SCIENCE, ENGINEERING & TECHNOLOGY
ARENBERG DOCTORAL SCHOOL
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Mid Term Written Report

Name of the PD researcher:	Kai Craenen
Student Number:	0599553
Title of the PhD:	The effects of ionizing radiation and folic acid on neural tube closure, eye development and cognitive functions in mice
Name of the Supervisor:	Prof. Dr. Godelieve Moons

This report should include the following elements:

- State of affairs in relation to the research: results obtained and activities planned;
- Publication of the research results and/or mentions at meetings/conferences
  - you will need to be main author of at least 1 accepted international publication
  - you will need to have participated actively in an international conference
  - you will need to have presented at least 2 seminars
- Educational supervision already conducted and planned;
- Formal course units already followed and pending;
  - you will need to have followed formal training to an equivalent of 6 ECTS credits
  - of which at least 2 ECTS credits have been dedicated to skills courses
- General time schedule.

This is a good opportunity to run through the items in the doctoral training diary and identify potential problems!

The Supervisory Committee needs to approve this report by sending an email to the doctoral researcher or by signing the attached signature sheet:

The PhD researcher needs to upload this document (including approval emails if applicable) via his/her KULoket tool.

Written report by the PhD researcher:

1. Problem

Over the past decades, research on the effects of radiation on biological specimens (cells and animal models) has enhanced our knowledge on a number of industrial, clinical and research-oriented applications. Besides the clear advantageous socio-economical impact of ionizing radiation in the medical sector, it is important to note the related detrimental health effects as well. Next to its well-known role in cancer induction, a number of studies have shown non-cancer effects after irradiation, such as cardiovascular and cognitive detriment. For instance, irradiation of the embryo during early development (e.g. neurulation) can have a negative impact on proper development and thus lead to so-called birth-defects or congenital abnormalities, which are often associated with structural and functional aspects of the central nervous system (CNS)[1–8]. This PhD project will focus specifically on rostrally oriented neural tube defects (NTDs), eye defects (EDs) and abnormal behavior/vision in mice resulting from prenatal radiation exposure.

After the Chernobyl accident in 1986, large quantities of the radioactive isotope Cesium-137 were deposited throughout Ukraine with higher amounts found in the northern half of the country. The years following the disaster, an increase in NTD and ED occurrence was observed in a number of contaminated regions. It was suggested that this phenomenon originated from extensive maternal Cesium-137 exposure[5]. However, it should be noted that no clear-cut NTD increase was observed in western Europe the years following the disaster[9,10]. So far, increased occurrence of malformations and sub-optimal cognitive functioning in descendants exposed *in utero* to radiation are often reported in the literature about the Hiroshima and Nagasaki A-bombings[11]. Thus, suggesting a causative role of ionizing radiation in the increased prevalence of NTDs, EDs and cognitive malfunctioning. Currently, an important issue is the exposure of women during the earliest stages of pregnancy to ionizing radiation in the context of diagnostic or interventional procedures. The possibility of unexpected pregnancy between radiotherapy sessions and subsequent exposure of the embryo to low doses of radiation is a matter of concern. It would be of interest to assess exactly how prenatal ionizing radiation exposure may induce these congenital abnormalities. The discovery of afflicted cellular mechanisms may furthermore lead to the use of new compounds for the prevention and treatment of these defects.

Folic acid (FA) and folates have a known track-record in ameliorating birth defect prevalence: it is thus often advised for pregnant women to consume additional FA during gestation. Although FA food fortification is not common practice in any of the EU member states, the consumption of food supplements containing the compound by pregnant women has been advised by various EU governments in the past. Important to note is that a number of previous experimental findings have suggested a positive effect of FA on cognitive functioning if administered postnatally[12–16]. Furthermore, a link between abnormal folic acid metabolism and irradiation events has been reported in the past[17–19]. It is then imperative to determine the efficacy of postnatal FA administration in improving the standard of life after prenatal exposure to radiation.

2. Strategic aims

Aim 1.1.: Mechanisms of prenatal radiation-induced NTDs and EDs

In the first aim, the development of NTDs and EDs is assessed after prenatal irradiation at embryonic day (E)7.5, using different doses of X-rays (up to 1.0 Gy). To this end, (immuno)histochemistry (IHC) and subsequent morphometric analyses are being performed at different developmental stages following radiation exposure. In addition, to unravel underlying mechanisms responsible for radiation-induced NTDs and EDs, a series of biotechnological techniques such as microarray, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) are being employed.

Aim 1.2.: Assessing the efficacy of FA in ameliorating radiation-induced prenatal anomalies

A possible link between prenatal irradiation and FA activity is being evaluated. FA will be administered during pregnancy and possible amelioration in radiation-induced anomalies at the height of the rostral NT and embryonic eye will be assessed. Custom made food is to be used as the vehicle to supply the animals with high FA intake. Initially, the FA content of the food has been validated with LCMS both directly by the manufacturer and indirectly, using LCMS of plasma/milk/brain samples of fortified pregnant mice. In addition, the impact of irradiation at E7.5 on the plasma content of various FA metabolites will be assessed, to better understand a possible link between X-irradiation and folate bioavailability. After food validation, a macroscopic study will be initiated where the prevalence of NTDs and EDs will be assessed in FA fortified pregnant females.

Aim 2.1.: Role of prenatal ionizing radiation in the development of postnatal persistent defects

The second aim will shift the focus towards postnatal anomalies and adult behavioral defects as a result of early prenatal exposure to radiation. Analyses will be performed both at the behavioral and morphological level. Animals irradiated at E7.5 will be subjected to an array of visual and behavioral tests to assess both visual acuity as well as cognition, learning and memory. The visual acuity tests will be performed first, to assess differences in visual ability that might be due to an abnormal, radiation-induced, eye development. In addition, Magnetic Resonance Imaging (MRI) and IHC will be employed to determine detailed eye and brain morphology, whereas optical coherence tomography (OCT) will be employed to assess the structure of the retina.

Aim 2.2.: Assessing the efficacy of FA in ameliorating radiation-induced prenatal anomalies

Finally, FA will be administered pre- and/or postnatally to assess its efficacy in counteracting radiation-induced anomalous adult eye and brain morphology and a possible behavioral decline. To keep in mind the prenatal experiments, FA will be implemented in the experimental design only if its beneficial role in preventing X-ray induced congenital defects has been established (see Aim 1.2.).

3. Results obtained during the first two years

3.1. Prenatal studies

3.1.1. Macroscopic study

Data obtained at the SCK●CEN Radiobiology Unit before the onset of the PhD was subjected to an in-depth analysis to support future experimental design. Based on previous studies indicating an increase in the occurence of exencephaly and microphthalmos after prenatal exposure to ionizing radiation (see chapter '1. Problem'), it was hypothesized that irradiation during neurulation may affect both eye and neural tube development. In turn, the radiosensitivity of various early developmental neurulation stages, in terms of ED and NTD development, was assessed. Acute single-dose irradiation was performed at embryonic gestational days (E7, E7.5, E8, E8.5 or E9) after conception using X-rays (0.5 Gy, 1.0 Gy).

Exposure to (1.0 Gy X-rays) at all irradiation days leads to a significant increase in left eye microphthalmos, with the highest incidence (7.9%, fig. 1A) in the E7.5 group, which is in turn followed up by the E7 group (7.7%). The incidence of microphthalmos in the control groups were left (L, fig. 1A) = 0.3%, right (R, fig. 1B) = 1.0% and left and right simultaneous (LR, fig. 1C) = 0.4%. Note that LR does not imply the summation of L and R, but is used to identify animals that exhibit microphthalmos at both eyes simultaneously. The right eye demonstrated a significant increase in all irradiated (1.0 Gy) groups (fig. 1B), except E9. Similar to the left eye, prevalence of the phenotype was most apparent in the E7.5 group (24.9%), closely followed by the E7 group (23.8%). Embryos with both eyes demonstrating the microphthalmic phenotype were most observed after irradiated at E7.5 (34.2%, fig. 1C), although all irradiated groups did demonstrate a significant increase. Irradiation at E7.5 significantly increased prevalences with doses above 0.5 Gy (fig. 1D) for left (1.1%), right (8.6%), both eyes (3.6%) and subsequently the summarized total (L+R+LR) (13.3%). Interesting to note is that development of the right eye appears to be more easily affected by radiation, with the prevalence of microphthalmos being much higher in the right eye as compared to the left eye after irradiation with equivalent doses. Looking at all animals that exhibit some form of microphthalmos phenotype (L+R+LR), we observed the highest incidence in the E7.5 (1.0 Gy) group. Here the prevalence was 67%, whereas the controls had a prevalence of 1.7%. Prevalence of exencephaly was significantly increased as compared to controls (0%) in the E7, E7.5 and E8 groups, with E7.5 (15.2%) showing the highest prevalence (fig. 1E). Exencephaly already appears significantly increased after 0.5 Gy exposure at E7.5 (0.9%, fig. 1F).

In addition to microphthalmos and exencephaly, malformations such as gastroschisis, agnatia, iris anomalies and jaw malformations were also increased after irradiation during neurulation (data not shown here). Furthermore, a significant increase in prenatal death and embryonic weight at E18 was observed as well after irradiation during neurulation (0.5 Gy, 1.0 Gy) (data not shown here).

In conclusion, these data suggest that **irradiation at E7.5** is the ideal experimental set-up to **induce microphthalmos and exencephaly**; the congenital defects of interest. Hence the decision to irradiate at said developmental stage during all experiments of the project. These data, in addition to cranial skeletal stainings, histological images of microphthalmic eyes and data on postnatal survival chances are being prepared for submission to the **peer-reviewed** journal **Neurotoxicology and Teratology** (Kai Craenen, Jasmine Buset, Mieke Verslegers, Sarah Baatout, Lieve Moons, Mohammed Abderrafi Benotmane; Elsevier, impact factor 2.488, 5-year impact factor 3.322).

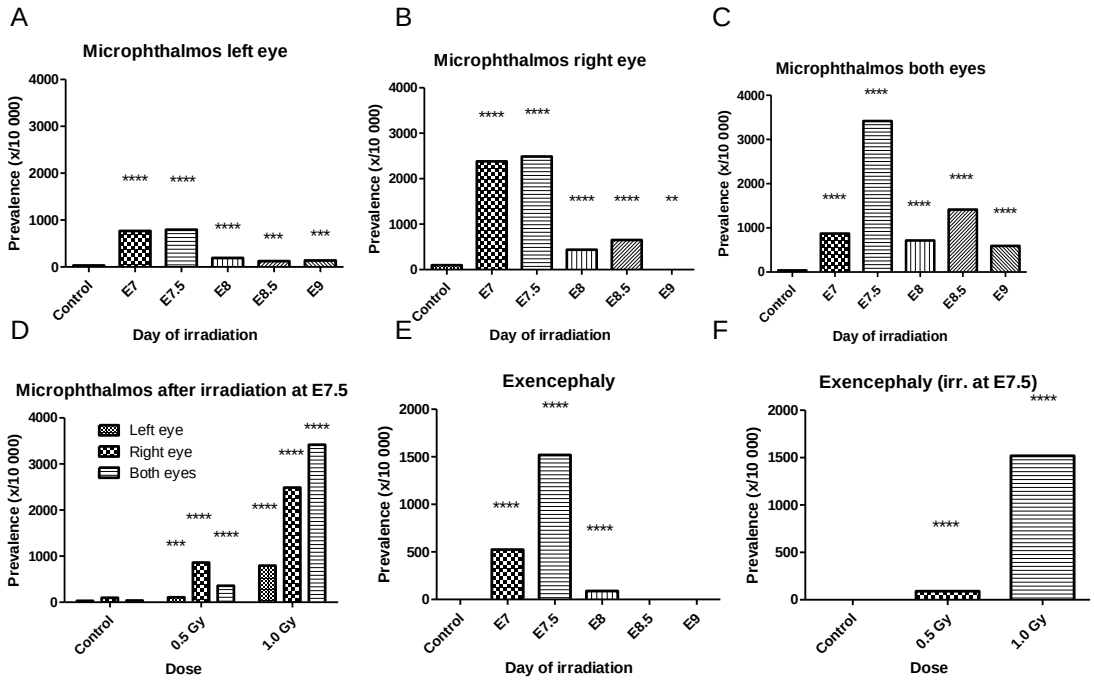

Fig 1. Prevalences of malformations after irradiation during neurulation. Significance according to Fischer's exact (two-tailed).

3.1.2. Embryonic morphology at E9 and E11

In order to gain more knowledge on the prenatal phenotypes that occur after irradiation at E7.5 and to better understand the origin of the X-ray induced exencephaly and microphthalmos phenotypes, pregnant females were sacrificed at E9 followed by a scoring of the embryos according to their Theiler stage (TS). Theiler staging is a classification system based on visual qualities; with one factor being the initiated fusion of the neural folds and the associated turning of the body axis. For more details, consult EMAP eMouse Atlas Project (http://www.emouseatlas.org)[20]. Embryos at TS14 have passed the closure initiation phase and are mostly in the process of closing the anterior neuropore. Embryos in TS 12 - 13 have yet to initiate the turning of the embryo and TS11 specimens are even more rudimentary, consisting mostly out of the neural plate. Our results indicate that irradiation (1.0 Gy) significantly decreases the number of embryos in TS14 (fig. 2), the most prevalent group. In addition, increases in all prior TS stages can be observed albeit not significant. This may serve as a first indicator that **proper closure** of the **neural tube** is already **affected at E9** after **irradiation**, which is an important factor in the development of exencephaly. After the macroscopic scoring, the embryos collected in this study have been assigned to gene expression analysis (3.1.3. Gene expression analysis at E9) using either microarray or rt-qPCR (4.1.3. Validation microarray data). As sample collection continues, data will continue to be added to this prenatal macroscopic study.

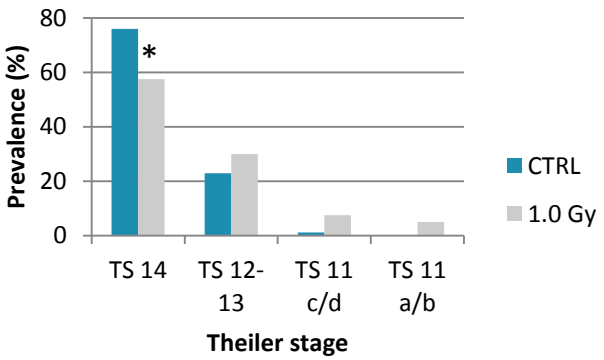

Fig 2. Distribution of the different Theiler stages to which the embryos belong. TS: Theiler stage. Statistical significance according to Fischer's exact (two-tailed) (litters ≥ 7, embryos ≥ 80).

Using a similar experimental approach as discussed above, embryos were also collected and scored at E11. However, here Theiler staging was not used, but rather an in-house developed scoring system using categorical observations. This scoring system has a strong focus on the developing eyes, with the starting development of the pigment epithelium being considered a hallmark of the eye at E11[21]. After irradiation (1.0 Gy, fig. 3A) at E7.5, a **significant decrease** in the number of eyes with **pigment epithelium** could be observed at **E11**, which is a similar response in both the left and right eyes. Referring to the macroscopic observations made at E18 (discussed in '3.1.1. Macroscopic study'), it is interesting to note that there is **no significant difference** between the **radiosensitivity** of the **right and left eye** at **E11**. This suggests that **not all eyes with retarded growth at E11**, based on the onset of pigment epithelium development, **result in microphthalmos** or anophthalmos at **E18**. In addition, irradiation leads to a **decreased closure** of the **anterior neuropore (fig. 3B)**. The abnormal phenotype that was observed has been discussed in literature before as being associated with exencephaly in Shmt1 knockout mice[22]. Crown rump length, a general indicator for embryonic growth[23–28], was measured at E11 after irradiation at E7.5 (0.5 Gy, 1.0 Gy). A very **significant increase** in the number of **growth retarded** (CRL smaller than the mean of the control group minus two times the standard deviation) embryos was observed with **1.0 Gy** (fig. 3C), but **no negative effect** was observed with **0.5 Gy**. These data appear to suggest a **threshold effect** occuring between 0.5 Gy and 1.0 Gy. The embryos collected during this macroscopic study have been embedded for sectioning and subsequent IHC analysis. Irradiated specimens with anomalous features will be compared to control embryos and assessed for various developmental markers discussed in '3.1.5. IHC'.

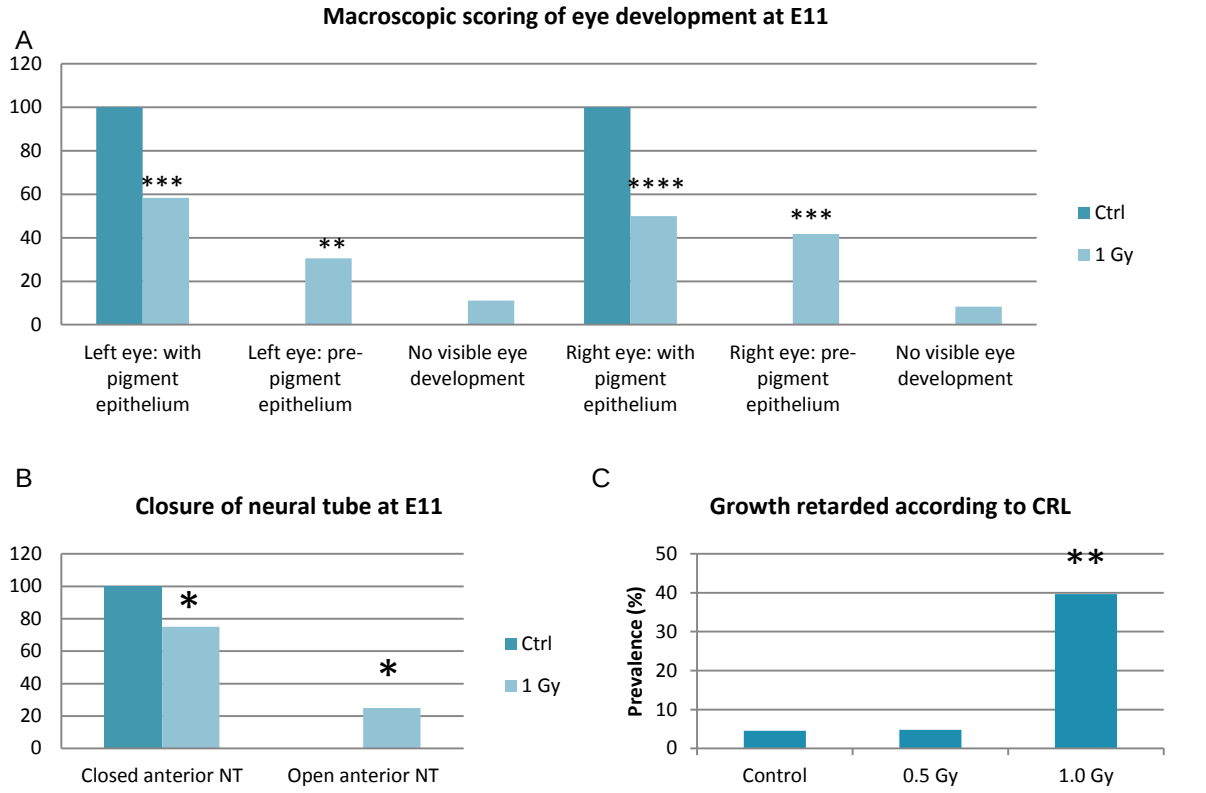

Fig 3. Macroscopic observations made at E11. A: Eye assesment, B: Closure of the anterior neural tube, C: CRL measurements and growth retardation. Significance according to Fischer's exact (two-tailed). Sample size: litters ≥ 3, embryos ≥ 18.

3.1.3. Gene expression analysis at E9

As a first screening for genes that may be involved in the radiation response leading to the congenital defects discussed above, pregnant dams were irradiated at E7.5 and embryos were collected at **E9**. E9 was selected due to its critical role in neural tube development and considering the fact that anomalous development was already observed at this stage as was discussed in chapter '3.1.2. Embryonic morphology at E9 and E11'. RNA from the heads of entire litters was pooled to prevent selection bias and subjected to microarray analysis of control versus 1.0 Gy in utero irradiated embryos. The heat map (fig. 4) obtained from a first analysis shows a series of genes that were the **most significantly up- or downregulated** after **irradiation**. **Gene picking** resulted in the selection of e.g. Pax6, Wnt2b and Slc38/a11. Both **Pax6** (transcription factor)[29–31] and **Wnt2b** (signaling factor)[32–35] are known to be involved in the development of NTDs and EDs, whereas **Slc38/a11** (amino acid transporter) is of interest due to its role in folate/amino acid housekeeping[36,37] of the embryo. Important to note is that there was a lot of variation on the results and no false discovery rate (FDR) adjustments were made. Future analysis will include the exclusion of specific subsets of genes from the analysis to make the FDR less stringent. Furthermore, splice variants and gene ontology will be investigated in more detail. The variance may be the result of pooling

RNA from the entire litter, which may be problematic considering the existing inter-embryonic difference in radiosensitivity. In order to reduce the variation, future validation experiments may consist of comparing healthy control TS 14 embryos with growth retarded irradiated TS 12 - 13 embryos, but this remains a topic of discussion.

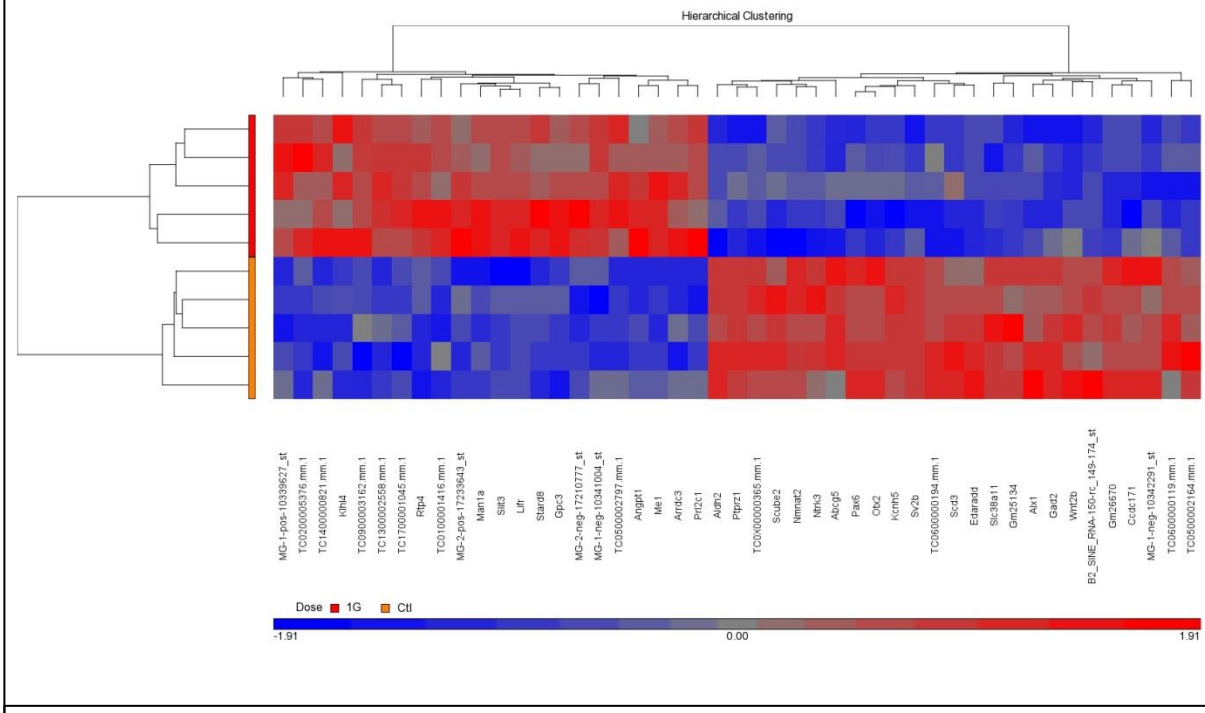

Fig 4. Gene expression analysis heat map with the top significantly changed genes after irradiation. Red shows upregulated genes, whereas blue indicates downregulated genes. The Y-axis shows the doses: red = 1.0 Gy, orange = Ctrl. Litters = 5. P < 0.001, FC = 1.2 /– 1.2

3.1.4. Validating FA fortified food and assessing the impact of X-rays on plasma metabolites

Knowledge on fluctuations of maternal mice plasma FA (related) metabolite levels during pregnancy is extremely limited at best. Thus, the experiment discussed here was performed to get a better understanding on the role of folates during gestation. Female mice were impregnated and had their blood sampled using a repeated measurements methodology, after which plasma was extracted and processed using LCMS[38] at the collaborating AMC laboratory of Dr. Henk Schierbeek (Division of Mother and Child) with the help of Efraim Oosterink. Blood sampling started a week before coupling at – E7. Next, blood was sampled shortly after coupling (E0) to prevent stress-induced coupling difficulties. Continuing, blood was sampled at E7 and E9 (important stages during neural tube closure) and at E18 (shortly before delivery). A significant increase of concentration could be observed at the E18 time point (fig. 5J), which may be related to the change in maternal metabolism, associated with beginning lactation. However, to our knowledge there have been no previous studies on this phenomenon. To validate a metabolic impact of the FA fortified food (pilot batch) on maternal 5-methyl tetrahydrofolate (5-MTHF, a metabolited derived from FA) plasma levels, animals were placed on the custom-made FA fortified diet at –E7, immediately after blood sampling. We see a significant increase at E0 (fig. 5A), one week after the onset of fortification, which is in line with previous studies. However, this significant increase disappears at E7 and E9. Of note is that the neural tube is closing at this stage, a metabolically highly active period during development, considering the short cell cycle duration at this stage (less than 10 hours). Several research groups have speculated in the past that it is the massive replication of genetic material, and thus the use of one-carbon donors such as 5-MTHF, during neural tube development that makes FA fortification prevent neural tube defects. This in turn may potentially explain the trend of 5-MTHF in plasma at E7 and E9, although this has not been described before in literature. Interestingly, when animals are irradiated (control food) they no longer show the significant increase (fig. 5J) of 5-MTHF at E18 (fig. 5D). This may be the result of radiation-induced metabolic anomalies[17–19]. The baseline 5-MTHF measurements performed at –E7 in animals that were both on the fortified food diet and were later irradiated at E7.5 are much higher than the comparable values in the other treatment groups. This anomaly made it impossible to perform proper statistical analysis, comparing the later repeated measurements to the baseline measurement. This anomaly may be the result of different storage conditions (darker, colder) and the date at which the analysis were performed (most in the same two days, unlike all the other samples). It is thus important to interpret this graph with the necessary caution.

Measurements of methionine (fig. 5B/E/H/K) did not show any significant changes in the different repeated measurements groups, whereas homocysteine increased significantly at E0 and E18 (fig. 5I) when the animals were on the fortified diet and irradiated at E7.5. The biological significance of these results remains unclear.

We can conclude from the results that the **food forification** has a small but **significant impact** on the maternal 5-MTHF plasma levels one week after fortification. Furthermore, in control females a significant increase of 5-MTHF was observed at E18, which was no longer present in the irradiated females. This suggests a **possible impact** of **X-ray** exposure at E7.5 on **folate bioavailability**. Currently, discussions are ongoing with the food manufacturer to order a new batch for the follow-up experiments.

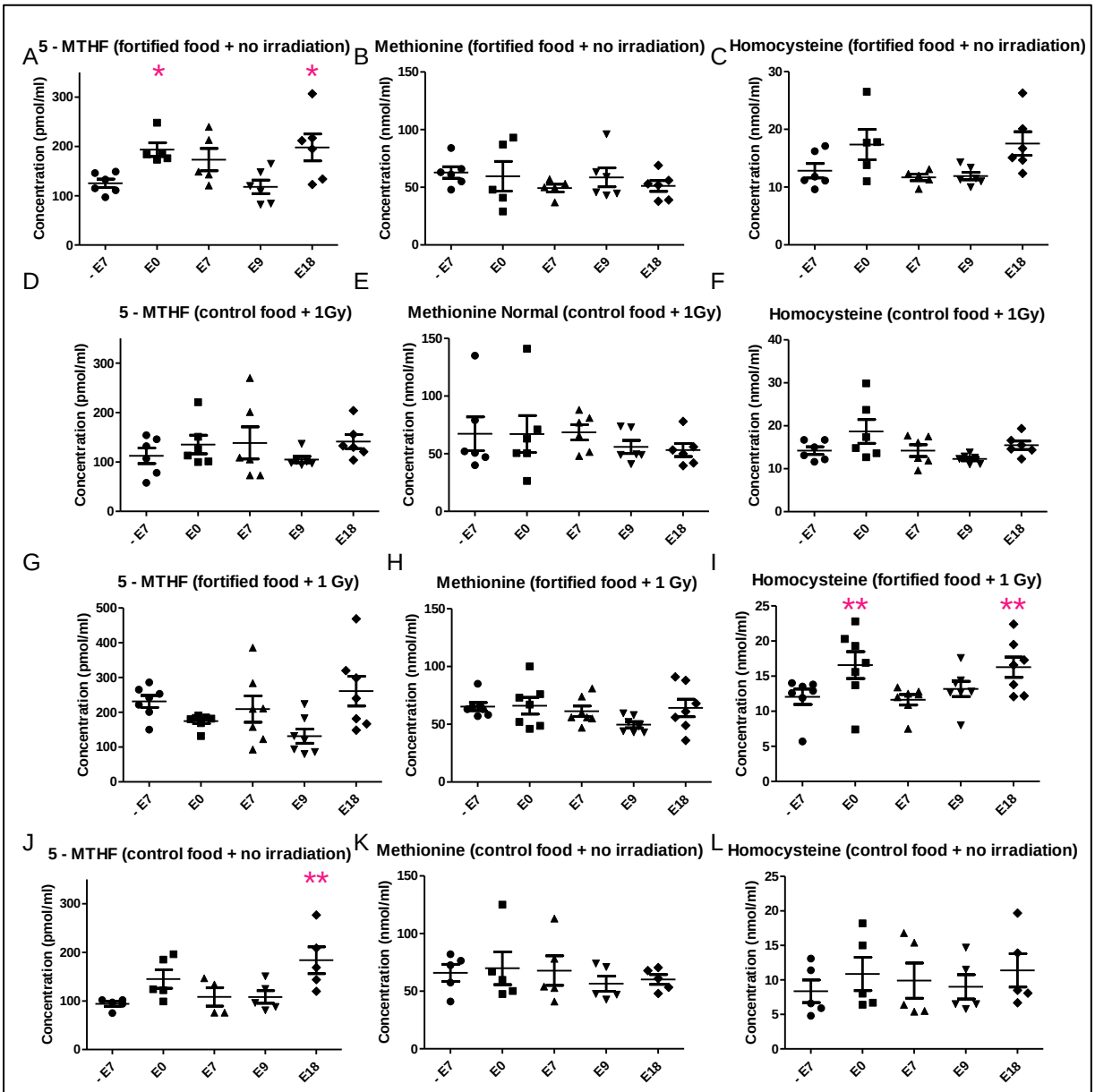

Fig 5. Maternal plasma measurements of 5-MTHF, methionine and homocysteine. Statistical significance according to repeated measures ANOVA: contrast set between –E7 vs E0, E7, E9 or E18.

3.1.5. IHC

Considering that exposure to ionizing radiation may affect cellular proliferation, stainings for **Ki67** (proliferation, fig. 6A) and **PH3** (mitosis, fig. 6B) were **optimized** for **paraffin sections** of **E9 – E11 – E18** embryos. In addition, stainings for cells in apoptosis (CC3, TUNEL) are currently being optimized. Both anomalous proliferation and apoptosis are key cellular reactions to ionizing radiation exposure and are known to play a role in the development of neural tube defects[39–43].

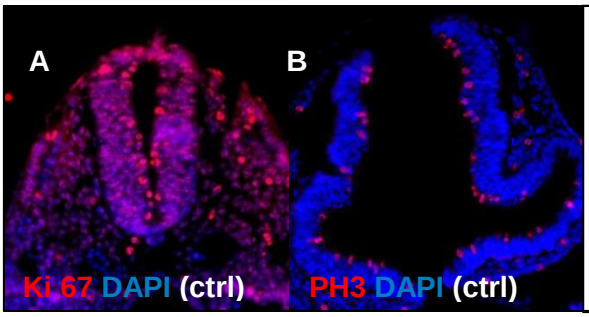

Fig 6. Exemplary IHC images optimized for E9 embryonic stainings in the transverse plane of the neural tube. A: Ki67 staining for proliferating cells, B: PH3 staining for cells in mitosis.

3.2. Postnatal studies

3.2.1. Viability study

In order to determine a dose limit for the experiments discussed in chapter '4.2. Postnatal/adult studies', pregnant females were irradiated (0.1 Gy, 0.5 Gy or 1.0 Gy) and survival chances of the progeny was followed up until W5. Irradiation at E7.5 with 1.0 Gy significantly decreased survival chances by W5, but the lower doses did not significantly alter survival chances in the other groups (fig. 7A). As a follow up, animals from the 0.1 Gy and 0.5 Gy groups were further grown until W10 (training age for the vision/behavioral tests), when their brain and body weights were measured (fig. 7B-E). These weights were not significantly altered in neither males nor females in any of the irradiation conditions (≤ 0.5 Gy). These data suggest a **dose limit** of **0.5 Gy** for the **adult studies**, since here viability is most optimal.

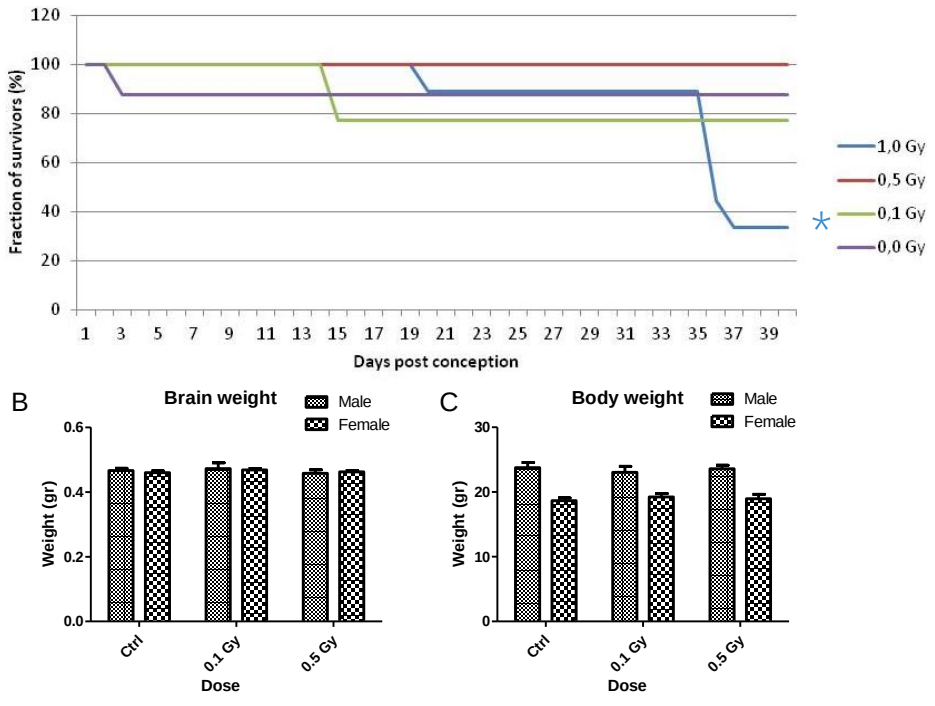

Fig 7. Postnatal/adult survival and body/brain weights. A: survival after irradiation at E7.5 (litters ≥ 2, pups ≥ 8, statistical significance according to t-test), brain (B) and body (C) weights after irradiation at E7.5 (litters = 4).

3.2.2. Eye morphology

In order to assess the impact of prenatal X-ray exposure at E7.5 on adult eye morphology, the right eyes of progeny that were irradiated *in utero* were sectioned (fig. 8D) and subsequently subjected to an in depth histological analysis. General eye structures were measured (axial length diameter, paraxial length diameter, retina diameter, axial eye diameter, paraxial eye diameter, anterior chamber depth, lens circumference) as well as the thickness of the nuclear layers of the retina; the inner nuclear layer (INL) and the outer nuclear layer (ONL). The dose limit for this experiment was set at 0.5 Gy, based on the observations made in the viability study discussed in the previous chapter. No significant changes were observed in any of the conditions, illustrating that prenatal irradiation (E7.5) with **0.5 Gy and lower** does **not affect** overall **eye morphology** (fig. 8A-C). Linking these observations to the macroscopic study discussed above, it is interesting to note the low prevalence of microphthalmic eyes at 0.5 Gy. This may explain why no significant changes are observed in the measurements. However, these data also suggest that there are no subtle morphological changes happening, such as those at the level of the retinal layers. This supports the hypothesis that ionizing radiation-induced microphthalmos is an **all-or-nothing phenotype**. In addition, there may be a **dose threshold at** 0.5 Gy or above, **under which no** subtle morphological **changes** may be observed. Such a threshold has been observed in previous radiobiological experiments. Finally, it is important to take into consideration that **there may still be functional anomalies** present due to abnormal connectivity. This however remains to be experimentally proven, as is discussed in chapter '4.2.1. vision and behavior'.

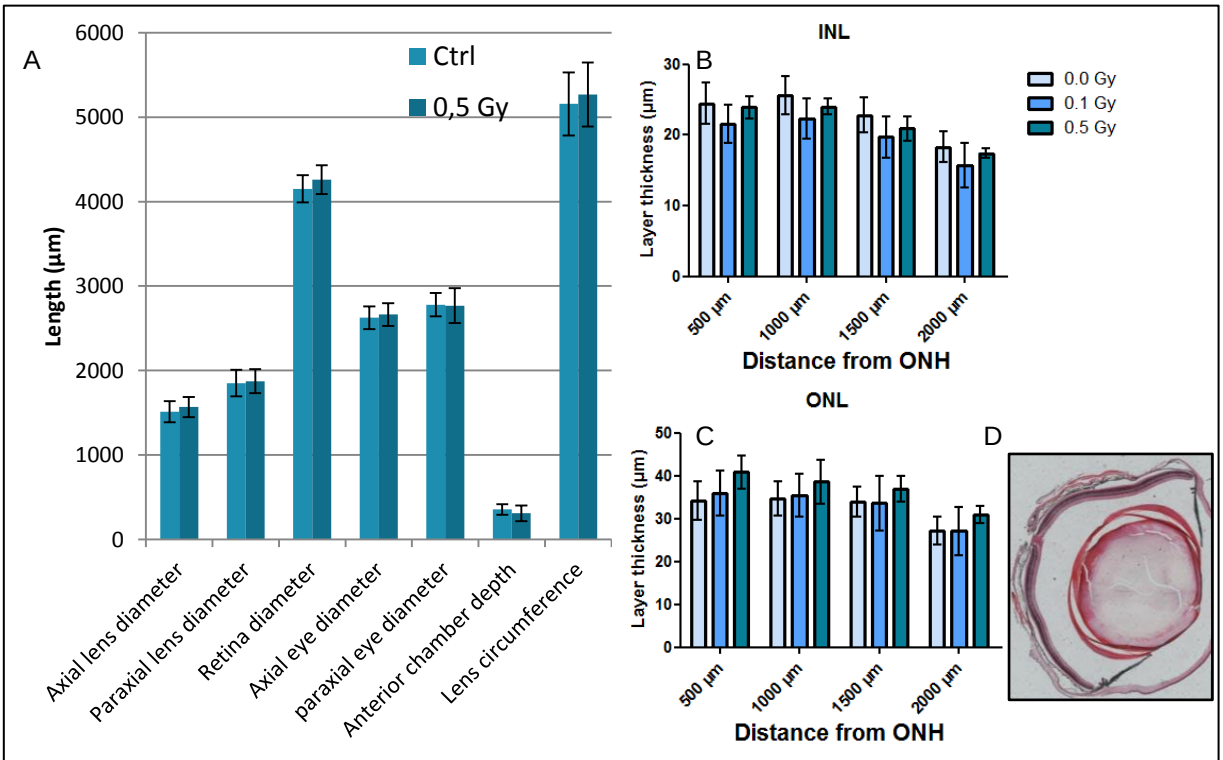

Fig 8. A: Measurements of the eye after *in utero* X-ray exposure (litters = 4, pups ≥ 20). Thickness of the nuclear layers (B-C) is depicted in function of the distance from the optic nerve head (ONL). D: Example of an H&E staining of the eye (control) used for the analysis.

4. Project outline for the second two years and schedule

4.1. Prenatal studies

4.1.1. Macroscopic study

Based on the observations made in chapter '3.1.4.' where a small pilot batch of food (10 kg) was used, we plan to order a larger quantity to perform a follow-up of the macroscopic study discussed at '3.1.1.'. This is in order to assess the efficacy of folic acid in ameliorating radiation induced congenital defects such as exencephaly and microphthalmos. Due to the nature of the customized food, the expiration date falls 6 months after delivery at the SCK-CEN animalarium. To this end, it would be ideal to perform all FA-related experiments within the 6-month window of time to prevent the need for multiple batches and thus possible inter-batch variations. In this experiment, pregnant animals will be irradiated (1.0 Gy positive control dose) at E7.5 and may or may not receive the folic acid fortified diet. At E18, macroscopic observations will be performed of the congenital defects. The prevalences will be compared to those of control animals (sham-irradiated, standard food). Half of the obtained fetuses will be dehydrated and embedded in paraffin for IHC, whereas the other half may be used for skeletal stainings.

4.1.2. IHC

Sham-irradiated and irradiated (1.0 Gy at E7.5) litters have been collected at (E9 and E11) and embedded in paraffin for the IHC study. As a first approach, stainings for Ki67 (proliferation marker) and PH3 (mitosis marker). In addition, CC3 (early apoptosis) and TUNEL (late apoptosis) stainings will be further optimized and used on the experimental sections. Based on the observations made in the validation of the gene expression analysis, more markers may be added.

4.1.3. Validation microarray data

The observations made in the microarray experiment discussed at '3.1.3.' will be subjected to more in depth analysis to suppress the large variation on the data. Subsequently, the set of genes of interest (GOIs) discussed before will be updated. Primers will be designed for GOIs and rt-qPCR will be performed to assess the validity of the microarray data. These results may in turn be subjected to protein analysis/proteomics if technically achievable, considering the very small sample size of the embryos.

4.2. Postnatal/adult studies

4.2.1. Vision and behavior

In order to establish the persistant effects of prenatal irradiation in adult mice, pregnant females will be irradiated at E7.5 and the progeny will be grown until W10. It is at this stage that visual acuity will be assessed using the optokinetic drum. Previously, a behavioral test battery including the morris water maze, dry Y maze, social interaction was suggested. This would allow for profound testing of cognition and memory, although these tests are based on visual cues. Thus, depending on the visual performance of the irradiated animals, the

behavioral test battery may be adjusted to prevent vision-based bias and thus misinterpretation of cognition-oriented data.

The experiments will start immediately after the macroscopic study has been finished. This is in order to assess whether or not the folic acid had a beneficial effect on the radiation induced congenital defects and whether it should thus be included in the functional studies. If folic acid is to be given pre-/postnatally, the visual/behavioral tests are to be performed within a 6-month time frame, similar to that discussed in chapter '4.1.1. macroscopic study' due to the short shelf life of the customized food. This situation has been depicted in the time schedule shown below.

4.2.2. Adult brain and eye morphology

In parallell to the functional experiments, a separate group of animals irradiated *in utero* at E7.5 will be subjected to morphological MRI imaging (ventricle size, cortical thickness, etc.) to assess adult brain morphology. To assess possible anomalies in brain development, repeated scans are to be performed. Using the same argumentation as that in chapter '4.2.1. vision and behavior', folic acid may or may not be included in the experimental set-up.

Schedule for the third and fourth year of the PhD project

<table>
<tr><td colspan="4">Year 3 of the PhD project</td><td colspan="4">Year 4 of the PhD project</td></tr>
<tr><td colspan="2">4.1.1. Macroscopic study</td><td colspan="2">4.2.1. Vision and behavior</td><td colspan="4">Article and thesis writing</td></tr>
<tr><td colspan="2"></td><td colspan="2">4.2.2. Adult brain morphology</td><td colspan="4"></td></tr>
<tr><td colspan="3">4.1.3. Validation microarray data</td><td colspan="3">4.1.2. IHC</td><td></td></tr>
<tr><td colspan="4">Master thesis student</td><td colspan="4"></td></tr>
<tr><td>Submit 1st article</td><td colspan="3"></td><td>Submit 2nd article</td><td>Submit 3rd article</td><td></td><td>Submit PhD thesis</td></tr>
</table>

Prenatal studies = blue highlight, postnatal/adult studies = green highlight, writing = orange highlight

6. Education and scientific output

National and international conferences with poster as output

- BSN meeting: K. Craenen, M. Verslegers, J. Buset, S. Baatout, L. Moons, M. A. Benotmane - *Assessing the impact of prenatal irradiation in a mouse model: a focus on eye/neural tube development and postnatal viability* – (U Mons, May 22nd 2015)
- BSCDB meeting: K. Craenen, M. Verslegers, J. Buset, S. Baatout, L. Moons, M. A. Benotmane - *The impact of in utero irradiation on mouse embryonic development: neural tube formation, eye development and postnatal viability* – (U Liège, June 5th, 2015)
- SCK•CEN Day of the PhDs: K. Craenen, M. verslegers, J. Buset, S. Baatout, L. Moons and M. A. Benotmane - *The impact of prenatal radiation exposure on eye/neural tube development and postnatal viability in a mouse model* – (SCK•CEN, October 29th 2015)
- 42nd Annual Meeting of the European Radiation Research Society: K. Craenen, J. Buset, M. Verslegers, S. baatout, L. Moons, M. A. Benotmane - *Exploring radiosensitivity during neurulation: impact of X-irradiation on the incidence of various malformations, prenatal mortality and adult health* – (Royal Tropical Institute in Amsterdam, 4 - 8th September 2016)

Conferences attended without presentation

- BSCDB - BSBMB Joint autumn meeting (U Antwerpen, November 6th-7th 2014)
- Is the Brain smart enough to understand who we are? (Palais des Académies, Brussels, 2nd April 2015)

Projects written

- IWT: K. Craenen, M. Verslegers, A. Benotmane - *The effects of ionizing radiation and folic acid on neural tube closure, eye development and cognitive functions in mice* (Brussels, submitted September 12th 2014).

Courses/presentations given

- European master in radiation biology: K. Craenen - *Practical: sectioning of the mouse eye* (SCK•CEN, March 17th 2015)
- DoReMi workshop: K. Craenen – *Animal models in neurulation research* (SCK•CEN, June 17th 2015)
- Summer school in radiobiology – *Practicals in microscopy: neuron cells and the eye* (SCK•CEN, August 17th 2015)
- 2 days of practical sessions – *Vergelijkende biologie* (Prof. Huybrechts) (KUL, 2015)
- 2 Week course in radiobiology – *The brain: a mysterious conductor* (SCK•CEN, April 27th – May 12th 2016)

Exams supervised

- Dierkunde II, Prof. V. Darras (KUL, 16th June 2015)
- Biologie van seks, Prof. L. Moons (KUL, 13th January 2016)

Courses attended

- Assistentenopleiding (KUL, October 17th 2014, November 24th and Juli 2nd 2015);
  ECTS = 0.5 skill
- Scientific integrity for starting PhD's (KUL, November 24th 2014);
  ECTS = 0.2 thematic
- Day of the PhD's (SCK•CEN, April 28th 2015);
  ECTS = 0.6 thematic
- DoReMi radiation sensitivity (Institut Curie, Paris, December 8th -12th 2014);
  ECTS = 3.0 thematic
- Partek: microarray and NGS (ERASMUS, Rotterdam, March 24th-25th);
  ECTS = 0.7 skill
- *.be creative* competition for young entrepreneurs: *Impacter M. 1* (October 14th- 31th 2014);
  ECTS = 0.5 skill

Students guided

- Bachelor student: Lise Balcon, ONIRIS college (Nantes, France) (Aug. – Sept. 2015)
- Bachelor student: Bria Lacour, University of Texas (Austin, US) (Jan. – May. 2016)
- Master student: Livine Craeghs, KUL (academic year 2016-2017)
  - The student will be closely involved in the validation of the microarray data (4.1.3. Validation microarray data) and the experiments discussed in '4.1.2. IHC'. The student will spend most of her master thesis time at the SCK-CEN Radiobiology lab.

7. References

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14. Prado, E. L., Ullman, M. T., Muadz, H., Alcock, K. J. & Shankar, A. H. The effect of maternal multiple micronutrient supplementation on cognition and mood during pregnancy and postpartum in Indonesia: a randomized trial. *PLoS One* **7,** e32519 (2012).

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The PhD researcher needs to upload this form and the signed signature sheet via his/her KULoket tool.
The members of the Supervisory Committee can approve by signing the signature sheet which the PhD researcher downloaded via KULoket or by signing the next page:

Names and signatures of the members of the Supervisory Committee:

Prof. Dr. Lieve Moons	
Dr. Rafi Benotmane	
Dr. Mieke Verslegers	
Prof. Dr. Rudi D'Hooge	
Prof. Dr. Uwe Himmelreich	

Craenen Kai

From: Lieve Moons <lieve.moons@kuleuven.be>
Sent: woensdag 6 juli 2016 7:56
To: Craenen Kai
Subject: Re: Midterm report

I hereby agree to submit the report.
Best, Lieve

Prof. Dr. Lieve Moons
Research Group Neural Circuit Development and Regeneration
Zoological Institute, Naamsestraat 61, 3000 Leuven
Tel: 32-16-32 39 91
Fax: 32-16-32 42 62
Email: lieve.moons@kuleuven.be

From: Craenen Kai <kai.craenen@sckcen.be>
Date: Tuesday 5 July 2016 13:20
To: Godelieve Moons <lieve.moons@bio.kuleuven.be>, Benotmane Rafi <rafi.benotmane@sckcen.be>, Verslegers Mieke <mieke.verslegers@sckcen.be>, Rudi D'Hooge <Rudi.DHooge@ppw.kuleuven.be>, Uwe Himmelreich <Uwe.Himmelreich@med.kuleuven.be>
Subject: Midterm report

Dear All,

Considering the fact that I'm almost halfway my PhD project, I have to submit my midterm report at the Arenberg Doctoral school.
Please read the report in attachment and send me a confirmation mail (or a signed scan of the final page) if you agree with its contents and thus the continuation of the PhD project.
In case you do not agree with the contents, please inform me so the document can be adapted.

Thank you for your continued support,
Sincerely,
Kai

Kai Craenen, PhD student
Molecular & Cellular Biology MCB
Institute for Environment, Health & Safety EHS
SCK-CEN, Boeretang 200
BE-2400 Mol

kcraenen@sckcen.be
Tel: +3214332386
www.sckcen.be

Craenen Kai

From: Rudi D'Hooge <rudi.dhooge@kuleuven.be>
Sent: woensdag 10 augustus 2016 12:45
To: Craenen Kai; Uwe Himmelreich; Rudi D'Hooge; Benotmane Rafi; Verslegers Mieke
Cc: Lieve Moons
Subject: RE: Deadline Mid term report passed!

Dear Kai,

I support submission of your midterm report. Sorry for the late reply.

Rudi D'Hooge

Prof. Dr. Rudi D'Hooge
PhD (BIOMED), PhD (PSYCHOL) | Professor of Biological Psychology

University of Leuven (KU Leuven) | Biological Psychology | http://ppw.kuleuven.be/lbp
PO Box 3714 – Tiensestraat 102, B-3000 Leuven, Belgium
tel +32 (0) 16 326142 | tel secr. +32 (0) 16 326145 | fax +32 (0) 16 326099

From: Craenen Kai [mailto:kai.craenen@sckcen.be]
Sent: woensdag 10 augustus 2016 10:22
To: Uwe Himmelreich <Uwe.Himmelreich@med.kuleuven.be>; Rudi D'Hooge <Rudi.DHooge@ppw.kuleuven.be>; Benotmane Rafi <rafi.benotmane@sckcen.be>; Verslegers Mieke <mieke.verslegers@sckcen.be>
Cc: Lieve Moons <lieve.moons@kuleuven.be>
Subject: FW: Deadline Mid term report passed!
Importance: High

Dear all,

I still need confirmation by mail from **all the addressees of this message** (except Lieve) that I can upload my midterm report to the doctoral school.
The deadline has now passed, so please do not wait much longer to contact me. **It is becoming quite urgent.**
The midterm report can again be found in attachment.

Sincerely,
Kai

From: Lieve Moons [mailto:lieve.moons@kuleuven.be]
Sent: maandag 1 augustus 2016 11:25
To: Craenen Kai
Subject: FW: Deadline Mid term report passed!

Dag kai,

Ik dacht dat dit in orde was? Je bent keer op keer te laat. Probeer daar wat aan te doen aub.

Mvg,
Lieve

Prof. Dr. Lieve Moons
Research Group Neural Circuit Development and Regeneration
Zoological Institute, Naamsestraat 61, 3000 Leuven

Tel: 32-16-32 39 91
Fax: 32-16-32 42 62
Email: lieve.moons@kuleuven.be

From: "phd@wet.kuleuven.be" <phd@wet.kuleuven.be>
Date: Monday 1 August 2016 04:00
To: Kai Craenen <kai.craenen@student.kuleuven.be>, Lieve Moons <lieve.moons@kuleuven.be>, Kai Craenen <kai.craenen@kuleuven.be>
Subject: Deadline Mid term report passed!

English version below.

Beste Kai Craenen,

Wij volgen de voortgang van iedere doctoraatsstudent aan onze faculteit van nabij op. Bij nazicht van je dossier werd onlangs vastgesteld dat je de mijlpaal 'Mid term report' nog niet hebt behaald. Behoudens een vergissing onzentwege had je deze mijlpaal moeten voltooien tegen 01.07.2016.

Gelieve daarom zo snel mogelijk het nodige te doen om deze mijlpaal te behalen. Meer informatie over hoe je dit doet, vind je terug in KU Loket onder de rubriek 'Doctoraatsopvolging'. We geven nog mee dat dit het laatste geautomatiseerde bericht is dat je krijgt in verband met deze mijlpaal, die momenteel als 'onvoltooid' in je dossier staat aangegeven.

Wij danken je alvast voor je medewerking en wensen je veel succes met het voltooien van deze mijlpaal en het verdere verloop van je doctoraat.

Vriendelijke groeten,
je facultaire doctoraatsverantwoordelijke.

Dear Kai Craenen,

We closely track the progress of each doctoral student at our faculty. We recently checked your file and noticed that you did not yet complete milestone 'Mid term report'. Barring a mistake in our records, the deadline (01.07.2016) of this milestone has passed.

May we therefore ask you to take the necessary action to complete this milestone as soon as possible. More information on how to do so is available inthe KU Loket application 'PhD progress'.

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We thank you for your cooperation and wish you the best of luck with achieving this milestone and continuing your doctoral project.

Kind regards,
the PhD administration of your faculty.

Craenen Kai

From: Benotmane Rafi
Sent: woensdag 10 augustus 2016 14:03
To: Rudi D'Hooge; Craenen Kai; Uwe Himmelreich; Rudi D'Hooge; Verslegers Mieke
Cc: Lieve Moons
Subject: RE: Deadline Mid term report passed!

Dear Kai

I agree for the submission of your midterm report,

Kind regards

From: Rudi D'Hooge [mailto:rudi.dhooge@kuleuven.be]
Sent: woensdag 10 augustus 2016 12:45
To: Craenen Kai; Uwe Himmelreich; Rudi D'Hooge; Benotmane Rafi; Verslegers Mieke
Cc: Lieve Moons
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Rudi D'Hooge

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Cc: Lieve Moons <lieve.moons@kuleuven.be>
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Importance: High

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From: Lieve Moons [mailto:lieve.moons@kuleuven.be]
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Lieve

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Email: lieve.moons@kuleuven.be

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English version below.

Beste Kai Craenen,

Wij volgen de voortgang van iedere doctoraatsstudent aan onze faculteit van nabij op. Bij nazicht van je dossier werd onlangs vastgesteld dat je de mijlpaal 'Mid term report' nog niet hebt behaald. Behoudens een vergissing onzentwege had je deze mijlpaal moeten voltooien tegen 01.07.2016.

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Wij danken je alvast voor je medewerking en wensen je veel succes met het voltooien van deze mijlpaal en het verdere verloop van je doctoraat.

Vriendelijke groeten,
je facultaire doctoraatsverantwoordelijke.

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Kind regards,
the PhD administration of your faculty.

Craenen Kai

From: Uwe Himmelreich <uwe.himmelreich@kuleuven.be>
Sent: woensdag 10 augustus 2016 16:05
To: Craenen Kai; Uwe Himmelreich; Rudi D'Hooge; Benotmane Rafi; Verslegers Mieke
Cc: Lieve Moons
Subject: Re: Deadline Mid term report passed!

Dear Kai,
Yes, it is ok with me if you upload the midterm report as circulated in your email.
Best regards,
Uwe.

From: Craenen Kai <kai.craenen@sckcen.be>
Sent: 10 August 2016 10:21
To: Uwe Himmelreich; Rudi D'Hooge; Benotmane Rafi; Verslegers Mieke
Cc: Lieve Moons
Subject: FW: Deadline Mid term report passed!

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Kind regards,
the PhD administration of your faculty.

Craenen Kai

From: Verslegers Mieke
Sent: donderdag 11 augustus 2016 15:57
To: Craenen Kai
Subject: RE: Deadline Mid term report passed!

Ok, bij deze dan!

Tot snel,
Mieke

From: Craenen Kai
Sent: donderdag 11 augustus 2016 15:48
To: Verslegers Mieke
Subject: RE: Deadline Mid term report passed!

Dag Mieke,

Neen, een e-mail met bevestiging is voldoende volgens de doctoral school. ;)

Mvg,
Kai

From: Verslegers Mieke
Sent: donderdag 11 augustus 2016 15:44
To: Craenen Kai
Subject: RE: Deadline Mid term report passed!

Kai, voor mij ook in orde, maar heb je geen handtekening nodig dan?

Mieke

From: Craenen Kai
Sent: woensdag 10 augustus 2016 10:22
To: Uwe Himmelreich; Rudi D'Hooge; Benotmane Rafi; Verslegers Mieke
Cc: Lieve Moons
Subject: FW: Deadline Mid term report passed!
Importance: High

Dear all,

I still need confirmation by mail from **all the addressees of this message** (except Lieve) that I can upload my midterm report to the doctoral school.
The deadline has now passed, so please do not wait much longer to contact me. **It is becoming quite urgent.**
The midterm report can again be found in attachment.

Sincerely,
Kai

From: Lieve Moons [mailto:lieve.moons@kuleuven.be]
Sent: maandag 1 augustus 2016 11:25

To: Craenen Kai
Subject: FW: Deadline Mid term report passed!

Dag kai,

Ik dacht dat dit in orde was? Je bent keer op keer te laat. Probeer daar wat aan te doen aub.

Mvg,
Lieve

Prof. Dr. Lieve Moons
Research Group Neural Circuit Development and Regeneration
Zoological Institute, Naamsestraat 61, 3000 Leuven
Tel: 32-16-32 39 91
Fax: 32-16-32 42 62
Email: lieve.moons@kuleuven.be

From: "phd@wet.kuleuven.be" <phd@wet.kuleuven.be>
Date: Monday 1 August 2016 04:00
To: Kai Craenen <kai.craenen@student.kuleuven.be>, Lieve Moons <lieve.moons@kuleuven.be>, Kai Craenen <kai.craenen@kuleuven.be>
Subject: Deadline Mid term report passed!

English version below.

Beste Kai Craenen,

Wij volgen de voortgang van iedere doctoraatsstudent aan onze faculteit van nabij op. Bij nazicht van je dossier werd onlangs vastgesteld dat je de mijlpaal 'Mid term report' nog niet hebt behaald. Behoudens een vergissing onzentwege had je deze mijlpaal moeten voltooien tegen 01.07.2016.

Gelieve daarom zo snel mogelijk het nodige te doen om deze mijlpaal te behalen. Meer informatie over hoe je dit doet, vind je terug in KU Loket onder de rubriek 'Doctoraatsopvolging'. We geven nog mee dat dit het laatste geautomatiseerde bericht is dat je krijgt in verband met deze mijlpaal, die momenteel als 'onvoltooid' in je dossier staat aangegeven.

Wij danken je alvast voor je medewerking en wensen je veel succes met het voltooien van deze mijlpaal en het verdere verloop van je doctoraat.

Vriendelijke groeten,
je facultaire doctoraatsverantwoordelijke.

Dear Kai Craenen,

We closely track the progress of each doctoral student at our faculty. We recently checked your file and noticed that you did not yet complete milestone 'Mid term report'. Barring a mistake in our records, the deadline (01.07.2016) of this milestone has passed.

May we therefore ask you to take the necessary action to complete this milestone as soon as possible. More information on how to do so is available inthe KU Loket application 'PhD progress'.

This is the last automatic email you will receive about this milestone, which is currently recorded as 'overdue' in your file.

We thank you for your cooperation and wish you the best of luck with achieving this milestone

and continuing your doctoral project.

Kind regards,
the PhD administration of your faculty.