette, Mulgrew, & Kramer, 1982; Mulhern et al., 2005). Children below the age of 4 years very seldom get CRT in Sweden due to the high risk of developing severe cognitive deficits. In contrary, in adults, the risk of cognitive dysfunction after brain irradiation is increasing by age (Brandes et al., 2000; Swennen et al., 2004).

A similar age dependent pattern of sensitivity to cranial radiation is seen in rodents, with increased sensitivity of the juvenile brain in younger age and increased sensitivity of the adult brain in older age (A. Fukuda, Fukuda, Swanpalmer, et al., 2005; Lamproglou, Baillet, Boisserie, Mazeron, & Delattre, 2002; Schindler, Forbes, Robbins, & Riddle, 2008). The reason for this is probably multifactorial. One important factor could be the difference in inflammatory response to cranial radiation, which has been reported in the juvenile rodent brain compared to the adult (Kalm, Fukuda, et al., 2009; Kalm, Lannering, Bjork-Eriksson, & Blomgren, 2009; Mizumatsu et al., 2003; Monje et al., 2002; Moravan et al., 2011).

In **paper II** we addressed this question in a comparative study, showing different effects of cranial radiation in the juvenile and the adult rat hippocampus. As reported by others (Amrein, Isler, & Lipp, 2011; Bostrom et al., 2013; Kuhn, Dickinson-Anson, & Gage, 1996), we found a higher baseline level of BrdU-incorporation (proliferation) in the juvenile rats compared to adult ones. This explained the higher levels of acute cell death in the juvenile hippocampus compared to the adult following cranial radiation,

which has also been demonstrated previously (H. Fukuda et al., 2004; Roughton, Kalm, & Blomgren, 2012). In the juvenile rat, the progenitor pool is likely more important than in the adult, since the hippocampus has still not developed to its full size. This was also demonstrated in our study by the smaller volume of GCL assessed in the irradiated juvenile brain compared to control four weeks after irradiation, an effect which was not seen in the adult animals. For comparison with humans, a smaller hippocampus has also been demonstrated in children after CRT and further correlated to cognitive functions (Riggs et al., 2014).

In **paper II,** we also reported more activated (CD68+) microglia in adult compared to juvenile rats, regardless of irradiation. An increased activation of microglia with age in adult brains has been shown by others both in rodents (Schindler et al., 2008) and in humans (Sheng, Mrak, & Griffin, 1998). Microglia have many functions and play a vital role in maintaining homeostasis of the CNS. Apart from scanning and defending the CNS from infections, microglia are important in phagocytosis of dead cells, clearance of cell debris and trophic support for neurons and macroglia through secretion of growth factors (Ousman & Kubes, 2012). The phenotypes of activated microglia has been classified into M1 (e.g. iNOS+, CD16+) and M2 (e.g. CD206+), where M1 is the classical pro-inflammatory phenotype and M2 generally is considered to be a more neuroprotective, regeneration-promoting phenotype (Kohman & Rhodes, 2013). Hence microglia have the potential of being both good and bad for neurogenesis. In **paper II**, we assessed the general activation (CD68+) of microglia but did not phenotype them into M1 and M2. This would be interesting to do when further exploring the radiation-induced differences in the juvenile and adult rat brain. However, since activated microglia are the key regulators of the inflammatory response, it is tempting to speculate that inflammation plays a larger role in cognitive dysfunction in adult rats than in juvenile rats.

Further, in **paper II** we could demonstrate different cytokine/chemokine profiles in the juvenile rat hippocampus compared to the adult. One interesting finding was that the growth factor *macrophage colony stimulating factor* (M-CSF) was only transiently increased followed by a decrease in the juvenile rats, whereas it was constantly increased in adult rats, following irradiation. M-CSF increases the proliferation of microglia in both humans and rodents (Smith et al., 2013; Yamamoto, Nakajima, & Kohsaka, 2010) and could thereby influence the inflammatory response. Further, M-CSF interplays closely with *insulin like growth factor 1* (IGF-1) which is known to be very important for brain development (Gow, Sester, & Hume, 2010). Future studies are warranted to clarify the role of M-CSF and IGF-1 in

neuroinflammation and neurogenesis after cranial radiation. In **paper II** we could also confirm the paradoxical reactions of the pro-inflammatory cytokine *interleukin 6* (IL-6), which earlier has been reported to increase in adult rats (Monje et al., 2002) and decrease in juvenile rats (Kalm, Fukuda, et al., 2009) in response to cranial radiation. Further, we detected higher levels of chemokine (C-C motif) ligand 2 (CCL2) both before (in controls) and after radiation in adult rats than in juvenile rats. CCL2 has been shown to peak 6-12 hours post irradiation (Kalm, Fukuda, et al., 2009; S. W. Lee et al., 2013), which we could confirm. In the CNS, CCL2 is produced by microglia, astrocytes and endothelial cells (Luo et al., 1994) and it binds to the CCR2 receptor, which is expressed by monocytes/macrophages (Sica et al., 1997), microglia (Boddeke et al., 1999), neural precursor cells, as well as neurons in the hippocampus (Banisadr et al., 2002; Tran, Banisadr, Ren, Chenn, & Miller, 2007). It is known that CCL2/CCR2 signaling is important for recruitment of immune cells and neural progenitors to sites of neuroinflammation (Belmadani, Tran, Ren, & Miller, 2006; Fife, Huffnagle, Kuziel, & Karpus, 2000). In addition, two recent studies further elucidated the relationship between CCL2/CCR2 signaling post irradiation and the development of cognitive dysfunction. In CCR2-deficient mice, loss of hippocampal-dependent learning after irradiation was prevented through preserved neuronal plasticity rather than through altered neurogenesis (Belarbi, Jopson, Arellano, Fike, & Rosi, 2013) and in CCL2-deficient mice, a near-normal fraction of progenitor cells generated neurons after irradiation, i.e. the differentiation and maturation was preserved (S. W. Lee et al., 2013). How these results relate to our findings of higher CCL2 levels and higher numbers of activated microglia in adult than in juvenile brains remains to be further investigated.

In conclusion, we demonstrated different responses to cranial irradiation in the juvenile and the adult rodent brain in many aspects, suggesting different injury mechanisms responsible for the cognitive dysfunctions seen after CRT. We propose a combination of massive apoptosis resulting in ablation of the neural progenitor pool and a decreased production of growth factors, rather than sustained inflammation, to be responsible for the cognitive impairments seen after cranial radiation in the juvenile rodent brain. Further research is needed to investigate if this holds true also for humans. If so, this will demand different strategies to ameliorate the cognitive deficits after CRT in children and adults.

The importance of age in cognitive decline after cranial radiation was also reflected in **paper I**. In spite of the very low dose, we were able to detect a very small but significant difference in verbal test scores, most likely because

the radiation was given at a very early age (median 5 five months for the first treatment).

In a juvenile rodent model, females have been shown to be more sensitive to cranial radiation than males, both in terms of white matter growth, neurogenesis and behavior (Roughton, Bostrom, Kalm, & Blomgren, 2013; Roughton et al., 2012). Also in children, CRT impairs hippocampal development more in girls than boys (Nagel et al., 2004) and cognitive functions are known to be more affected in girls than boys post cranial irradiation (Lahteenmaki et al., 2007; Waber et al., 1990). The knowledge of different radiation sensitivity between genders is very important to bear in mind in future studies. In this respect **paper I** is limited, since only boys/men were included. This was due to the study design, discussed in the section of material and methodological considerations, but nevertheless a similar kind of study on girls would be of great interest for future guidelines in diagnostic radiation. Likewise **paper II** is based only on male rats and hence has some limitations. Mixing genders in the experimental design could obscure the outcome, according to the known differences in sensitivity to radiation between genders. The preferable way of dealing with the problem would be to perform studies of both genders in parallel.

4.2 Modulation

4.2.1 Technical

The theoretical foundation for sparing the hippocampus from irradiation during CRT in order to ameliorate later cognitive dysfunctions has been discussed in the introduction. The question as to whether this was feasible was addressed in **papers III and IV**. **Paper III** is a retrospective *in silico planning comparative study*, focusing on the component of adjuvant cranial whole brain irradiation during treatment of MB in children. In this study we investigated the possibility of sparing the neurogenic niches of the hippocampus and the SVZ from radiation with different radiation techniques, without compromising the CTV. Four different techniques were compared; standard opposing fields, IMRT, IMAT and IMPT. We could show that the CTV target was least affected by the IMPT technique, followed by the IMRT- and lastly the IMAT technique. No sparing was possible with the standard opposing field technique. This implied that the use of protons would offer the best possibility to spare the neurogenic niches from radiation during treatment. In **paper III**, we deliberately did not consider the dose contribution to the neurogenic niches from the boost treatment of the posterior fossa in the MB treatment course. However, in reality this could be

important depending on several factors like the treatment technique, the size and location of the tumor and the CTV and corresponding PTV size, which was further explored in **paper IV**. In this paper we did not include SVZ as an organ at risk (OAR) for several reasons. The role of SVZ neurogenesis in cognition is unclear and a recent prospective study did not see any correlation between dose to the SVZ and neurocognitive outcome (Redmond et al., 2013). Since IMAT had offered the lower sparing possibility than IMRT and IMPT (**paper III)**, we chose not to include IMAT in **paper IV**. In **paper IV** we could show, not unexpectedly, that a larger boost target volume gave rise to a higher hippocampal dose. More interesting was that the use of different techniques influenced the dose to the hippocampus to a much higher extent, than the use of smaller treatment volumes. Also in this study protons showed the best potential of sparing the hippocampus from radiation. We also found a correlation between the mean hippocampal dose and the distance between the closest point of PTV and the center of the hippocampus (average between left and right) for each of the radiation techniques, standard opposing fields, IMRT and IMPT respectively. This correlation was abundant up to around 1.5-2 cm of distance. For larger distances the delivering technique did not make much impact on the hippocampal dose. By measuring this distance for a MB patient, the mean hippocampal dose could be estimated for each technique and hence be a guide for choice of technique before start of treatment planning.

Our dosimetric data were put into a clinical perspective by estimating the potential cognitive benefits from sparing the neurogenic niches. To be able to perform these estimations, we derived a mathematical model based on dose-response data from a large retrospective cohort study on neuropsychological outcome in adult survivors of CNS malignances (Armstrong et al., 2010). In **paper III**, we estimated the risk for developing memory impairment after a dose of 23.4 Gy of CRT to be 47%, 41%, 44% and 33% with opposing fields, IMRT, IMAT and IMPT, respectively. Hence the hippocampal sparing techniques were estimated to lower the risk of cognitive impairment after CRT, with protons offering the largest reduction. Similarly the proton technique was estimated to offer the best opportunity of decreasing the risk of cognitive dysfunction in **paper IV**. Notably, in our model we made the assumption that sparing the hippocampus was equivalent with sparing the temporal lobe. However, since the hippocampus is located in the temporal lobe and has been demonstrated to be important for memory, it seems reasonable to hypothesize that the hippocampus is the main critical structure for radiation-induced cognitive dysfunction, as already pointed out in the introduction. Sparing the entire lobe in treatment of MB would likely yield an unacceptable high risk of relapse, which still has to be considered and

possibly be investigated in more detail if hippocampal sparing radiation therapy should be a clinical reality. Further, our estimates are limited by the uncertainties in the study of Armstrong et al. (Armstrong et al., 2010)

Hippocampal sparing radiation therapy without compromising target coverage by the use of IMRT or tomotherapy has been demonstrated previously (Gondi et al., 2010; Gutierrez et al., 2007; Marsh, Godbole, Diaz, Gielda, & Turian, 2011; Redmond et al., 2011). Our studies were based on children (**papers III and IV)** and to our knowledge this has not been done before. Further we included IMPT in our analysis, thereby expanding the current knowledge. The importance of our contribution in this field is well illustrated by the fact that IMPT actually is the technique that offers the best option for sparing neurogenic niche of the hippocampus.

To further emphasize the relevance of sparing the hippocampus, we could also in **paper I** confirm that the hippocampal dose was a better predictor of late cognitive side effects than the anterior or the posterior brain doses, which has also been suggested for doses in the spectrum of radiotherapy (Redmond et al., 2013). This highlights the importance of defining the hippocampus as an OAR, to be further considered and evaluated in future clinical studies.

Hyperfractionation means that lower doses are delivered each time but more often, for example 1 Gy twice daily, instead of 2 Gy once daily. This approach was tested for average risk MB patients and resulted in preserved cognitive functions assessed up until 2 years after completion of radiation, tough, the study was small and the time to follow-up was premature, considering the progressive nature cognitive dysfunction (Gupta et al., 2012). The PNET4 study showed that hyperfractionation was associated with better executive functions, however not mirrored in better health status, behavior or quality of life assessed at median 5.8 years after diagnosis (Kennedy et al., 2014). Hyperfractionation schedules are more resource demanding than standard regimens and with the modest improvements on cognitive outcomes reported so far, there might be more favorable technical solutions, like IMPT, which on the other hand is more expensive.

To validate the estimated benefits in cognitive functions from sparing the hippocampus from radiation, a prospective study is needed. We hope that **papers III and IV** will constitute the basis for, as well as inspire to, such future studies.

4.2.2 Biological

Amifostine (WR-2721) and WR-1065

Amifostine is a clinically available radio protective compound that is used for protection of salivary glands during irradiation of head- and neck cancer among other diagnosis (Brizel et al., 2000). It is a sulfhydryl-containing agent, readily converted to its active metabolite WR-1065 by alkaline phosphatases and then taken up by cells. The conversion to WR-1065 is less effective in malignant tumors due to the lower amount of alkaline phosphatase in tumors and also the uptake of WR-1065 into tumor cells is lower (Calabro-Jones, Aguilera, Ward, Smoluk, & Fahey, 1988). Amifostine protects against radiation in the tissue mainly by scavenging free radicals and stabilizing of DNA (Hospers, Eisenhauer, & de Vries, 1999). Under normal physiological conditions, it has been debated whether amifostine and WR-1065 can pass through the BBB (Millar, McElwain, Clutterbuck, & Wist, 1982; Spence et al., 1986; Utley, Seaver, Newton, & Fahey, 1984) and efforts to increase the passage have been made by modulating the BBB, for example through the use of hypertonic arabinose (Lamperti et al., 1988). Amifostine has been reported to protect vessels from radiation-induced damage in adult rats and chickens (Giannopoulou, Katsoris, Parthymou, Kardamakis, & Papadimitriou, 2002; Plotnikova, Levitman, Shaposhnikova, Koshevoj, & Eidus, 1988). In young adult rodents, amifostine was reported to ameliorate cognitive impairment and reduce apoptotic cell death after cranial irradiation (Lamproglou et al., 2003; H. J. Lee et al., 2010). To investigate the potential radioprotective effect in the juvenile brain, we performed a pilot study on P9 Wistar rats. WR-1065 (300 mg/kg) was administered as an i.p. injection 30 minutes prior to a single fraction of 6 Gy. The animals were sacrificed 6 hours post radiation for evaluation of apoptosis in the SGZ. At this time point 60% of the animals had already died from toxicity of the drug, although similar dose-levels of the drug had been used by others (Plotnikova et al., 1988). However, we could show a significant protection from WR-1065 ($p<0.001$), ***fig 4*** (unpublished data). When we repeated the experiment with lower doses of WR-1065, no protection from the drug was observed, ***fig 4*** (unpublished data). We speculate that because of the low penetrance of WR-1065 through the BBB, the lower doses did not reach a sufficient concentration inside the brain to protect it from the radiation. The reason why Lamproglou et al could show radiation protection from lower concentrations of amifostine might be due to their fractionated delivery of radiation (3 Gy x 10), thereby inducing a more permeable BBB. The microenvironment of the neurogenic niche is dependent on preserved microvessels, which have been reported to be protected from radiation-induced damage by amifostine (Plotnikova et al., 1988). By measuring the volume of the hippocampal GCL

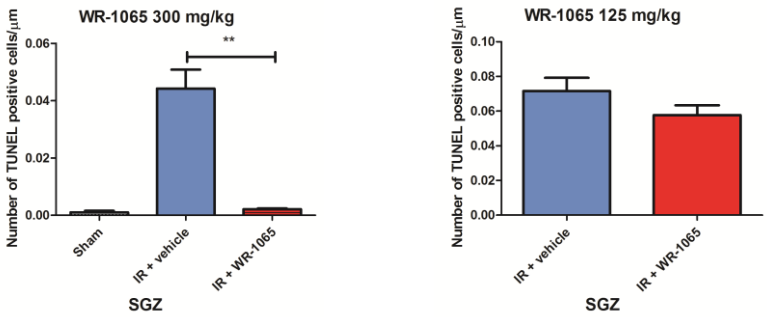

*Figure 4. The mean number of TUNEL positive cells (apoptotic cells) /µm in the subgranular cell zone (SGZ), 6 hours after cranial irradiation of 6 Gy. Treatment with WR-1065 300 mg/kg n=3-6 in the groups (left) and WR-1065 125 mg/kg n=7-10 in the groups (right). Error bars show SEM. **p<0.01.*

2 weeks after cranial radiation, as an indirect measure of functioning neurogenesis, we hypothesized that the total potential of amifostine as a protector of the neurogenic niche during irradiation could be evaluated. This endpoint would not be dependent on BBB penetrance, but rather the effect of the microenvironment associated to the vessels. This pilot study was performed in a similar way to the study mentioned above but instead amifostine (200 mg/kg) was administered 15 minutes before radiation. The use of amifostine instead of WR-1065, was due to the inability of purchasing this substance at the time beeing. As can be seen in ***fig 5*** (unpublished data), we did not observe any protection from amifostine.

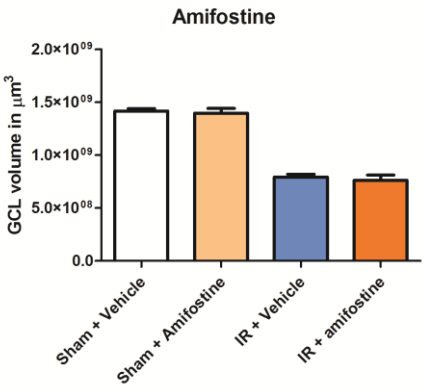

Figure 5. Volume of the granule cell layer (GCL) measured 2 weeks after cranial irradiation of 6 Gy ± treatment with amifostine. n=5-6 in the groups. Error bars show SEM. No statistical significant treatment effect was found.

In conclusion we were not able to show any radiation protection from amifostine or WR-1065 to the juvenile rat brain by using tolerable doses of the drug and relevant clinical doses of radiation.

N-acetylcysteine (NAC)

NAC is a free radical scavenger and also acts as a precursor of glutathione (GSH), a natural free radical scavenger in the body. It readily penetrates the BBB and has been reported effective in reversing memory impairment and brain oxidative stress in aged rodents (Farr et al., 2003). In other trauma models of the brain, believed to be dependent on damage from formation of free radicals, like head trauma and hypoxia/ischemia, NAC has been reported to be of protective value (Hicdonmez, Kanter, Tiryaki, Parsak, & Cobanoglu, 2006; Khan et al., 2004; Wang et al., 2007). We designed a study with similar doses and timing of administration of NAC that had been used in those injury models. NAC was given ip 5 minutes prior to a single fraction of 6 Gy, as described in the section of material and methodological considerations, at a dose of 100 mg/kg, 200 mg/kg, 300 mg/kg, or 200 mg/kg 5minutes + 2 hours before radiation. The results are shown in ***fig 6*** (unpublished data).

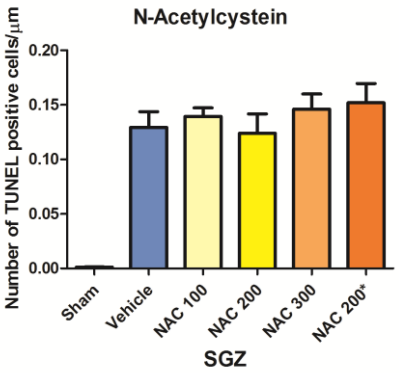

Figure 6. The mean number of TUNEL positive cells (apoptotic cells) /µm in the subgranular cell zone (SGZ), 6 hours after cranial irradiation of 6 Gy ± treatment with NAC. n=4-5 in the groups. NAC 200 means NAC 200 mg/kg 2 hours + 5 minutes before radiation. Error bars show SEM. No statistical significant treatment effect was found.*

In our setting, NAC offered no protection to the juvenile brain from radiation, as measured by apoptosis in the SGZ, 6 hours after radiation. One reason for the different outcome could be that the scavenging potential of NAC is not enough to prohibit the massive production of free radicals during radiation compared to the other injury models or that NAC has other effects not yet fully elucidated. Though, a known effect of NAC is the ability to inhibit

production of IL-1, TNF-α and NFκB, illustrated in a brain trauma model (Chen, Shi, Hu, & Hang, 2008). Considering the role of these factors in inflammation, which is also important for cognitive dysfunction in the adult brain (Mizumatsu et al., 2003; Monje et al., 2002; Moravan et al., 2011), NAC could still be a potential candidate for radioprotection. Chronic administration of NAC after cranial irradiation would probably be more efficient in hampering radiation-induced inflammation, than acute administration of NAC as in our study. However, a recent study in rodents, indicating a potential tumor-enhancing effect of NAC, raises concerns to further investigate the role of NAC as a potential radio protecting agent, at least in the setting of radiotherapy (Sayin et al., 2014).

Other substances of interest for ameliorating cognitive impairment of adult rodents after cranial irradiation are peroxisomal proliferator-activated receptor (PPAR)-agonists (Ramanan et al., 2009; W. Zhao et al., 2007) and renin angiotensin system (RAS) blockers (T. C. Lee et al., 2012; Robbins et al., 2009). Their protective effect has been reported to act by modulation of synaptic plasticity and neuroinflammation, independent of protection of neurogenesis (Greene-Schloesser et al., 2013). PPAR agonists and RAS blockers do not protect tumor cells, and even inhibits tumor growth (George, Thomas, & Hannan, 2010; Grommes, Landreth, & Heneka, 2004), making them very attractive as potential radioprotectors during radiotherapy. The role of PPAR agonists and RAS blockers are yet to be proved in the protection of the juvenile brain, especially considering our results in **paper II**, suggesting other mechanisms than chronic inflammation to be important for the cognitive dysfunction seen after CRT. Human phase I/II studies on these substances are ongoing. Lithium is also a promising substance that has been shown to reduce apoptosis of neural precursor cells in the hippocampus, as well as ameliorate cognitive impairments of juvenile mice and rats after cranial radiation (Huo et al., 2012; Yazlovitskaya et al., 2006). The substances discussed in this section are examples that are promising. Apart from these substances, numerous others have been tried with variable success.

So far, treatment of cognitive impairment after CRT in humans has been of limited benefit and more of symptomatic than prophylactic nature. For example methylphenidate and donepezil has been used to improve cognition, function and mood in patients post CRT (Meyers, Weitzner, Valentine, & Levin, 1998; Shaw et al., 2006). Recently a larger placebo-controlled, double-blind, randomized trial on memantine was performed by the Radiation Therapy Oncology Group (RTOG). Memantine is an NMDA receptor antagonist which is in use for treatment of vascular dementia,

especially small vessel disease. Although the primary endpoint (delayed recall at 24 weeks) was not reached, the study successfully showed a better cognitive function over time for patients treated with memantine than placebo after whole brain radiotherapy (P. D. Brown et al., 2013). The role of memantine in treatment of children remains to be investigated.

5 CONCLUDING REMARKS

In response to specific aims

I. We did not find an effect on highest obtained education when we investigated the risk of cognitive dysfunctions at the age of 18 years after exposure to low-doses of cranial radiation in infancy. There was no decrease in logical, spatial or technical test scores after ionizing radiation doses up to the highest dose category (median 680 mGy). Verbal tests displayed a significant trend for decreasing scores with increasing doses to the hippocampus, though the absolute mean difference between the zero dose and the highest dose category was very small, only 0.64 stanine points. For comparison this corresponds to 4.8 points by using a scale with a mean of 100 and a standard deviation of 15, like the WAIS and other IQ-tests. Further the significance was highly dependent on the highest dose category, containing few subjects, warranting cautiousness before jumping into conclusions about safety aspects. Hence we concluded that there was no clinically relevant effect on cognitive outcomes after low doses of radiation to the infant brain. Thus our results indicate that doses equivalent to a few diagnostic CT scans to the brain in infancy might not be as harmful as suggested in previous studies. Our data included only boys, and therefore further studies on girls are warranted.

II. We found that the hippocampal dose better predicted the outcome of late cognitive side effects than the doses to the anterior or the posterior brain. This further inspires our struggle to define the hippocampus as an organ at risk that should always be delineated during radiotherapy of the brain and adjacent target volumes.

III. We found essentially different reactions to irradiation in the juvenile hippocampus compared to the adult hippocampus in rats, regarding levels of apoptosis, specific cytokines, chemokines and growth factors as well as density of resident microglia and number of activated microglia. This implies different injury mechanisms in juvenile and adult rats after

cranial irradiation. However, the different mechanisms remain to be further investigated and also have to be demonstrated in children to be of clinical interest. If they can be verified in humans, our results open up for different protection as well as rehabilitation strategies for children and adult patients in the future.

IV. We concluded that sparing of the hippocampus in children receiving cranial radiotherapy is feasible by the use of modern and more conformal techniques. In comparison with opposing fields, IMRT and IMAT, IMPT offered the best option. We also found the choice of technique to have greater impact on the dose to the hippocampus, than the size of margins of the tumor boost, even more emphasizing the potential gain from more modern radiation therapy techniques. Further we predict that sparing of the hippocampus would ameliorate the cognitive deficits seen after CRT. However, a future prospective study is needed to confirm our estimates.

V. We found no protection of the neurogenic niche from cranial radiation by the use of amifostine, WR-1065 or N-Acetylcystein in tolerable doses, measured as decreased apoptosis in the SGZ or growth of the GCL.

6 FUTURE PERSPECTIVES

It would be interesting to further explore the differences between radiation-induced reactions in the juvenile- and adult rodent brain. A special focus will be on the growth factors, which have the potential of playing a large role in restoration of neurogenesis after radiotherapy. The multiple functions of CCL2 in the setting of radiotherapy make it an exciting candidate for further studies. Further, it is of great interest to find ways of measuring the above-mentioned cytokines in children and adult, in order to verify the animal data.

A medical center has recently been set up by pediatric oncologists for evaluation of late toxicity induced by cancer treatment in childhood. This is a perfect foundation for future prospective studies. By defining the hippocampus as an OAR in the clinical routine for all patients receiving cranial irradiation, dose-data would be much easier to retrieve. These data could be combined with psychological testing, which would result in expanded knowledge on the dose-response relationship in cognitive decline. It is of great interest to expand the knowledge of cognitive deficits in the range of 1 to 18 Gy, to be able to define a desired dose-level to the hippocampus during hippocampal sparing radiotherapy. In collaboration with other professions like physicists, radiologists, clinical physiologists, clinical chemists and pediatric oncologists, it could be possible to work out additional non-invasive measurements of brain dysfunctions, to be correlated to cognitive outcome.

For MB, it would be interesting to investigate the radiation-sensitivity of different subtypes, in order to pick a suitable group for a prospective study on hippocampal sparing radiotherapy in the future. This could be done by in-vitro analysis of differences in radiosensitivity between different MB subgroups as a first step. Also the International Society of Paediatric Oncology (SIOP) PNET 5 study will hopefully generate some data. More data on patterns of failure are also requested.

A new proton facility, Skandionkliniken, will open in 2015. Here spot-scanned protons will be available. It is owned by the seven university hospitals in Sweden. Since more or less all children will potentially benefit from proton- compared to photon radiotherapy, all children from the whole country will be treated there. This will offer great possibilities for collaborations and hopefully it will be an excellent forum for prospective clinical studies.

The different sensitivity to radiation between girls and boys needs to be further explored in order to tailor the strategies of protection against cognitive decline.

Being a clinician, I will probably be more involved in clinical trials than in experimental projects in the future. However, I appreciate the knowledge I got from working with preclinical research very much. My hope is to be a bridge-builder between the preclinical- and the clinical world in the future, since their cross-talk is essential for good and interesting research.

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