# 1. General information

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<th>Coordinating investigator 1</th>
<th>Prof. Dr. L. Koenderman</th>
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# Title of project:
The lifespan of human eosinophils and basophils under homeostatic and allergic asthmatic conditions

# Time schedule:
- **Start of project**: 01-07-2010 (dd-mm-yyyy)
- **Duration of project**: 48 (months)
- **Period of funding**: from 01-07-2011 (dd-mm-yyyy) till 01-07-2014 (dd-mm-yyyy)

# Grant
- € 240.150.-

# Short description of the project for public information (in Dutch, see guidelines)

Allergisch astma wordt gekenmerkt door een allergische ontsteking in de long. Er is nog steeds veel onbekend m.b.t. de mechanismen die deze chronische ontsteking veroorzaken. Interessant genoeg worden er verschillen gevonden in aanwezigheid van verschillende ontstekingscellen gevonden in de long van patiënten met verschillende vormen van astma. Echter het is erg onduidelijk welke mechanismen hieraan ten grondslag liggen. Een belangrijk lacune in kennis is nu door ons project beantwoord: hoe lang blijven verschillende ontstekingscellen aanwezig in het bloed en in het weefsel in gezonde mensen en patiënten met astma. Dit is belangrijk omdat het ontwerpen van nieuwe medicijnen die hier op aan gaan grijpen er anders uitzien al naar gelang het mechanisme. De vraag is namelijk gaan er steeds nieuwe cellen naar het weefsel toe of blijven de cellen hier lang in leven. Deze op het eerste gezicht eenvoudige vraag was moeilijk te beantwoorden Maar nieuwe technologie heeft dit nu mogelijk gemaakt. Wij hebben kunnen aantonen dat de verschillende ontstekingscellen in astmapatiënten verschillende tijden in het weefsel verblijven. Onze studie heeft vooral aangetoond dat een subpopulatie van een van de ontstekingscellen, eosinofiele granulocyt genaamd, veel langer aanwezig is dan de andere veel meer voorkomende cel, de neutrofiele granulocyt. Dit betekent eigenlijk dat nieuwe medicijnen gericht op astma gekenmerkt door neutrofiele granulocyten meer het bewegen naar het weefsel toe zouden moeten remmen, terwijl geneesmiddelen gericht op de eosinofiele granulocyt meer gericht zouden moeten worden op de overleving van deze cellen in het weefsel.

# Report

## Summary:
The lifespan of human neutrophils, eosinophils and basophils under homeostatic and allergic asthmatic conditions

**Authors**
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**Keywords (max. 6)**
eosinophil, basophil, life span, asthma, in vivo labelling, inflammation

**Abstract (max. 250 words):**
The lifespans of neutrophils, eosinophils and basophils were determined in the blood of healthy controls individuals as well as of patients with allergic asthma. In addition, the retention times of neutrophils and eosinophils were determined in the sputum of asthma patients with sufficient numbers of cells in this compartment. Unfortunately, basophils could not be studied in sputum because of their extremely low numbers. The lifespans were determined via stable isotope labelling with the use of short term labelling with deuterated glucose for 6 hrs. During this pulse the DNA in all cycling cells was enriched in deuterium that could be determined
mass spectrometry. Composite enrichment curves of isolated cells were analysed. In parallel a new mathematical tool was developed to fit the measured curves with models predicting similar enrichment. The model was built on the premise that granulocyte differentiation follows a conveyor belt type. Our data demonstrate that neutrophils, eosinophils and basophils live for 72-96 hrs, 7 days and 6.2 days in the peripheral blood, respectively. This is much longer as stated in the textbooks. Neutrophils found in the sputum do not show any delay in labelling implying that the cells directly go to the sputum with no retention in the tissue. On the other hand at least a subpopulation of eosinophils is long lived in the tissue (> 15 days). Our data indicate that targeting neutrophil asthma would benefit from inhibitors preventing tissue homing whereas treatment of eosinophil asthma would benefit from inhibitors of tissue retention.

2.2 Description of original question/aim (max. 150 words):

The overall question was the analysis of the kinetics of neutrophils, eosinophils and basophils in the peripheral blood and sputum during healthy homeostasis and in patients with allergic asthma during active disease. The specific research questions were:

1. What is the circulatory lifespan of eosinophils and basophils under homeostatic conditions?
2. What is the time for transit through the post-mitotic pool?
3. Does allergic inflammation influence half-lives and transit times through the post-mitotic pool?
4. What is the retention time of cells in the sputum in those patients with eosinophils and neutrophils present in this compartment (active disease/immunologically challenged).

2.3 Results (max. 2500 words, please submit a maximum of 4 figures and diagrams separately):

1. Neutrophils: design of the study, proof-of-principle and results

Pulse chase after short term $^2H$-glucose labelling

In total, 32 healthy volunteers received twelve oral doses of $6,6^2H_2$–glucose over the course of six hours. Availability of label in plasma was determined prior to and at 3 moments during intake of labeled glucose and $^2H$-enrichment in the DNA of blood neutrophils was followed over time (figure 1). The first labeled neutrophil DNA was detected in the circulation at day 6 after intake of label, representing the maturation time from progenitor to release in the blood (the post-mitotic pool transit time, PMPtt). This was in agreement with previously published results obtained from healthy volunteers, but slightly longer than observed under non-homeostatic conditions (1-4). Due to this delay between label incorporation and release into the bloodstream, re-utilization of labeled nucleotides cannot occur before day 6 after intake of label and it will take an additional 6 days before the re-utilized label can be found in the DNA of circulating neutrophils. In addition, we show that approximately 66% of the adenosine moiety is de novo synthesized in a promyelocytic cell line (not shown), further indicating that re-utilization of label should not be a problem in this study. Deuterium enrichment reached its peak at day 8 after intake of label, and decreased almost to baseline at the end of the study (day 17/18 see figure 1).

Computational modelling
Traditional models based on ordinary differential equations modelling the enrichment in blood and bone marrow such as published previously (4,5) proved unable to produce adequate fits. There are several possible explanations why this might be the case: ODE models assume rapid uplabelling in the BM, followed by an exponential decay of the amount of label in the BM. However, as stated above, myelocytes divisions have been shown to occur very strictly every 18 hours (7,8) which makes an exponential decrease of label in the BM unlikely (7,8).

Secondly, these models assume a fixed PMPtt for each cell, a notion has been challenged previously (3) and may not be biologically plausible.

Therefore, an agent-based method was used to analyze the pooled data from all volunteers. The agent-based model describes individual cells based on a conveyor-belt model as first described by Cartwright et al (9) (Figure 2A). The model takes into account BM and blood kinetics, as well as a PMPtt (figure 2) during which no proliferation takes place. Cells dividing in the BM when label is present incorporate this label at a maximum of 50%, as only one strand of DNA is newly synthesized for each division. Both labelled and unlabeled BM cells keep dividing. The time between divisions is described as t(div). After a division, cells can either remain a progenitor cell or start maturation towards a mature neutrophil and enter the PMP. The average time cells stay in the PMP until they enter the bloodstream as mature cells is the PMPtt.

In the circulation, neutrophils can be either circulating or marginated. Since the marginated pool is in equilibrium with circulating neutrophils (10) and the amount of label in cell is assumed to be similar in the two pools (11), it does not affect labelling kinetics and for simplicity was not included in the model.

Since it is unlikely that each cell behaves exactly similar, we allowed for heterogeneity between cells by adding a normally distributed variation of around each parameter. I.e. the mean PMPtt of the population is described in the parameter PMPtt, but some cells will exit the BM earlier or later. This amount of variation is described as SD_PMPtt.

This model was run ~375,000 times with different values for the PMPtt, circulatory lifespans, t(div) and different SDs for each of these parameters. These values were based on the wide range of previous estimates on neutrophil lifespan (5-192h) (12) and neutrophil progenitor turnover (10-92h). For the average PMPtt a wide range around the observed appearance of the first labeled neutrophil DNA was chosen (4-8 days). SD’s ranged from 0 (no variation between cells) to a maximum of 40% of the parameter it is applied to. For each tested lifespan, the exact same set of extra parameters was run, so for each lifespan the same number of runs was performed.

For each run, the quality of the fit to the experimental data was determined as the sum of squares of the residuals (SSR), with a low SSR indicating a better fit (Figure 2B). As the model is stochastic, two different runs with the same set of parameters can have a slightly different outcome. Therefore, several of the best fits were re-run 10 times and plotted against the measured data (Figure 2C-J). which show a too rapid uplabelling for short lifespans and a too slow downlabelling for long lifespan, with a best fitting estimate at a 72h lifespan. This too slow uplabeling with short neutrophil lifespans seems to be caused by a SD for the PMPtt. To allow more insight into which parameter combinations were used, and which showed the best fits to the experimental data, all possible combinations were plotted, with the values of the best fits (not shown). These indicate that longer neutrophil lifespans in our model require the smaller the standard deviations for PMPtt and tDiv, and the best fits have a tDiv ranging from 48h to 96h.

Our estimate of the circulatory neutrophil lifespan is 3 – 9 times longer than that obtained using ^3H-TdR or DF^32P labelling, which can be explained by the use of toxic labelling methods or non-homeostatic conditions (12). On the other hand, the estimated circulatory lifespans are
shorter than those previously presented by our group. As stated above, $^2$H$_2$O labelling of neutrophils was only capable of determining the slowest turnover, either in blood or BM. Nonetheless, our model clearly demonstrates that a short neutrophil lifespan is not in agreement with the results obtained by pulse-chase $6,6^2$H$_2$–glucose labelling.

**Bone marrow kinetics**

Even though we did not acquire BM from our volunteers, our computer model made predictions regarding the turnover of neutrophil progenitors. Fitting of the data obtained after short term $^2$H-glucose labeling shows a relatively long tdiv (72h) but does not allow for short circulatory neutrophil lifespans (see figure C-F). This relatively long tdiv is in the same order of magnitude as the hypothetical one proposed by Li et al in response to the $^2$H$_2$O study (6). Our results do not seem in line with other in vivo and in vitro methods described above (7,13,14), which indicated shorter neutrophil progenitor division times ranging from 18-30 hours. These data were obtained in vitro or from patients with hematological disorders. It is unknown whether division times of myelocytes in vitro represent the situation in vivo. In addition, BM has been shown to undergo increased turnover during inflammation (15,16). It is possible that these data reflect the BM kinetics under non-homeostatic conditions, whereas our predictions reflect homeostatic conditions. Of importance is that restricting division times of myelocytes in the BM to a maximum of 30h is associated with longer blood lifespans. Therefore, the estimates of our model will be conservative as our model predicts a relatively long tdiv.

In conclusion, our study applying short-term labeling with $^2$H-glucose has circumvented problems regarding long-term labeling with $^2$H$_2$O and suggests that a relatively long division time of progenitors in the BM is not necessarily associated with a short (<1 day) life span of neutrophils in peripheral blood. Our study supports a long (2-4 day) lifespan of neutrophils in the peripheral blood under homeostatic conditions.

**II. Eosinophils and basophils in peripheral blood**

In addition to determining the neutrophil lifespan, we determined the circulatory lifespan of eosinophils and basophils in healthy volunteers (see figure 3). Labelling kinetics are very different in cell type: Labelled DNA is seen in eosinophils at day 5 after intake of label, whereas basophils take at least 6 days. Subsequently, eosinophils show a rapid uplabelling curve similar to that of neutrophils. Basophils labelling kinetics appear far slower, with maximum labelling being reached at day 12 after intake of label. Using a mixed effects model we have estimated the lifespan, maturation time and tdiv for both eosinophils and basophils for each individual separately (figure 3). These results show that basophils have a circulatory lifespan of 6.2 days and that the slow basophil uplabelling is due to slow kinetics in the bone marrow with a tdiv of >5 days. In contrast, eosinophil progenitor cells have a faster turnover in blood, but a relatively long lifespan in the circulation of 7 days.

**III. Complex kinetics of blood and sputum eosinophils in patients with eosinophilic asthma.**

Our data shows that the kinetics of eosinophils in sputum of EA patients is complex. When we model the enrichment data in peripheral blood (see figure 4) it turns out that eosinophils from normal donors exhibit a blood lifespan of eosinophils of around 7 days. This might underestimate the real lifespan as the data seems to suggest that there are two populations of
eosinophils; one slow and one fast (figure 4). Counterintuitively, eosinophils in the blood of
eosinophil asthma patients show a significant delay (≈ 2 days) in kinetics, where an
acceleration was expected (figure 4). This expectation was based on the assumption that pro-
inflammatory cytokines would accelerate the production of eosinophils in the bone marrow and
thereby an increased flux through the eosinophil lineage.

However, this was not found and at least part of the answer lies in the complex kinetics for
eosinophils in the sputum. As can be seen in figure 4 the enrichment of eosinophil DNA in the
sputum precedes the enrichment of DNA of cells in the peripheral blood. Interestingly, the
uplabeling in sputum follows the same kinetics as the eosinophils in normal individuals in
blood (figure 4B). This can be explained by the hypothesis that a rapid mobilizable pool
of eosinophils is present in peripheral blood under homeostatic conditions that can be
recruited very fast to tissues with eosinophilic inflammation leaving cells in the blood
that exhibit slow homing to the tissue (this data is consistent with the modeling data
presented in figure 4C).

The enrichment of the DNA of sputum cells is low and is persistent. This is in marked contrast
to neutrophils that show similar kinetics as found in the peripheral blood in the same patients
(results not shown). These data are consistent with a model of a slow population of
eosinophils in the sputum with a lifespan > 15 days. These long-lived eosinophils (already
present at the start of the analysis) will “dilute” the ²H-signal of newly recruited labelled cells
explaining the lower enrichment in sputum eosinophils (see figure 4).

It is now tempting to speculate that the fast cells in the blood end up as the fast cells in the
sputum. This situation is similar as found for blood and sputum neutrophils (results not shown).
Next to these fast cells, there is a population of eosinophils that are characterized by long term
presence in the sputum. At present it is unclear whether these “slow” cells originate from the
fast cells in blood and survive for a long time in sputum, or whether they comprise of a second
wave of slow cells migrating to sputum considerably later.

In conclusion. Our project has led to new important insights regarding the kinetics of
inflammatory cells. These kinetics are consistent with the hypothesis that in eosinophilic
asthma patients eosinophils are characterized by a shorter lifespan in peripheral blood
compared to normal and a long residence time in the sputum. These findings are consistent
with the mepoluzimab studies which already suggested longevity of eosinophils in the tissue of
eosinophilic asthmatics (17, 18). This has important implications as new therapy should focus
on resolution of eosinophils in the tissue rather than antagonism of homing. The latter takes as
long as the longevity of the eosinophils is. The situation with neutrophils is reverse. We could
not show any appreciable delay in the sputum. This leads to two mutual exclusive hypothesis:
neutrophils are only responding to tissue damage and are not cells involved in the
pathogenesis of asthma or a high turnover of neutrophils in tissue causes tissue damage. The
latter seems less likely as there is no real correlation between neutrophil numbers and disease
severity.

References:
1. Cronkite EP, Fliedner TM, Bond VP, and Rubini JR. Dynamics of hemopoietic proliferation
in man and mice studied by H3-thymidine incorporation into DNA. Annals of the New
W, van der Woerd-de Lange JA, et al. Estimation of kinetic parameters of neutrophilic,
3. Dancey JT, Deubelbeiss KA, Harker LA, and Finch CA. Neutrophil kinetics in man. The
Did the study solve the original question? yes (explain) (max. 250 words):

1. What is the circulatory lifespan of eosinophils and basophils under homeostatic conditions?

**Eosinophils:** 7.0 days
**Basophils:** 4.6 days

2. What is the time for transit through the post-mitotic pool?
### Eosinophils: 4.9 days
### Basophils: 5.9 days
3. Does allergic inflammation influence eosinophil circulatory lifespan and transit time through the post-mitotic pool?
   **Yes:** Lifespan: 7.0 days
   **Yes:** Transit time: 6.2 days
4. What is the retention time of cells in the sputum in those patients with eosinophils and neutrophils present in this compartment (active disease/immunologically challenged).
   **Eosinophils:** > 15 days at least for a subpopulation of cells
   **Neutrophils:** no increased retention time

#### Papers (see instructions)

3.1 All publications (published or submitted peer-reviewed manuscripts):


3.2 All publications (not peer-reviewed like abstracts, newspapers, websites, etc.):


4. Implementation (see instructions):

The results of the project are important for the design of new anti-asthma drugs. It is now clear that such approach for neutrophil dominated asthma is different than for eosinophilic asthma. As eosinophilic asthma is now a main target for new biologicals it is now clear that particularly biologicals affecting the life span of eosinophils in the tissue are required. This fits nicely with the finding that only prolonged therapy with anti-IL-5 leads to required results. It is clear that IL-5 is important in the production of eosinophils, but it is questionable whether IL-5 is an important survival factor in the tissues. With our new technology it is now possible to answer the important question: “Does my new anti-inflammatory drug affects the lifespan of inflammatory cells in the tissue?.”
An important next study would be to determine whether the new anti-inflammatory drugs now being tested in eosinophilic asthma affect the life-span of these cells in the sputum.
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Figure 1. Plasma glucose and DNA enrichment. (A) Deuterium enrichment of plasma glucose during intake of 5.6-2H5-glucose, which determines the availability of label for each volunteer. In every individual 4 time points were studied (mean ± SD, n=32). (B) Neutrophil DNA enrichment over time is plotted, with 6 time points for 12 volunteers and an additional single time point for 20 volunteers (92 data points in total). Dashed line represents mean enrichment, error bars indicate SD.

Figure 2. (A) Schematic representation of the neutrophil compartment as used in the computational model. The model is a linear conveyor-belt model, in which cells subsequently go through each phase: division, maturation in the post-mitotic pool and residence in circulation followed by clearance from circulation. To allow for biological variation, cells go through each phase at a mean speed with a normally distributed variability. The SD of the variability is an independent parameter for each phase. This model was run >375,000 times and the quality of the fit was determined by the SSR method, in which a lower SSR indicates a better fit. For each run, the SSR was plotted against the lifespan (E), with a cutoff above 10 (indicating irrelevant fits) for clarity. The best fitting parameter combinations for a lifespan of 7, 15, 24, 42, 60, 72, 96 and 120 hours (C-J) were rerun 10 times and plotted against the measured enrichments, normalized for enrichment at day 8 (blood) or 6h (BM). Information on the values for the other parameters can be found in supplementary figure S2. Green lines represent BM DNA enrichment, red lines represent fits of blood neutrophil enrichment +/- 95% CI (pink area) and black dots represent the measured DNA enrichments +/- SD.
Figure 3. DNA $^2$H-enrichment of eosinophils (A) and basophils (B) of healthy controls. Computational modelling shows a longer circulatory lifespan for eosinophils (C), whereas basophils have a longer maturation time (D) and a slower BM turnover (E).
Figure 4.
A. Pooled data of $^3$H-DNA enrichment in blood eosinophils obtained from asthma patients (red dots n=9) and healthy individuals (green dots n=9).
B. Pooled data of $^3$H-DNA enrichment in blood (red dots) and sputum (blue dots) eosinophils obtained from the same asthma patients. Blood samples are obtained from 9 patients (multiple venapunctures) and sputum cells from 6 of these patients with sufficient numbers of eosinophils (>3%).
C. Estimations of eosinophil kinetics by mathematical modeling of the data obtained in figure 5, for EA patients (n=9) and healthy individuals (n=12). The data indicate a short lifespan in peripheral blood and a low turnover in the bone marrow.