In the introduction the pros and cons of the most important cell separation techniques have been outlined. Briefly, the techniques that are applicable in humans are in vitro techniques. These techniques make use of the assumption that red blood cells (RBC) of different cell age have different physical properties, such as a difference in cell density, mainly determined by the hemoglobin concentration in the red cell, or cell volume. The premise that differences in hemoglobin concentration correlate with cell age is not undisputable. Furthermore, even though the mean hemoglobin concentration of a fraction containing RBCs may correlate with the mean cell age of these RBCs, it is still debatable whether this relationship applies to the age of the individual RBC as well. The assumption that hemoglobin concentration correlates with mean cell age is mainly based on investigations in animals, and have, due to this limitation, limited applicability in humans. The most often used in vitro techniques are density separation, mostly with the use of a discontinuous density gradient (Percoll), or volume separation with the use of counterflow centrifugation. Both techniques have serious limitations. The fractions that are analyzed still have a wide red cell distribution width and a wide hemoglobin distribution width. With all these imperfections in mind, a considerable amount of research is still performed with these in vitro techniques, because of the easiness with which RBCs can be obtained and studied in all sorts of apparatus, such as the flow cytometer, ektacytometer and FACS scan.

In vivo techniques can only be applied in animals, with the possible exception of studies in which a low dose of radioactive iron is used. One technique that is used in the research of old RBCs, is the hypertransfusion technique. In these experiments, a lot of rodents are used. It relies on suppression of hematopoiesis by inducing a polycythemia in animals through hypertransfusion. Briefly, half of a population of rodents are bled to death and the blood is used for transfusion in the other half of the population. This is repeated a number of times, until all RBCs of the rodents are old RBCs. Even though these RBCs have all aged in vivo, it is still a matter of some debate whether the characteristics of the RBCs are the same as the characteristics of RBCs that have aged in a normal animal. The most promising in vivo technique is
the technique that makes use of the tight binding of avidin and biotin. RBCs can be biotinylated and reinfused. Later, during the life of the animal, these RBCs can be isolated and studied. Biotin seems not to interfere with the processes of the RBCs.

In the second part of the introduction, attention is focused on the RBC markers that can be used to determine the age of a RBC. An ideal marker is an intrinsic property of the RBC with a predictable change during the RBC life and easy to measure in the laboratory. Furthermore, it is necessary that the marker can be measured in small amounts of RBC. Used most often are mean corpuscular hemoglobin concentration (MCHC) and RBC enzyme content. Both are simple to determine, but there are some doubts. By some, the MCHC is regarded as an imperfect measure of cell age. The decline in RBC enzyme content is probably not linear, but tends to be biphasic with a rapid decrease in the first part of the life-span of the RBC and levelling off thereafter.

In our studies, we have used the percentage of hemoglobin A1c (HbA1c) as a parameter of cell age. The glycation of hemoglobin is rather well studied. The determination of the HbA1c concentration with high pressure liquid chromatography is easy and used in daily practice in the treatment of patients with diabetes mellitus. HbA1c is the most abundant of the minor hemoglobins. The HbA1c concentration is probably the best available marker of RBC age. One other promising marker of cell age is the ratio of two forms of membrane protein 4.1 (a and b). This marker has not yet gained acceptance, mostly because the determination is technically cumbersome.

The third part of the introduction concerns itself with the mechanism that may cause RBC death. Many hypotheses have come forward. Broadly, they can be divided into two groups; one suggesting that a functional impairment in RBC metabolism occurs. Examples of these mechanisms are a decline in RBC enzyme activity, leading to exhaustion of the metabolic processes in the RBC, an increase in hemoglobin concentration and a decrease in RBC volume, leading to a decreased deformability and subsequent removal of the
RBC in the spleen. The other hypotheses suggest that changes occur in the membrane-composition, leading to adhesion of immunoglobulins to the cell surface and recognition of these cells by the mononuclear phagocytic system with subsequent destruction of the RBC by these cells.

Chapter 2 describes our results with the combination of counterflow centrifugation and Percoll separation. HbA1c was used as a parameter of cell age. It was confirmed that this combination resulted in a superior separation when the results with regard to mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCHC, and HbA1c were compared to either separation technique alone. Furthermore, the limitations of density separation were evident as HbA1c increased only slightly in the density separated fractions. MCV was inversely correlated to HbA1c concentration. MCV and MCH decreased with increasing HbA1c, while the MCHC increased. The conclusion was that during the life of the RBC, there is an ongoing loss of cellular water and hemoglobin. As can be deduced from the increasing MCHC, the loss of water is proportionally greater than the loss of hemoglobin. Furthermore, when MCV and MCHC were correlated to HbA1c concentrations, MCV appeared to correlate better to mean cell age than MCHC.

In chapter 3 studies are reported on the changes in the hemoglobin content and the subsequent glycation of the subfractions HbA1a-c. It is shown that the decrease in HbA0 content is due to a combination of loss of hemoglobin from the RBC and a modification to glycated subfractions. The decrease in HbA0 was estimated to be 25% of the total amount in young RBCs. The other hemoglobin fractions HbF, HbA2, and a not yet identified fraction X decreased also. An increase in all glycated subfractions could be observed. This study strengthened our supposition that HbA1c is a reliable marker of RBC age.

In chapter 4 studies which looked at young RBCs are reported. The combination of separation techniques proved to be superior in the enrichment of fractions with young RBCs. Reticulocytes were counted by the new methy-
lence blue staining technique and by the thiazole orange staining technique in a FACS scan. Correlation between these two counting methods was very good, confirming many other studies. One consistent finding was, however, that the number of thiazole-orange staining cells was consistently higher than the number of reticulocytes in the fractions that contained only small amounts of young RBCs. After counterflow centrifugation young RBCs and reticulocytes were seen in all fractions; after Percoll separation, they were mainly concentrated in the top fractions. This suggests that reticulocytes have a low density, but can have large differences in volume when they leave the bone marrow.

After labelling with radioactive iron ($^{59}$Fe) RBCs were transfused in one individual. At intervals, blood was drawn and separated in the three separation techniques. After Percoll separation, radioactivity could already be seen in fractions containing cells with a high density after a short time period (approximately 24 days), suggesting that a substantial increase in MCHC occurs in young RBCs. After counterflow centrifugation fractions containing cells with a high MCV had a higher amount of radioactivity during the first seventy days compared to fractions containing RBCs with a low MCV. After combination separation a two-fold enrichment with young cells in the fraction with the lowest HbA1c was seen in comparison with either counterflow centrifugation or Percoll separation. The amount of radioactivity increased in the fraction containing RBCs with the highest MCHC, the lowest MCV and the highest HbAc after approximately 50 days.

Besides loss of their RNA-material, young cells already seemed to loose water and hemoglobin like older cells, resulting in a decrease of MCV and MCH and an increase in MCHC. The loss of volume is probably a continuous process.

In chapter 5 studies regarding whole red cell deformability are reported. Deformability was measured with an ektacytometer, using a laser diffraction technique in which erythrocytes were studied in suspension using rotational stress. Deformability was decreased after Percoll separation, but not after counterflow centrifugation. After the combination the deformability was much
more decreased than in any other study. This was probably due to a combination of an increase in intracellular viscosity (≈MCHC) and a decrease in membrane elasticity. Interestingly, the surface area to volume ratio increased somewhat. The conclusion was that during the life of the RBC there is a decrease in deformability.

During the aging of the RBC many processes occur simultaneously. These processes include at least an increase in hemoglobin concentration, an decrease in hemoglobin content and cell volume, a decrease in enzymatic activity of the RBC, and a decrease in deformability. In analyzing the processes that trigger the RBC for destruction, the underlying assumption is that the mechanism that is responsible for cell death, is the same in every RBC. This assumption is not necessarily true. In looking at disease states, it is clear that RBCs may perish due to several mechanisms. Auto-immune hemolytic anemia is due to a derangement in the immune system, leading to covering of RBCs with autoimmune antibodies and subsequent removal from the circulation of these cells in the spleen. Hereditary spherocytosis is an example of a disorder in which the membrane of the RBC is deficient, leading to a decreased deformability and early destruction. Glucose-6-phosphate dehydrogenase deficiency is an example of enzyme deficiency, leading to hemolysis. It may well be that normal old RBCs are removed from the circulation due to several mechanisms, depending on which aging process is the first to mark the cell for destruction.

One very important aspect of our studies was the conclusion that the utmost importance must be given to the separation technique that is chosen to analyze processes concerning life and death of the red cell. The results that we obtained with many studies, were very different between different separation techniques. Each separation technique resulted in the isolation of different fractions of RBCs. The best in vitro technique, nowadays available, is the combination of counterflow centrifugation and Percoll separation, but even this technique has many limitations.